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Variation and Adaptation of *Pseudomonas aeruginosa* Toxicity to HeLa Cells and Fibroblasts

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The toxic components of supernatants from *Pseudomonas aeruginosa* cultures directed against HeLa cells and *Staphylococcus aureus* were evaluated with the aim of discovering interactions. Supernatants of eight different strains of *P. aeruginosa* were assayed for cytotoxic activity. All were active against HeLa cells; seven were toxic for *S. aureus*. On repeated suspension of *P. aeruginosa* in 0.9% sodium chloride solution, a shift from HeLa cell toxicity to staphylococcal lytic activity occurred along with a change of toxic activity from a high (50,000 ± 5,000) to a low (8,000 ± 400) molecular weight (MW) range on gel filtration. Addition of protein to the minimal medium of cultures producing material toxic only for *S. aureus* reactivated the generation of HeLa cell-toxic material. Cultivation of *P. aeruginosa* in the presence of HeLa cells and a chloramphenicol supplement produced suppression of the generation of material toxic for *S. aureus* but facilitated that of HeLa-toxic material of high MW. Adaptation of toxicity against fibroblasts developed only on cocultivation of *P. aeruginosa* together with *S. aureus* and in the presence of fibroblasts. Under these conditions a strong lytic activity for *S. aureus* appeared, even in the presence of chloramphenicol. Chloramphenicol caused the material toxic for fibroblasts to elute at a low MW well separated from that toxic for HeLa cells. In contrast to the high-MW toxic substances, the low-MW material did not induce antibodies after injection into rabbits. This may explain failures of vaccination against *P. aeruginosa* infection and of serum therapy of homologous sepsis in humans.

Pseudomonas aeruginosa culture supernatant contains a staphylolytic toxin, in addition to exotoxin A, which is toxic to HeLa cells (4, 5, 13, 27, 40, 51). Their concomitant appearance suggests pathogenetic significance in mixed infections in humans. In our earlier investigations both activities appeared either together or separately (25, 40). An interrelationship between the two might be indicated by a repeatedly observed change of toxicity.

This shift of toxicity can be induced in vitro by suspension of *P. aeruginosa* in sodium chloride (0.9%) solution. To eliminate HeLa cell toxicity, it was necessary to suspend the bacteria in sodium chloride solution at least three times. Reappearance of the original toxin production was effected by the addition of protein to the minimal medium. More specific alterations were observed on cultivation of *P. aeruginosa* either alone or in mixed cultures with *Staphylococcus aureus* in the presence of HeLa cells or fibroblasts with a chloramphenicol supplement. For this phenomenon the term adaptation has been chosen to describe (i) the selective production of HeLa cell toxicity whereby production of staphylolytic toxicity is suppressed and (ii) the formation of specific substances toxic to fibroblasts by coincubating *P. aeruginosa* with culture cells and chloramphenicol.

Subsequent to toxicity of *P. aeruginosa* directed against human culture cells, a permanent change of HeLa cell and staphylolytic toxicity was observed. From this stage new substances were isolated, which may play an important role in the pathogenic mechanisms of gram-negative bacteria with special reference to human cells.

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MATERIALS AND METHODS

Eight *P. aeruginosa* strains were obtained from clinical isolates at the Institute for Hygiene at the University of Heidelberg, Heidelberg, Federal Republic of Germany. They produced a blue-green pigment during isolation, and their characteristics were in accordance with taxonomic guidelines laid down elsewhere (3). Data regarding the specific origins of the strains as well as their susceptibilities to antibiotics in agar diffusion tests (2) are summarized in Table 1.

Cultivation and preparation of *P. aeruginosa* for toxin production. Culture media were DST agar (Oxoid Ltd., Basingstoke, England), which contained (grams per liter) proteose peptone (10.0), extract of veal (10.0), glucose (2.0), sodium chloride (3.0), disodium phosphate (2.0), sodium acetate (1.0), adenine sulfate (0.01), guanine hydrochloride (0.01), uracil (0.01), xanthine (0.01), aneurine (0.00002), and bacteriological agar (12.0, pH 7.4), and Trypticase soy broth (without glucose; BBL Microbiology Systems, Cockeysville, Md.), which was supplemented with 1% potassium nitrate and 1% glycerol.

