Virulence of a Nutritional Mutant of *Vibrio comma.* (25518)

SAMUEL B. FORMAL, HOWARD BOHNER, HERMAN SCHNEIDER AND LOUIS S. BARON
(Introduced by J. P. Ransom)
Division of Immunology, Walter Reed Army Inst. of Research, Washington, D.C.

It was first shown by Bacon *et al.*(1) that purine requiring mutants of *Salmonella typhosa* are less virulent for mice than their prototrophic parents when injected intraperitoneally. The mutants which they employed were obtained by the technic of Lederberg and Zinder(2) and Davis(3). Using similar procedures, Garber *et al.*(4) reported that a similar situation existed in strains of *Klebsiella pneumoniae.* Subsequently Formal *et al.*(5) found that a naturally occurring mouse-avirulent typhoid mutant which on one occasion had been isolated from the Panama carrier also required purines for growth. The fact that purines are either absent or present in limiting concentration in peritoneal cavity of the mouse offers a logical explanation for the relative avirulence of purine requiring strains when administered intraperitoneally to mice. For some time it has been known that laboratory strains of *Vibrio comma* differ in their capacity to infect mice fatally(6). Our purpose was to determine whether this difference might be attributed, at least in part, to the fact that an avirulent culture has a purine requirement for growth while a more virulent strain does not. In addition, we considered it pertinent to compare ability of strains to infect mice fatally following challenge by intraperitoneal route and guinea pigs after oral administration.

Materials and methods. Two strains of *V. comma,* obtained from the type culture collection of Walter Reed Army Inst. of Research, were employed in our work; both were Inaba types. Strain 20-A-67 requires only glucose and inorganic salts for growth while strain 20-A-47 requires purines in addition to this minimal medium. A mutant of strain 20-A-47 which proved purine independent, was obtained by plating 10^9 cells of this strain on minimal medium and selecting a colony which grew out. This purine-independent variant has been designated 20-A-47 PI. Virulence tests in mice were performed by injecting 0.5 ml amounts of known numbers of organisms suspended in 5% hog gastric mucin into the peritoneal cavity of Bagg strain mice weighing 12 to 16 g. Equal numbers of males and females were used to test each dilution. When the effect of purines in virulence tests was examined, 10 mg xanthine and 3 mg adenine contained in 0.5 ml saline was injected intraperitoneally immediately after the challenge suspension. Animals not receiving purines received an equal volume of saline. Hartley strain guinea pigs were rendered susceptible to a fatal enteric infection following oral challenge by depriving animals of food for 4 days and feeding 125 mg calcium carbonate prior to and injecting 1 ml tincture of opium intraperitoneally following orally administered challenge(7,8). Mucinase tests were carried out using a procedure previously described (9).

Results. Results of virulence tests employing intraperitoneal challenges in mice are summarized in Table I. The LD_{50} of strain 20-A-47 which requires purines for growth is approximately 700 x 10^5 cells; when purines are inoculated into the peritoneal cavity at the same time as the challenge suspension, the LD_{50} is reduced to 4.1 x 10^5. The purine-independent mutant of strain 20-A-47 has an LD_{50} of 2.5 x 10^5 and as can be seen, the simultaneous inoculation of purines does not significantly increase its ability to cause a fatal infection. The LD_{50} of strain 20-A-67, which does not require purines to multiply, is 10x10^5 cells. The difference in the LD_{50} of strain 20-A-67 and the purine-independent mutant of strain 20-A-47 may be due in part to experimental error and perhaps to the fact that the wild-type culture of 20-A-67 employed, might have contained a fair proportion of cells requiring purines or similar substances for growth, thus decreasing the overall mouse virulence of the strain. On the other hand, the purine-independent mutant of strain 20-A-47 had been isolated from a mini-

TABLE I. Effect of Inoculation of Purine and Reversion to Purine Independence on Mouse Virulence of *Vibrio comma* Strain 20-A-47.

Challenge	Requires purines	No. of organisms inj.* 5×10^8	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	LD_{50}	S.E.
20-A-47	Yes	33/40	18/40	1/40	0/40	0/40		700×10^5	270×10^5
20-A-47 + purines†	"		37/40	36/40	22/40	8/40		4.1 "	1.5 "
20-A-47 PI	No		32/40	26/40	23/40	11/40	4/40	2.5 "	.9 "
20-A-47 PI + purine†	"		39/40	33/40	30/40	20/40	6/40	1 "	.4 "
20-A-67 (purine independent)	"		30/40	27/40	20/40	9/40	5/40	10 "	45×10^4

* Organisms suspended in 5% hog gastric mucin.
† 10 mg xanthine and 3 mg adenine administered intraper. at time of challenge.

mal glucose plate and its population was perhaps more homogenous. We have had occasion over a period of a year to perform experiments similar to those summarized here and have noted that the actual LD_{50} values could vary considerably—perhaps as much as a thousand fold—but the values relative to each other were always approximately the same.

Table II gives a summary of results of experiments comparing ability of these strains to infect Hartley strain guinea pigs by the oral route. A single dose level of approximately $3x10^7$ cells was employed, since it was known from experience that a dose of this magnitude would fatally infect a large percentage of the animals when strain 20-A-67 is used as a challenge. The purine-dependent, mouse-avirulent 20-A-47 strain caused a 33.9% mortality while the purine-independent, mouse-virulent 20-A-47 PI strain produced a 30% mortality. On the other hand, 68.6% of the animals challenged with the wild-type purine-independent mouse-virulent strain 20-A-67 succumbed.

