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Relation of Capsular Materials and Colony Opacity to Virulence of *Vibrio vulnificus*

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Colonies which varied in opacity were isolated from the four strains of *Vibrio vulnificus*. Opaque and translucent colonial types of the strains were distinguished from the corresponding parent strains. Variation in the opacity of colonies formed by each strain was accompanied by variation of capsular material formation, which was clarified by electron microscopy of the organisms stained with ruthenium red. The opaque-type colonies of the strains had capsular materials. On the other hand, three translucent-type colonies had no observable capsular materials, and one had incomplete capsular materials, in contrast to the corresponding opaque type. The corresponding opaque and translucent types of the strains were compared for points of virulence in mice and guinea pigs. By having capsular materials, the bacterial strains acquired resistance to serum bactericidal action, antiphagocytic activity, high lethality for mice, and strong invasiveness in the subcutaneous tissue of guinea pigs. Capsular materials of *V. vulnificus* were considered to be important for the expression of virulence.

Vibrio vulnificus, a halophilic marine vibrio, is an occasional pathogen for humans. Hollis et al. (8) described two methods of infection that are observed in humans. One is that the bacterium enters through the mouth, and sepsis develops in immunodepressed humans, especially those with liver cirrhosis, hepatoma, and hemochromatosis. The other one is that cellulitis develops by infection of wounds on the occasion of fishing, swimming, and so on. In the former type of infection, it is suggested that the presence of free Fe^{2+} in serum (19), lack of complement, functional defects of reticuloendothelial system, or all three (1) are important for the development of sepsis. It is also reported that the organism produces hemolysin (10), cytotoxin (12), and proteases (16), and these toxins are considered to be important virulence factors. Severe hemoconcentration was also observed before the death of mice with lethal infections (2).

Capsule is an important virulence factor of the pathogenic bacteria, such as *Streptococcus pneumoniae* and *Klebsiella pneumoniae*. Its importance was also shown by the studies of *Escherichia coli* mutants with and without capsules (3, 6, 7).

In the course of testing the serum sensitivity of *V. vulnificus* strains, we noticed that the sensitivity was associated with variations in colony opacity of the organism. This variation in opacity was also accompanied by variation of capsular materials. By comparing isogenic strains with and without capsular materials, we found that capsular materials were important for the virulence of *V. vulnificus*.

MATERIALS AND METHODS

Bacterial strains. *V. vulnificus* 371 was isolated from seafood and was donated by Sunao Dohke. Strain 374 was isolated from the blood of a patient with septicemia and was donated by Tadao Tanabe. Strains L-1 and L-180 were isolated from bone marrow fluid and blood, respectively, and donated by Sumio Shinoda. They were identified as *V. vulnificus* by standard clinical microbiological techniques, such as Gram stain, growth on selective media, fermentation patterns of appropriate sugars, and tolerance of NaCl.

Media and culture. For preparation of the liquid medium, brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was supplemented with NaCl to a final concentration of 0.9% (wt/vol). For the solid medium, lactose broth (Eiken Chemical Co. Ltd., Tokyo, Japan) was supplemented with NaCl and agar to final concentrations of 2% (wt/vol) and 1.5% (wt/vol), respectively (LB agar). Bacterial strains were cultured at 37°C overnight in 5 ml of brain heart infusion broth in L-shaped test tubes with shaking in a Monod incubator, and 0.1 ml of the culture was transferred to new brain heart infusion broth and cultured for 3 to 6 h at 37°C before use.

Animals. Female mice of an outbred ddY strain, male mice of an inbred C3H/He strain, and male guinea pigs of an outbred Hartley strain were purchased from a local breeder. Ten-week-old mice and guinea pigs weighing 500 to 700 g were used for experiments.

Capsular staining and electron microscopy. The ruthenium red staining method was used for the observation of bacterial capsules by electron microscopy. Staining and fixation of the bacterial pellets were performed according to the method described by Luft (14). Then, the bacteria were encapsulated in 0.7% (wt/vol) agarose, and the blocks were dehydrated in alcohol. After the alcohol was replaced by propyrene oxide, the blocks were enbedded in Epon 812. The thin slices were stained by uranyl acetate and lead citrate and examined with a JEM (Japan Electron Optics Laboratory, Tokyo, Japan) model 100-CX electron microscope.

LD_{50} for mice. ddY mice were injected intravenously with 0.2 ml of serial 10-fold dilutions of the bacterial suspensions ($\sim 2 \times 10^9$ to 2×10^5 cells), and the total number of deaths were counted. One group consisted of five mice. The 50% lethal dose (LD_{50}) was calculated by the Reed-Muench method.