For toxin production, *P. aeruginosa* (Table 1) was cultivated on DST agar, in Trypticase soy broth, or both. For inoculation, *P. aeruginosa*, when not derived from liquid cultures, was suspended in sodium chloride (0.9%) solution. Usually approximately 100 DST agar plates (diameter, 9.5 cm) were each inoculated with 10^9 bacteria or, in the case of Trypticase soy broth (2, 52), with 10^{10} bacteria.

The cultures were incubated for 20 h at 37°C and maintained for 3 days at 6°C; cultures on DST agar plates were overlaid with 5 ml of sodium chloride (0.9%) solution, and

TABLE 1. Origins and susceptibilities to antibiotics of the eight strains of *P. aeruginosa* studied

Strain no.	Material	Disease	Resistance or susceptibility to[a]:			
			Azlocillin	Ticarcillin	Gentamicin	Polymyxin
1	Uricult[b]	Cystitis (catheter)	ND	R	R	S
2	Uricult	Cystitis (catheter)	ND	S	S	S
3	Swab from tympanum	Otitis media	ND	S	S	S
4	Tracheal catheter	Seizure disorder	R	S	S	S
5	Uricult	Cystitis (catheter)	S	S	S	S
6	Swab from an abscess	Osteomyelitis	R	R	S	S
7	Swab from a pustule	Dermatitis	S	S	S	S
8	Swab from the tubes	Salpingitis	R	ND	S	ND

[a] S, Susceptible; R, resistant; ND, not done.
[b] Uricult was obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany.

the bacteria were removed with a glass spatula. The bacteria were not harvested completely to avoid contamination by agar particles.

The bacteria suspended in sodium chloride (0.9%) solution, as well as broth cultures, were maintained for up to 28 days at 6°C. Finally, they were centrifuged for 60 min at 9,000 × *g* and the supernatant was designated S_0. Without an intervening growth phase, the bacterial sediment was suspended in 200 ml of sodium chloride (0.9%) solution and maintained for 4 weeks at 6°C. Centrifugation as described above resulted in a supernatant designated $1S_0$. A new suspension of bacterial sediment with 100 ml of sodium chloride (0.9%) solution and further treatment as described above produced the supernatant $2S_0$.

Cultivation and preparation of HeLa cells and human embryo lung fibroblasts. HeLa cells were cultivated in minimum Eagle medium with 10% calf serum; fibroblasts were grown in Dulbecco modified minimum Eagle medium with 10% fetal calf serum in plastic petri dishes at 37°C in a humidified CO_2 incubator (5% CO_2). All cell lines were free of mycoplasms. For assay of mycoplasms, cells were cultured in PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 20% horse serum and 10% yeast extract (Difco) for 3 weeks at 37°C in the presence of 5% CO_2 in air (95% humidity). In addition, cell sediments were stained with Hoechst stain no. 33258 and checked microscopically. Contamination of cells by mycoplasms was not detectable by these two assays.

Two days after plating, exponentially growing cells were removed from the dishes with 0.06% trypsin solution (in phosphate-buffered saline) and centrifuged for 10 min at 200 × *g*. Cells were suspended, washed three times in phosphate-buffered saline, and adjusted to 3 × 10^6/ml, and their numbers were confirmed by electronic cell counting (Coulter Counter model B [Coulter Electronics, Inc., Hialeah, Fla.]).

Incubation of *P. aeruginosa* with HeLa cells and chloramphenicol supplement. Five tubes were inoculated with 3 × 10^6 HeLa cells in 1 ml of phosphate-buffered saline and supplemented with Leinbrock (28) solution–0.9% NaCl (1:1) to a final volume of 6 ml of liquid. Chloramphenicol was added to four tubes to final concentrations of 8, 16, 32, and 64 μg/ml. *P. aeruginosa* (3 × 10^6) in the logarithmic growth phase was inoculated into each suspension and incubated for 24 h at 37°C and for 16 h at 22°C. From each culture, 130 DST agar plates were inoculated with 10^9 *P. aeruginosa*. Growth, harvesting, and further treatment of bacteria for toxin production were as described above.