We have not been able to detect any other significant biological differences among strains 20-A-67, 20-A-47, and the purine-independent mutant of strain 20-A-47. They are similar antigenically and all have the capacity to produce mucinase in brain-heart infusion broth shake cultures; whether they have the ability to produce the enzyme *in vivo* is not known. When tested for their ability to produce mucinolytic enzymes in nutrient broth shake culture, however, mucinase was only detectable in the 20-A-67 supernatant. While this observation may be significant, additional work is necessary to establish its importance.

Discussion. The data presented demonstrate that the nutritional requirements of a culture of *Vibrio comma* may influence its ability to cause a fatal infection in mice following intraperitoneal inoculation. Thus, in this respect the requirement for purines by cholera strains affects their virulence for mice just as it influences the mouse virulence of strains of *Salmonella typhosa* and *Klebsiella pneumoniae*.

The results also indicate that the capacity of cholera cultures to infect mice fatally by the intraperitoneal route and guinea pigs by the oral route need not necessarily go hand in hand. It is likely that the organism must possess attributes additional to those needed

TABLE II. Deaths in Starved Hartley Strain Guinea Pigs Following Oral Administration of a Purine-Requiring Strain of *Vibrio comma* and a Purine-Independent Mutant Derived from It.*

Strain	Requires purines	Virulent for mice†	Deaths‡ Total	% mortality	95% confidence interval, % Upper	% Lower
20-A-47	Yes	No	21/62	33.9	22.1	45.7
" PI	No	Yes	18/60	30	18.4	41.0
20-A-67	"	"	35/51	68.6	55.9	81.4

* Data pooled from 5 exp. † See Table I. ‡ Animals starved 4 days and given calcium carbonate *per os* before and opium intraper. following oral challenge with approximately 3×10^7 cells.

to infect mice in order to bring about a fatal infection in guinea pigs. Assuming—and we agree that it is a large assumption—that the pathogenesis of the infection as it occurs in guinea pigs more closely resembles the human than does the mouse infection, it would necessarily follow that the mouse test cannot measure all of those factors which may be of potential importance in establishing the disease in humans. If these factors happen to be antigens, their presence or absence might not be detected or evaluated in a mouse protection test. Yet it is this type of test which is now generally used to measure the potency of cholera vaccines. Thus, in this regard, the implications of the present work are obvious.

Summary. Mouse virulence of a purine-requiring strain of *Vibrio comma* is significantly increased if purines are injected at the same time as the challenge suspension. A purine-independent mutant isolated from this strain was significantly more virulent for mice than the parent culture. A concomitant increase in ability to fatally infect guinea pigs by the oral route was not noted in the mutant strain.

1. Bacon, G. A., Burrows, T. W., Yates, M., *Brit. J. Exp. Pathol.*, 1950, v31, 714.

2. Lederberg, J., Zinder, N. J., *J. Am. Chem. Soc.*, 1948, v70, 4267.

3. Davis, B. D., *ibid.*, 1948, v70, 4267.

4. Garber, E. D., Hackett, A. J., Franklin, R., *Proc. Nat. Acad. Sci. U. S.*, 1952, v38, 693.

5. Formal, S. B., Baron, L. S., Spilman, W., *J. Bact.*, 1954, v68, 117.

6. Griffitts, J. J., *Pub. Health Rep.*, 1942, v57, 707.

7. Freter, R., *J. Infect. Dis.*, 1955, v97, 57.

8. Formal, S. B., Dammin, G. J., LaBrec, E. H., Schneider, H., *J. Bact.*, 1958, v75, 604.

9. Formal, S. B., Lowenthal, J. P., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 10.

Received November 20, 1959. P.S.E.B.M., 1960, v103.

TAMe Esterase Activity of Blood Thrombokinase after Repeated Electrophoretic Fractionations.* (25519)

J. H. MILSTONE
(With technical assistance of Nadia Oulianoff, Harriet F. Brown and Lawrence C. Taylor)
Dept. of Pathology, Yale University School of Medicine, New Haven, Conn.

In recent studies thrombokinase was prepared from bovine plasma(1) and purified further by continuous electrophoresis which separated most of the contaminating thrombin. TAMe esterase appeared in 2 peaks, corresponding respectively to thrombin and thrombokinase peaks(2). Material from the thrombokinase peak has now been subjected to repeated electrophoretic fractionations; and there has continued to be a close correspondence between esterase and kinase activities.

Methods and materials. Continuous paper electrophoresis was performed in a refrigerated Spinco Model CP cell. Buffer: veronal, pH 8.6; ionic strength 0.02. Current, 50 ma. Protein was estimated by the method of Lowry *et al.*(3), with crystallized bovine albumin as standard; and values are subject to limitations noted by them. Thrombokinase was assayed by its capacity to activate prothrombin in presence of cephalin, calcium and bovine barium carbonate serum(1). Activity was expressed in terms of a working standard which has been stored at −23°C and used at intervals for 6 years. A value of 10 indicated that the fraction showed 10 times as much activity/ml as did the standard kinase solution. Esterase activity on tosylarginine methyl ester (TAMe) was determined by the method of Sherry and Troll(4). Thrombin was estimated as described(1), with a dry sample of NIH thrombin as the ultimate standard. Beginning with thrombokinase obtained in a yield of 1.2 mg/liter of plasma, a series of

* This investigation supported by Research Grant from Nat. Heart Inst., P.H.S.