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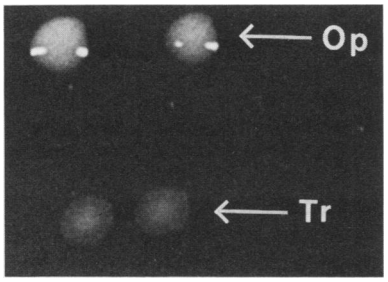

FIG. 1. Opaque (Op) and translucent (Tr) colony types of *V. vulnificus* 371 on a nutrient agar plate.

Susceptibility of serum bactericidal action. Blood was obtained by venipuncture of three normal, healthy, adult volunteers or by cardiac puncture of three guinea pigs and was allowed to clot at room temperature for 1 h. After centrifugation at 1,000 × *g* for 10 min at 4°C, the fresh human or guinea pig serum was removed. Bacteria were washed once with cold Dulbecco phosphate-buffered saline and resuspended in Eagle minimal essential medium (pH 7.4; GIBCO Laboratories). The concentration was adjusted spectrophotometrically to ca. 10^7 to 10^8 CFU/ml. The cell suspension (0.5 ml) was mixed with 0.5 ml of fresh serum in test tubes, and the mixture was incubated at 37°C for 30 min. The number of bacteria in serial 10-fold dilutions of the specimens before mixing and after 30 min of incubation was counted on LB agar after overnight incubation at 37°C.

Bacterial clearance from the bloodstream in mice. Organisms were suspended in phosphate-buffered saline and adjusted to a concentration of 10^6 to 10^7/ml, and 0.2 ml was injected intravenously in C3H/He mice. At intervals after the injection, 0.05 ml of blood was obtained from the retroorbital plexus by capillary tubes. After appropriate serial dilution in phosphate-buffered saline, viable cells in blood were counted after overnight culture on LB agar plates.

Assay for the spread of infection in subcutaneous tissue. To estimate the invasiveness of the organisms, serial 10-fold dilutions of bacterial suspension containing ca. 10^3 to 10^9 CFU/ml each were prepared in phosphate-buffered saline, and 0.1 ml was injected intradermally into the back skin of guinea pigs whose hair had been cut out by a hair clipper. At 1 and 4 h after the injection, the back skin with subcutaneous tissue was scissored off and cut down through the center of the injection sites by disposable trimming blades. The skin sections were stamped on LB agar plates and the spread of the colony formation was measured after overnight culture at 37°C.

Statistics. The statistical significance of the data was determined by analysis of variance. A P value of <0.05 was taken as significant.

TABLE 1. Comparison of the LD_{50}s for mice of isogenic opaque and translucent variants of *V. vulnificus*

Colony type	LD_{50}[a] (log_{10}) for ddY mice of strain:			
	371	374	L-1	L-180
Opaque	6.2	5.7	4.7	7.3
Translucent[b]	8.8	8.3	7.9	8.5

[a] LD_{50}s (CFU) were calculated by the method of Reed-Muench.
[b] By the analysis of variance, the mean of the LD_{50} values for the translucent group are statistically higher ($P < 0.01$) than that for the opaque group.

All experiments were repeated at least two or three times, and representative experiments are presented.

RESULTS

Colony opacity variants. Colonies which varied in opacity were isolated from four parent strains. One opaque variant was isolated among the translucent colonies of strain 374. From strains 371, L-1, and L-180, which consisted of opaque-type colonial variants, translucent variants were isolated (Fig. 1). The frequency of opaque to translucent variation of strains 371 and 374 was 2.65×10^{-4} and 2.26×10^{-4}, respectively, whereas the frequency of translucent to opaque variation of strains 371 and 374 was $<1.89 \times 10^{-4}$ and $<1.26 \times 10^{-4}$, respectively.

Capsular material formation. Bacterial pellets were stained by ruthenium red, and thin sections were observed by electron microscopy. Capsular materials were recognized as an electron-dense layer outside the outer membranes. Morphological features of the capsular materials of the opaque and translucent types of each strain were compared. All opaque-type strains had extracellular materials; the opaque type of strains L-1 and L-180 had continuous thick capsular materials, and the opaque type of strains 371 and 374 had a patchy capsule with a wavelike surface (Fig. 2). The reason for this difference is not known. The translucent type of strains L-1, 371, and 374 had no observable extracellular materials, but the translucent type of strain L-180 had a continuous thin layer of extracellular material, though the layer was thinner than that of opaque-type strain L-180. These capsular materials were not observed under light microscopy in an india ink preparation nor by the Hiss method for staining capsules.