Incubation of *P. aeruginosa* with fibroblasts in mixed cultures with *S. aureus* ATCC 511 with a chloramphenicol supplement. Suspensions of 2 × 10^6 fibroblasts in 1 ml of phosphate-buffered saline were filled into 5 tubes together with Leinbrock and sodium chloride (0.9%) solutions (1:1) to a final volume of 6 ml. Each tube was inoculated with 2 × 10^6 cells of *S. aureus* and *P. aeruginosa* in the logarithmic growth phase. Both kinds of bacteria had been transmitted before eight times into Leinbrock solution and incubated each time for 24 h at 37°C. The mixed cultures were incubated for 10 h at 37°C with the fibroblast suspensions prior to addition of chloramphenicol to final concentrations of 8, 16, 32, and 64 μg/ml. Further incubation for 15 h at 37°C was followed by maintenance for 10 h at 6°C. A total of 120 DST agar plates were inoculated from each culture. Growth, harvesting, and further treatment of bacteria were as described above. For this experiment, *P. aeruginosa* was chosen after long-term incubation with HeLa cells (6 weeks at 22°C with 16 μg of chloramphenicol per ml).

Concentration and partial purification of toxic substances. Low-molecular-weight (low-MW) substances were separated by dialysis in running water by using dialysis tubes (Kalle GmbH, Wiesbaden, Federal Republic of Germany), which excluded particles with an MW less than 4,000. The dialysate was lyophilized and fractionated on Sephadex G75 (Pharmacia, Uppsala, Sweden) at 7°C with 0.9% NaCl serving as the eluent. The column dimensions were 3.6 by 63 cm. A Uvicord S (LKB Instruments, Inc., Rockville, Md.) was used to register (280 nm) and collect 120-drop fractions.

Similarly, the hydrophilic Fractogel TSH HW-55 (F) (E. Merck AG, Darmstadt, Federal Republic of Germany) was used for fractionation of substances in the 10^3 to 10^6 MW range. NaCl (0.9%) was delivered through a column (2.1 by 113 cm) at 7°C by a peristaltic pump, and 100-drop fractions were registered at 280 nm as described above.

In addition, attempts were made to isolate the toxic proteins in their active form by preparative gel electrophoresis without sodium dodecyl sulfate (SDS). A protean double-slab electrophoresis cell (Bio-Rad Laboratories, Munich, Federal Republic of Germany) was used with an 11.7% polyacrylamide gel and a 0.025 M Tris–0.15 M glycine buffer (pH 8.3). The gels were cut perpendicular to the direction of migration into 1-cm-wide strips and eluted overnight with 0.9% NaCl.

Determination of MW via electrophoresis. Polyacrylamide gel (15%) was used for the procedure (63). The reference proteins were bovine serum albumin (MW, 68,000), chicken egg albumen (MW, 43,000), chymotrypsinogen A (MW, 25,000), myoglobin (MW, 17,800), and cytochrome *c* (MW, 13,000). The proteins were stained with Coomassie blue G250. Glycoproteins were detected by the *p*-aminosalicylic acid reaction (65).

Identification and quantitative determination of pyocyanine.

Pyocyanine was separated from the protein component via Fractogel TSH HW-55 (F) (see above) and identified spectroscopically in H_2O at pH 2 to 2.5 as well as at pH 7 to 8 (66). In addition, the concentration was determined quantitatively in solution (pH 7.7) at 690 nm by using the extinction coefficient $E_{1\ cm}1\% = 164$ (23).