Virulence to mice. LD_{50} values for mice were compared for the opaque and translucent types of four strains (Table 1). We found that the opaque types were more virulent than the corresponding translucent types ($P < 0.01$ by analysis of variance).

Sensitivity to serum bactericidal action. Sensitivities of the strains to fresh human and guinea pig serum were investigated. In Table 2, the numbers of bacteria affected by treatment with serum are summarized. The opaque colonial types of the strains were all resistant to serum bactericidal action, but the translucent colonial types were all sensitive.

TABLE 2. Sensitivities of *V. vulnificus* strains to fresh human and guinea pig serum[a]

Strain	Colony type	Reduction of bacterial no.[b] (log_{10}) after treatment with sera of:	
		Humans	Guinea pigs
371	Opaque	0.65	0.59
	Translucent	5.81	3.73
374	Opaque	−0.31	0.03
	Translucent	4.92	3.03
L-1	Opaque	0.38	−0.08
	Translucent	4.12	3.42
L-180	Opaque	−0.09	0.02
	Translucent	4.90	2.77

[a] The starting inoculum size was ca. 10^7 to 10^8 CFU in each experiment.
[b] Numbers less than 0 mean an increase in the number of bacteria during the treatment with serum.

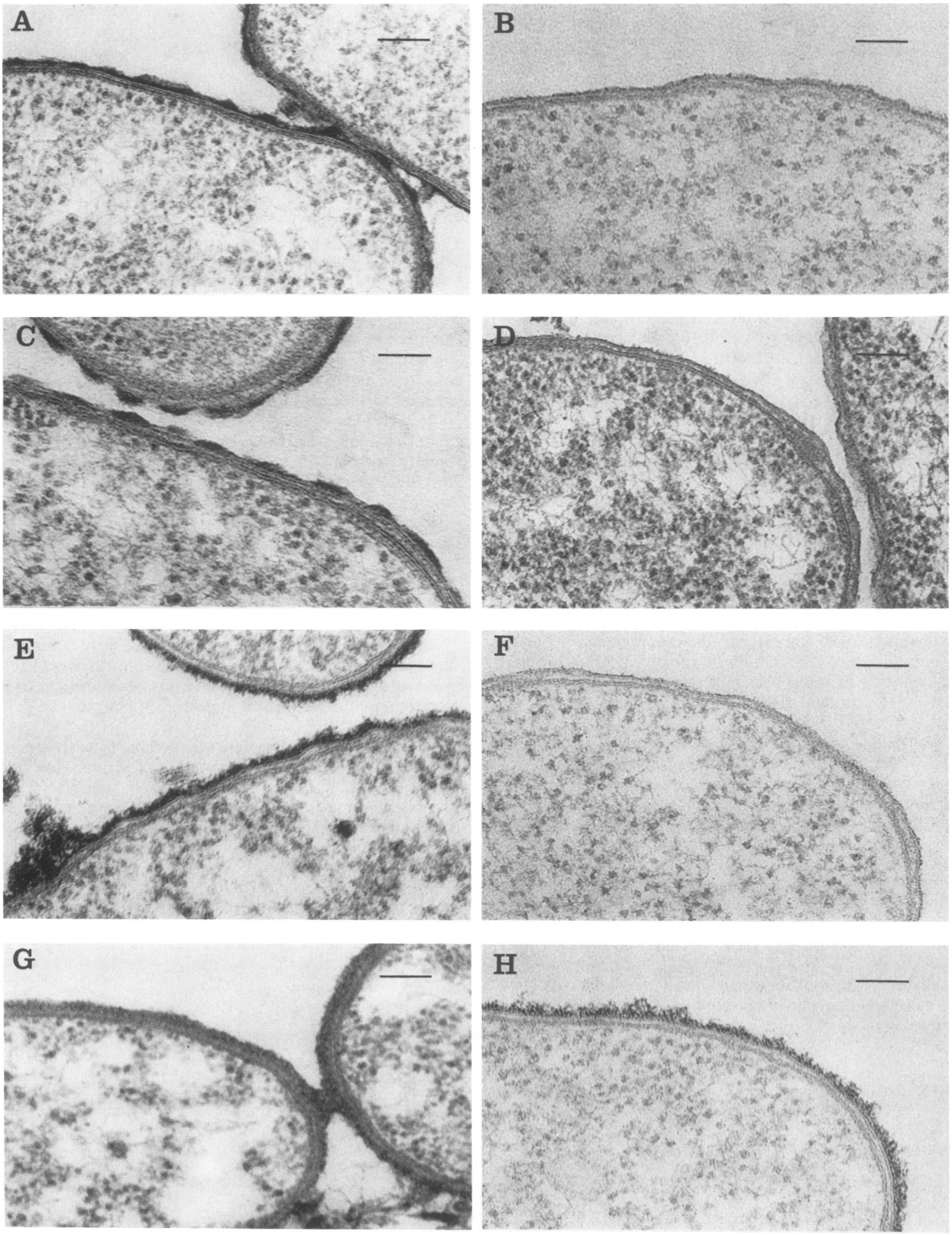

FIG. 2. Electron micrographs of *V. vulnificus* strains stained with ruthenium red. Morphological features of capsular substances of isogenic opaque and translucent types of the strains were compared. Strains: 371 opaque (A), 371 translucent (B), 374 opaque (C), 374 translucent (D), L-1 opaque (E), L-1 translucent (F), L-180 opaque (G), and L-180 translucent (H). Bars, 0.1 μm.