Purification and determination of proteolytic activity. The crude supernatant was dialyzed (see above) and fractionated on Sephadex G75. After exclusion of exoproteases, azocasein was determined to be a suitable substrate for endoprotease. Their activity was determined by a modified protease inhibitor test (14; P. Blosch, M.D. dissertation, University of Heidelberg, Federal Republic of Germany, 1982) (optimal conditions: 0.1 M sodium tetraborate, pH 8.0). The cleavage products were measured at 366 nm.

Determination of lipids. Ten milligrams of low-MW material (MW, <15,000) from lyophilized dialysate fractionated on Sephadex G75 columns was dissolved in 1 ml of methanol–2 ml of chloroform. After pipetting and evaporation of the chloroform phase in a stream of nitrogen, the residue was dissolved in 20 μl of chloroform and examined by thin-layer chromatography carried out in one dimension (34).

Determination of free fatty acids. Ten milligrams of low-MW material (MW <15,000) (see preceding paragraph) was dissolved in 0.1 ml of distilled water, and its enzymatic properties were examined by the commercially available NEFA kit obtained from Wako, Osaka, Japan (45, 58).

Analysis of amino acids. Samples (15 mg) of low-MW material (MW, <15,000) (see above) were hydrolyzed with 6 N hydrochloric acid at 105°C for 24 h in vacuo. The analysis was carried out in a Biotronik amino acid analyzer.

Sera and antigens. For immunization of rabbits (New Zealand race; weight, 3.0 kg) and for Ouchterlony assays (46, 47) the high- and low-MW materials toxic for HeLa cells, as well as staphylolytic substances, were separated by gel permeation chromatography (see above) and used as antigens. By an immunization scheme described elsewhere (8), rabbits received injections with 1 mg of high-MW protein (two animals for each substance) and 1.2 or 5 mg of low-MW protein (one animal for each amount). Concentrations of 0.25, 0.5, and 1 mg of high-MW protein per ml of sodium chloride (0.9%) and 0.25, 0.5, 1.2, and 5 mg of low-MW protein per ml of sodium chloride (0.9%) were used in Ouchterlony tests.

Agar diffusion test by the Ouchterlony plate method. Pure agar (1.5%) (Behringwerke, Marburg, Federal Republic of Germany) with a supplement of 1:10,000 merthiolate served as the diffusion medium (41).

Biological activity. The method used to determine biological activity (verification of toxicity to HeLa cells and fibroblasts and staphylolytic toxicity) has been described in detail previously (40).

RESULTS

Details regarding the origins and susceptibilities to antibiotics of the eight *P. aeruginosa* strains examined for exotoxin production are given in Table 1. The following observations were made when DST agar and Trypticase soy broth were used as culture media. (i) All strains exhibited toxicity to HeLa cells. (ii) Staphylolytic substances were detected in seven of the eight strains. (iii) The two types of toxicity could occur concomitantly or separately. (iv) Surface cultures were more suitable for extraction of staphylolytic substances.

Table 2 summarizes the biological activities of different culture fluids assayed with HeLa cells and *S. aureus* including the rinse and culture liquids S_0 and the resuspension liquids $1S_0$ and $2S_0$ obtained by suspending the bacteria twice in sodium chloride (0.9%) solution (without intervening phases of growth). This treatment changed the primary toxicity observed in the S_0 liquids. *P. aeruginosa* directly cultivated from specimens showed a reversibility of toxin production from staphylolytic substances to HeLa cell toxin each time it was suspended in 0.9% sodium chloride solution. Repeated suspension of strains previously cultivated several times on glucose agar caused only the change from HeLa cell toxicity to staphylolytic activity. The shift in toxicity was paralleled by altered elution on gel permeation chromatography. The biological activities initially found in the higher-MW range (50,000 ± 5,000) appeared more and more in the lower-MW range (8,000 ± 400; Fig. 1). Figure 2 shows the double-peaked fractionation curve resulting from a mixed-toxicity sample and the association of toxicity to HeLa cells and toxicity to staphylococci. The two types of toxicity migrated together in the high-MW range. A large initial peak suggested that high toxicity to HeLa cells was present. Proteolytic activity was found in the fraction of a small intermediate peak.

In exclusively HeLa cell-toxic or staphylolytic supernatants, the biological activity was associated mainly with either the high- or the low-MW substances. In the case of concomitant toxin appearance, both activities were present in both size classes. The primary or secondary appearance of strong or weak staphylolytic activity allows one to draw conclusions about the migration of toxicity in conjunction with the higher- or lower-MW substances (Table 3; culture 1, $1S_0$ and culture 2, S_0). If bacteria previously subjected to

TABLE 2. Comparison of the toxicities of supernatants of four different *P. aeruginosa* strains (cultivated as surface or suspension cultures) for HeLa cells and *S. aureus*[a]

Strain no. and supernatant	HeLa cell toxicity[b]		*S. aureus* toxicity[b]	
	DST agar	TSB[c]	DST agar	TSB
2				
S_0	++	++	++	(+)
$1S_0$	−	(+)	+++	−
$2S_0$	−	−	+++	−
4				
S_0	++	−	+	−
$1S_0$	−	+	+++	−
$2S_0$	+	++	++	−
6				
S_0	−	+	+	(+)
$1S_0$	−	++	(+)	+
$2S_0$	++	++	−	++
8				
S_0	+	−	++	+++
$1S_0$	++	−	+	++
$2S_0$	++	−	+	+++

[a] Unfractionated bacterial supernatant (0.1 ml) was added to HeLa cell cultures containing 3 ml of minimum Eagle medium with 10% calf serum in 3-cm (diameter) dishes and (1 ml) to *S. aureus* cultures containing 10^5 to 10^9 cells in 2.5 ml of Leinbrock medium (compare reference 40). S_0 = *P. aeruginosa* supernatant or rinsing fluid (from surface cultures); $1S_0$ and $2S_0$ correspond to the first and the second resuspension fluids, respectively.

[b] ++, + = Cytolytic alterations of over 80% of the HeLa cells 2 or 3 days after addition of supernatant; (+) = cytolytic alteration of over 20% of the HeLa cells; +++, ++, +, (+) = reduction of the germ number by 6, 4, 2, or 1 order(s) of magnitude, respectively.

[c] TSB, Trypticase soy broth.

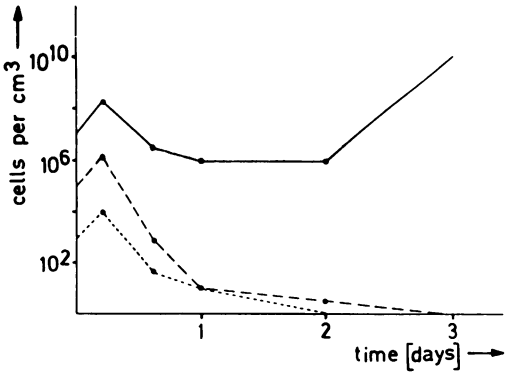

FIG. 1. Effectiveness of the small protein (MW, 8,000 ± 400) on *S. aureus*. Cultures with different germ contents received 125 μg/ml on day zero. ——, 10^7 CFU; – – –, 10^5 CFU; · · · · ·, 10^3 CFU.

repeated suspensions in sodium chloride (0.9%) solution (more than three times) were cultured in a medium supplemented with protein (e.g., human or calf serum), toxin production was promoted. The bacteria produced HeLa cell toxins and staphylolytic substances in the higher-MW range with toxicity to fibroblasts. Production of HeLa cell toxin was stimulated by incubating *P. aeruginosa* in Leinbrock solution together with washed HeLa cells at 37°C. Maximum toxin production was reached after a 12- to 18-h period of cocultivation. Chloramphenicol added to prevent contamination also affected the toxin spectrum generated by the bacteria (Table 4). At high concentrations of chloramphenicol, *P. aeruginosa* selectively produced substances toxic to HeLa cells. Bacteria maintained this quality on resuspension, and the toxic activity did not appear in the lower-MW range.