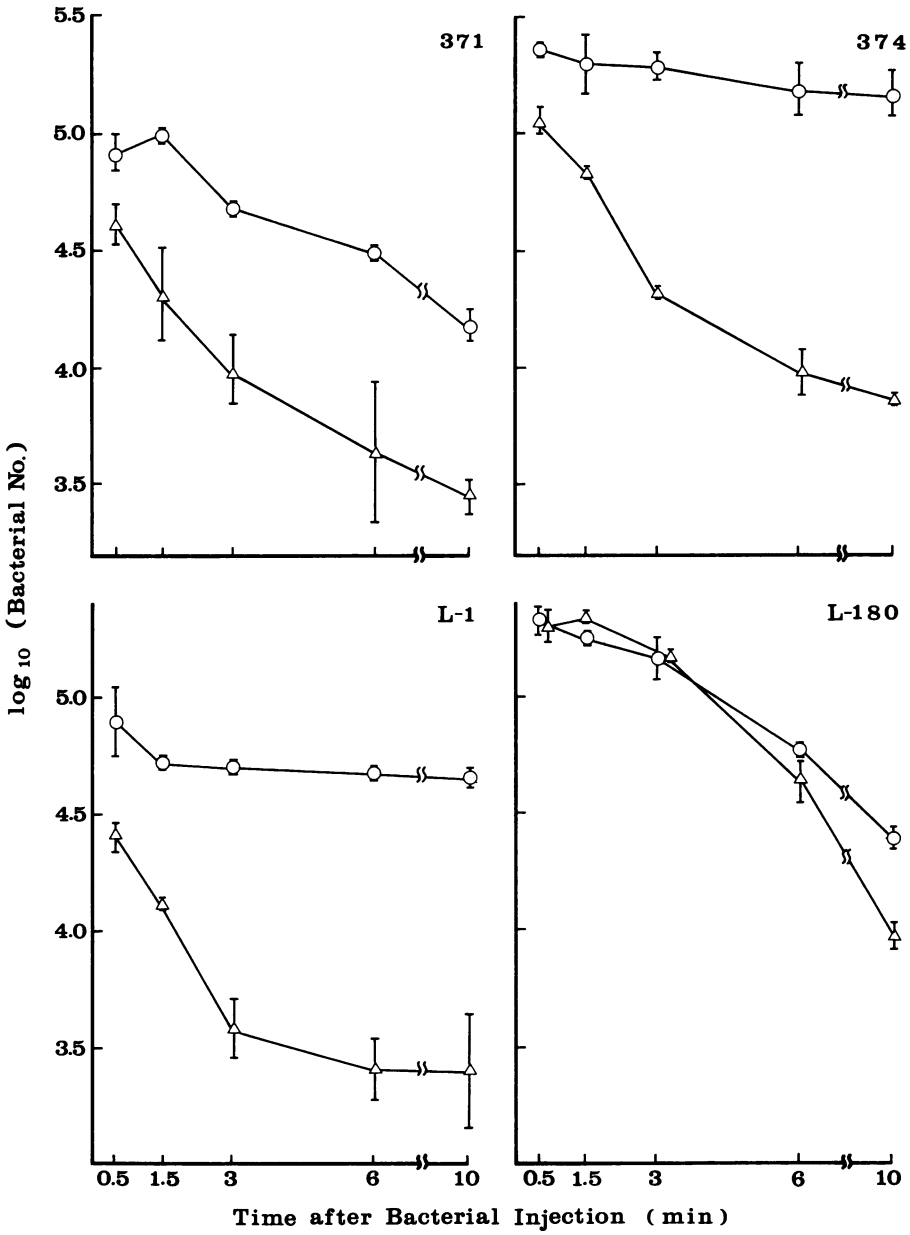

FIG. 3. Time course of bacterial clearance from the blood of mice. After the mice were injected intravenously with bacteria, blood samples were collected from retroorbital plexus, and CFU were counted. Each group consisted two or three male C3H/He mice. Vertical bars represent ranges. Symbols: ○, opaque variants; △, translucent variants.

The difference in sensitivity to serum between opaque and translucent variants was studied by analysis of variance. As a result, opaque variants were shown to be more resistant than translucent strains to both human ($P < 0.01$) and guinea pig ($P < 0.01$) serum.

These bactericidal activities of fresh sera were abrogated by heating at 56°C for 30 min (data not shown).

Bacterial clearance from the bloodstream of mice. Clearance rates of bacteria from the bloodstream were compared for opaque and translucent variants by using C3H/He mice (Fig. 3). Blood samples were obtained 30 s, 90 s, 3 min, 6 min, and 10 min after the bacterial injection. Recovery of opaque variants of strains 374 and L-1 did not diminish appreciably during the first 10 min, whereas recovery of their translucent variants fell 10-fold during the same interval. In contrast, counts of the opaque variant of strain 371 fell by 90% after 10 min, but its translucent variant was eliminated even more rapidly. There was no difference, however, between the clearance rates for opaque and translucent variants of strain L-180 by 6 min.