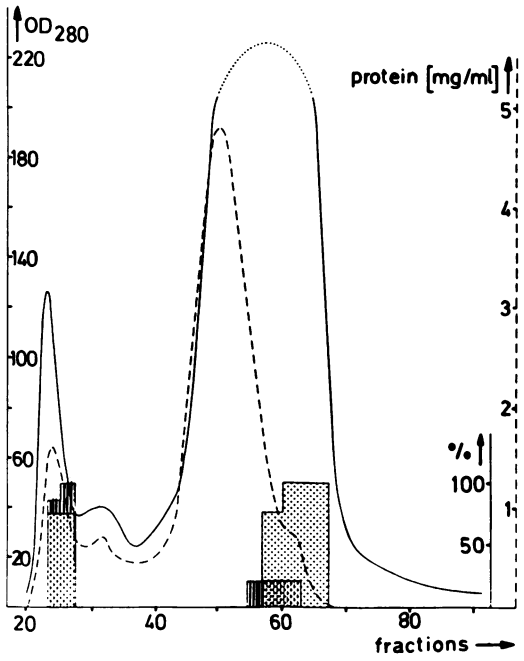

FIG. 2. Fractionation of material from the supernatant of *P. aeruginosa* strain no. 4 on Sephadex G75 (conditions: 10°C; column, 3 by 40 cm; eluent, 0.9% NaCl; elution, 33 ml/h; 5.5 ml per fraction). A total of 1.5 g of dialyzed and lyophilized material was separated. ——, Protein optical density at 280 nm (OD_{280}); – – –, protein determined as described in reference 37; ▥, toxicity for HeLa cells; ▨, toxicity for *S. aureus*.

P. aeruginosa cultured together with fibroblasts in the presence of 16 μg of chloramphenicol per ml produced neither HeLa cell- nor fibroblast-toxic substances but rather staphylolytic material. If *P. aeruginosa* and *S. aureus* were cultured together and fibroblasts were added, *P. aeruginosa* was stimulated to produce all three kinds of toxic activity. After the components were separated on Sephadex G75, a third peak appeared for the first time in the low-MW range. This peak showed toxicity to fibroblasts but not HeLa cells and appeared only in the supernatant of bacteria grown in the presence of 66 μg of chloramphenicol per ml and simultaneously exhibiting high staphylolytic toxicity.

Electrophoretic separation of high-MW (50,000 ± 5,000; see also reference 25) toxic products did not yield significant differences between HeLa cell-toxic and staphylolytic materials. A protein with endoproteolytic activity and high affinity to the substrate azocasein was isolated from the intermediate peak. The properties of the protease(s) of the intermediate peak obtained by gel permeation chromatography (Fig. 2) were as follows: MW, 18,500 ± 2,000; activity, 16,230 U/mg of protein; pH optimum, 8.0; inhibitor, calf serum; stability, freeze dried at −30°C for more than 1 year and kept in 0.9% sodium chloride solution at 4°C for more than 2 months.

The substances of the low-MW fractions (peak 2) migrated as one uniform band (Coomassie blue stain) in SDS gel electrophoresis; this corresponds to an MW of 8,000 ± 400. This protein exhibited a positive *p*-aminosalicylic acid reaction, which identified it as a glycoprotein (Fig. 3). In the gel stained with *p*-aminosalicylic acid, a second, faster-migrating substance was detected, which may contain significantly more carbohydrate and may therefore stain poorly with Coomassie blue. After hydrolysis, all common amino acids were present in the fractions containing the two toxic activities; significant quantitative differences between asparagine, glycine, alanine, and phenylalanine were noted.

The presence of lipids and free fatty acids in concentrations which could have an effect on staphylococci (20) was excluded. Pyocyanine particularly comigrated with less active fractions at concentrations which possessed no cytotoxic or bactericidal activity (0.29 μg/mg of fractionated material; 17, 19, 57).