Spread of infection in subcutaneous tissue. The invasiveness of the organisms in guinea pig subcutaneous tissue was estimated by measuring the colony diameter after stamping the skin sections on LB agar plates. Opaque and translucent variants from the four strains were compared in individual experiments. Results of experiments with strains 371 and 374 are shown in Fig. 4. Opaque types showed stronger invasiveness than did translucent types by 1 h. Moreover, the diameter of bacterial colonies measured 4 h after the inoculation was larger than that measured 1 h after the inoculation

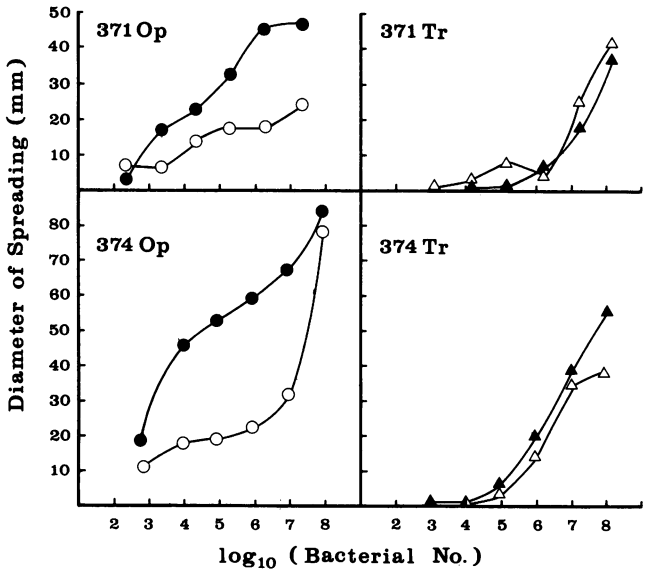

FIG. 4. Comparison of the invasiveness of isogenic opaque and translucent colonial types of two *V. vulnificus* strains. Guinea pig skin sections were stamped on LB agar plates 1 h (○, △) and 4 h (●, ▲) after the intradermal inoculation of bacteria. The diameter of the colonies formed was measured after the overnight culture.

for opaque strains, whereas no difference in invasiveness was found between 1 and 4 h for the translucent variants. Strains L-1 and L-180 gave results similar to those for strain 371.

DISCUSSION

In this study, variation in the colony opacity of four *V. vulnificus* strains was examined in relation to the virulence of the organism. Opaque and translucent variation was accompanied by the variation of capsular material. In three of four strains, translucent variants were morphologically devoid of capsular substance, and in the case of strain L-180, although capsular substance was present, it was decreased in the translucent mutant. We have not analyzed the biochemical nature of the variation between opaque and translucent colonies. However, it seems likely that the capsular material is an acidic mucopolysaccharide, since it reacted with ruthenium red (14). The variation between opaque and translucent colonies of *V. vulnificus* may not be plasmid determined because no plasmids were detected in preliminary study.