By immunization of rabbits with high-MW material toxic to HeLa cells and *S. aureus* (about 50,000 ± 5,000), antisera were produced which reacted with substances containing

TABLE 3. Toxic materials and their MW in supernatants of *P. aeruginosa* strain 4

Culture no. and supernatant	Toxicity against[a]:		MW[b]:	
	HeLa cells	*S. aureus*	50,000 ± 5,000	8,000 ± 400
1				
S_0	++	−	●	
$1S_0$	++	++		●○
2				
S_0	+	+++	●○	
$1S_0$	−	+++		●○
3				
S_0	−	+++		○
$1S_0$	−	(+)		○

[a] For definitions of symbols, see Table 2, footnote *b*.
[b] Symbols: ●, toxic against HeLa cells; ○, staphylolytic.

TABLE 4. Toxicity of the supernatants of *P. aeruginosa* in the presence of HeLa cells at different concentrations of chloramphenicol[a]

Cultures at 37°C:		Toxicity for HeLa cells (% dead cells) after day:			Toxicity for fibroblasts (% dead cells) after day:			Toxicity for *S. aureus* (reduction of germ count by the following order of magnitude)		
Concn of chloramphenicol (μg/ml)	Supernatant	1	2	3	1	2	3	6	4	2
0	S_0	50	100			NG[b]	NG	−	+	+
	$1S_0$							−	−	−
16	S_0	40	90	90	NG	NG	30	+	+	+
	$1S_0$	20	70	90			10	−	+	+
64	S_0	80	100		10	10	80	−	−	−
	$1S_0$	80	90	100		60	90	−	−	−

[a] Experimental conditions were as described in Table 2.
[b] NG, No growth.

both toxic activities. The HeLa cell-toxic fractions, together with the homologous sera, exhibited more intensely stained bands than did the staphylolytic fractions with their antisera.

HeLa cell-toxic and staphylolytic fractions within the MW range of 8,000 ± 400 did not cause rabbits to produce antibodies detectable by the Ouchterlony assay. They did not react with antisera against high-MW substances.

DISCUSSION

Although liquid culture media are usually used for extracting toxins (7, 8, 29, 31, 35, 62), information on the lethal toxin comes from surface cultures (30, 32). Our results with cultures grown in liquid and on solid media were nearly comparable (Table 2). The latter media were more suitable for extracting the staphylotoxin. Other authors have also reported difficulties in obtaining staphylolysin from cultures grown in liquid media (6). All *P. aeruginosa* strains examined in this study showed HeLa cell toxicity and, with one exception, staphylolytic activity. The incubation time in the suspension liquid played a critical role in this respect and also for the alternating appearance of the toxins (Table 2) (30). Others (24, 29, 33, 49) have reported that gel permeation chromatography of the culture supernatant resulted in a double-peaked fractionation curve similar to that shown in Fig. 2. The absolute and relative heights of the two peaks, as well as the position of an intermediate peak, were variable. Protein and carbohydrate content, as well as MW determinations, have been reported in previous studies (25, 26, 42).

P. aeruginosa suspended in 0.9% sodium chloride solution showed reversibility of toxicity to HeLa cells and staphylococci. The toxicity changed in parallel from the higher- to the lower-MW range. It is not known whether continuous protein cleavage on the external membrane of the bacterial cell (36) is involved. The presence of biologically active precursors of exotoxin A inside the bacterial cell has not been proven to date.

Fragment A of exotoxin A inhibits protein synthesis at two locations (9, 21, 22); on the other hand, staphylolysin is an endopeptidase which breaks pentaglycine cross bridges in the bacterial cell wall with limited residual activity on casein (6). If concentrated in old *Pseudomonas* cells, they are destroyed (6). The substances toxic to HeLa cells and the staphylolytic substances which were detected in the higher- and lower-MW ranges do not cleave casein. The situation is similar for hemolysin, leucocidin, and cytolysin (5). The latter was found in association with a protease(s) which was later isolated by others in the fractions comprising the first and the second peaks, as well as the fractions comprising the intermediate peak (5, 11, 24, 39, 44, 53, 64), as also found in this study. Preliminary investigations showed that proteolysis was not responsible for the conversion of HeLa cell toxin to staphylolytic activity. On change of the activity in the higher- and lower-MW ranges (Table 3), the two types of toxic substance from mixed-toxicity supernatants behaved in a complementary manner. The nature of the toxin conversion is still unknown.