Using isogenic capsular variant strains, we analyzed the role of capsular material in the sensitivity of the strains to fresh serum bactericidal action, bacterial clearance from the bloodstream, invasiveness in subcutaneous tissue, and LD_{50} for mice.

Studies to assess the serum sensitivity of colony type variants revealed that all of the opaque variants were more resistant to the killing activity of human and guinea pig sera than were the corresponding translucent strains, suggesting that the capsular substance played an important role in determining the serum resistance of the organism.

The relationship of colony opacity or capsular antigen to killing by serum has been described in other gram-negative bacteria (6, 9). It has also been suggested that serum resistance is an important determinant of virulence in such cases. Tamplin et al. (17) reported that *V. vulnificus* was much more resistant to killing by serum than was *Vibrio cholerae*. This difference may be due to the capsular materials around *V. vulnificus*.

The second characteristics associated with variation in colony opacity was a difference in the rate of clearance of the organisms from the bloodstream of mice. The mechanisms by which the organisms were eliminated from blood have not been yet fully analyzed. However, it appears that the rate of phagocytic activity by the reticuloendothelial system may be one of the most important factors, and serum resistance may also affect the rate of clearance. The opaque type of strains L-1 and 374 did not disappear from the blood by 10 min (Fig. 3). On the other hand, more than 90% of the translucent-type cells were eliminated from the blood by 10 min. There was also a slight difference in the clearance rate between the opaque and translucent types of strain 371. Therefore, it is likely that the opaque variants were more resistant to the phagocytic activity of the reticuloendothelial system than were the translucent variants. Kreger et al. (11) reported that a virulent strain of *V. vulnificus* has antiphagocytic surface antigen. Although they did not mention the structural identity of this surface antigen, it is assumed that it is the capsular substance. In the case of strain L-180, however, the clearance rate was the same for the opaque and translucent types by 6 min, and nearly 90% of the opaque type was eliminated from the bloodstream. In this regard, strain L-180 appears exceptional. The translucent variant of the strain possessed capsular material, although it was thinner than the opaque variant, and the opaque variant of strain L-180 was cleared from bloodstream to nearly the same extent as the translucent variant of other strains.

More direct evidence for the relationship between colonial type and virulence was obtained from the experiments to determine the LD_{50} for mice. The difference in LD_{50} by colony type was ca. 2 to 3 logs in the case of strains 371, 374, and L-1 and ca. 1 log in the case of strain L-180. Although there may be other virulence factors, such as hemolysin (10), cytotoxin (12), or protease (16), our present data suggest strongly the importance of capsular material in the virulence of *V. vulnificus*.

In case of *Shigella flexneri* 2a (4), the translucent type was more virulent than the opaque type, although the physicochemical characterization of the change in opacity has not been clarified (13). In *Neisseria gonorrhoeae*, the translucent colony type was also more virulent to chicken embryos (15), and a difference in the molecular weight of protein I is known to be the cause of the variation in opacity (9). It is interesting to note that in both cases, the translucent variants are more virulent than the opaque variants. However, the situation is reversed in *V. vulnificus*; opaque variants are more virulent than translucent variants.

Invasiveness in subcutaneous tissue may also reflect certain types of virulence. Again, a good correlation was found between invasiveness and colonial variation. To estimate the invasiveness of the organism, we measured viable cells located at the sections of the injected sites. This method, called the "stamp method," is useful to estimate the invasiveness of the bacteria in subcutaneous tissue. It is simple to prepare the specimens with a sharp blade, and the results can be read easily. Although fluorescent microscopy is also a useful technique to determine the spread of bacteria, it requires antibacterial sera and fluorescein-conjugated antibody; moreover, it cannot reliably distinguish between living and dead bacteria. We recommend the stamp method to estimate invasiveness of any bacteria in subcutaneous tissue.

The bacteria in blood and skin can express their virulence only after overcoming first-line host defense mechanisms, such as serum bactericidal activity (18), phagocytosis, and intracellular killing by phagocytes. So lethality for mice and invasiveness in guinea pig subcutaneous tissue are based on virulence factors which overcome these host defense mechanisms.

It has been reported that capsules of marine bacteria are involved in the adhesion of the bacteria to solid surfaces (5). Although adhesion of the strains used here to solid surfaces was not examined, the presence of capsular material may interfere with contact by phagocytic cells.

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