Bacteria grown in minimal media exhibiting staphylolytic activity developed toxicity to HeLa cells after the addition of serum. Incubation of *P. aeruginosa* with HeLa cells in Leinbrock medium at 37°C activated toxicity to HeLa cells. Chloramphenicol at a concentration of 64 μg/ml of culture medium suppressed the development of staphylolytic activity. Under these circumstances—as in the case of strains freshly isolated from patients—only material toxic for HeLa cells was detectable in the high-MW range. The mechanism by which chloramphenicol causes a preference for the production of material toxic for HeLa cells is unknown. This inhibitor is known to suppress formation of pyocyanine (56).

The possible significance of staphylolytic activity for human infections is indicated by the results obtained on coincubation of *P. aeruginosa* with fibroblasts (similar to that with HeLa cells). Toxicity to fibroblasts did not accompany high staphylolytic activity until *P. aeruginosa* was incubated together with *S. aureus*; this phenomenon was not

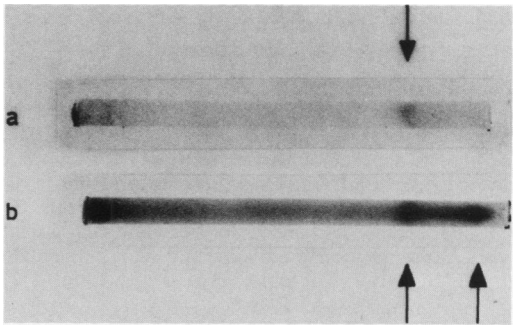

FIG. 3. Polyacrylamide gel (15%) electrophoresis in the presence of SDS (63) of material from peak 2. For this purpose 2 mg of the material was dissolved in 0.7 ml of sample buffer (0.125 ml of 250 mM Tris hydrochloride [pH 6.8], 0.4 ml of 5% SDS, 0.05 ml of 2-mercaptoethanol, 0.1 ml of glycerol) and heated for 1.5 min at 100°C. A sample (50 μl) of that solution was applied to each gel and separated (2 mA per gel) with 1 M Tris-glycine (pH 8.4)–0.2% SDS as the electrode buffer. (a) Coomassie blue-stained gel (25); (b) *p*-aminosalicylic acid-stained gel (65).

eliminated by chloramphenicol. Fractionation of culture supernatant obtained under these conditions resulted in a third peak; these substances were products of *P. aeruginosa* grown in 66 μg of chloramphenicol per ml of medium and exhibited toxicity to fibroblasts without toxicity to HeLa cells. Comparable investigations or information concerning the enzymatic processes which lead to variation and adaptation have not been carried out to our knowledge. To date only antagonism and synergism of *P. aeruginosa* and *S. aureus* in mixed cultures have been studied (10).

The toxic activities which occurred in the low-MW range could be classified as glycoproteins (glycopeptides). Based on their MW of 8,000 ± 400, these substances were clearly different from the toxins of *P. aeruginosa* previously detected by others. It is possible that the glycopeptides described in this investigation were also present in the cytotoxic dialysates recovered by others (60). Artificial toxicity due to lipids or free fatty acids could be excluded (20). The quantity of pyocyanine found was much lower than that required for bactericidal or cytostatic activity.

Generally, treatment of *P. aeruginosa* infections is based on antibiotics (15). Immunotherapy directed against polysaccharides (1, 54), lipopolysaccharides (55, 61), and proteins (16, 18, 43, 48, 50, 52, 59) has been attempted. Such immunotherapy in patients with cystic fibrosis (12, 38) has failed to prevent the overgrowth of mucoid strains. The use of alginatelike mucoid exopolysaccharides has obviated this difficulty (1). Successful immunizations with proteinaceous vaccines with 10,000 to 100,000 MW (59) and 16,000 MW (18) have been reported. The failure of the 8,000 MW proteinaceous material to induce antibodies in rabbits may explain deficiencies of immunotherapy.

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