THE VIRULENCE-ENHANCING EFFECT OF IRON ON NON-PIGMENTED MUTANTS OF VIRULENT STRAINS OF *PASTEURELLA PESTIS*

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MANY strains of *Pasteurella pestis* form pigmented colonies by absorption of haemin when grown on a suitable synthetic medium, as described in the foregoing paper. Non-pigmented secondary colonies subsequently appear, from which non-pigmented mutant strains can be cultivated. Non-pigmented mutants of virulent strains have reduced virulence for mice (corresponding mutants derived from avirulent strains are avirulent).

This paper deals with the peculiar virulence patterns of non-pigmented strains and the specific effect of iron compounds on them.

MATERIALS AND METHODS

Strains and cultures

As described in the foregoing paper.

Virulence tests

White mice (Porton strain) weighing 18–22 g. were used throughout.

Bacteria for injection were grown on tryptic digest meat agar slopes for 17 hr. at 28°, and then rotated for 3 hr. in tryptic digest meat broth at 37° (Burrows, 1955). They were then sedimented by centrifugation and re-suspended in phosphate buffer, pH 7·4.

The effect of additive substances was tested by dispersing them in arachis oil, using mechanical agitation with beads to produce an even suspension of small particles, and injecting 0·1 ml. of this suspension with 0·1 ml. of bacterial suspension.

Animals were observed for 14 days.

RESULTS

The virulence of strain M7

Strain M7 was derived from a non-pigmented (NP) sector developing on a pigmented (P) colony of the fully virulent strain M3. In contrast to its parent, M7 was not consistently lethal to mice injected with doses over the range 10^1–10^7 organisms. Such doses resulted in the death of approximately half the injected animals, and there was no proportionality between numbers of deaths and numbers of injected organisms. Deaths from M7 infection usually occurred later than from infection with M3. The results of a number of virulence titrations of strains M3 and M7 are summarised in Table I which illustrates the different virulence patterns of the two strains.

There was no enhancement of virulence of M7 after mouse passage, and all organisms recovered from mice dead from M7 infection were non-pigmented ; back mutation from NP to P was not observed either *in vivo* or *in vitro*.

TABLE I.—*Virulence of Strains M3 and M7 of* Pasteurella pestis

Dose (number of organisms).	Deaths per 10 mice. M3.	M7.
10^1	6	0 to 1
10^2	10	4
10^3	10	4 to 5
10^4	10	3 to 4
10^5	10	0 to 6
10^6	10	3 to 7
10^7	10	7 to 9
10^8	10	9 to 10

The range of the numbers of deaths in different experiments with M7 is given in the third column. In each experiment 10 mice received the stated dose.

In smears made from the organs of dead mice, a careful search was necessary to discover any organisms in M7 infection, in contrast with the teeming numbers immediately obvious with M3. A numerical assessment of this difference was made by taking viable counts of organisms recovered from blood, peritoneal and thoracic rinsings, and organs disintegrated by grinding with sand, after dissection of mice immediately after death. With M3, the bacterial population was always of the order of 10^{10} organisms, whereas with M7 the number was most frequently of the order of 10^6 and was never observed to exceed 10^8 organisms per mouse.

The effect on infections with strain M7 of nutrients essential for growth in vitro.

The low population of M7 *in vivo* suggested the possibility of nutritional limitation. Accordingly, substances found necessary for growth of *P. pestis in vitro* at 37° were tested for their effects on M7 infection.

The effect of doses of 4 mg. of each additive (suspended in arachis oil to retard elimination) was tested with a dose of 10^5 M7 bacteria; the results are compiled in Table II.

It was clear that none of the substances tested except haemin affected the lethality of M7. With haemin present, however, 9 or 10 mice per batch of 10

TABLE II.—*Effect of Substances Required* in vitro *when Injected into Mice with M7 Organisms*

Substances.	Deaths per 10 mice.
None	1 to 6
Glucose alone	5
Galactose alone	5
DL-Phenylalanine, L-Cystine, DL-Methionine, DL-Valine, DL-Isoleucine, Glycine, L-Arginine HCl (together)	2
Haemin alone	9 to 10

Each substance, whether injected alone or with other substances, was tested at a dose of 4 mg. Single or combined doses, suspended in 0·1 ml. of arachis oil, were injected intraperitoneally with a dose of 10^5 M7 organisms, suspended in 0·1 ml. of buffer.

were killed. Haemin at a level 8 times that used in these experiments was apparently non-toxic to mice in the absence of infection.

Further experiments showed that, under the same conditions, ferrous sulphate, in terms of iron content, was more active than haemin in promoting the lethality of M7 (Table III). Since 4 mg. of $FeSO_4.7H_2O$ was toxic (killing 3 out of 10 mice), the comparisons in Table III were made with smaller doses, which had no apparent effect on uninfected animals.

TABLE III.—*Comparison of Virulence-enhancing Effects of Haemin and Ferrous Sulphate for M7*

	Haemin.		$FeSO_4.7H_2O$.	
Dose (mg.).	Iron content of dose (μg.).	Deaths per 10 mice.	Iron content of dose (μg.).	Deaths per 10 mice.
4·0	340	10	—	—
0·4	34	0	80	10
0·2	17	3	40	10
0·1	9	1	20	10
0·0	0	0	0	0

Dose of M7 organisms = $6·5 \times 10^4$. The doses of haemin and ferrous sulphate used had no apparent effect on normal animals.

It was evident from microscopic examination of smears from the peritoneal exudate and spleen of dead mice that the number of M7 bacteria was enormously increased in the presence of haemin or ferrous sulphate, the numbers seen being then comparable with those observed in M3 infections. This was confirmed by making viable counts of organisms recovered from mice at death. The pattern of virulence also resembled that of M3 in the earlier occurrence of deaths. The enhancement of the peritoneal population of M7 in the presence of ferrous sulphate could be clearly demonstrated in mice sacrificed 40 hr. after infection, as shown in Table IV. At this stage, no deaths were imminent with the ordinary M7 mice,

TABLE IV.—*Effect of Ferrous Sulphate on the Growth of M7 or M3 Organisms in the Mouse Peritoneum*

	Peritoneal population 40 hr. after infection (millions).	
Strain.	Without $FeSO_4.7H_2O$.	With $FeSO_4.7H_2O$.
M7	$7·5 \times 10^{-2}$	643
	$6·1 \times 10^{-3}$	257
	$1·3 \times 10^{-2}$	196
M3	2,675	1,130
	7,050	1,340
	3,700	2,160

The dose of $FeSO_4.7H_2O$ was 0·4 mg., suspended in 0·1 ml. of arachis oil. In the parallel tests without $FeSO_4.7H_2O$, 0·1 ml. of arachis oil was injected. Doses of organisms—M3, $6·0 \times 10^4$; M7, $6·5 \times 10^4$. Three mice of each batch were sacrificed after 40 hr. The peritoneal cavity was rinsed with 1 ml. of citrated buffer, and serial dilutions of this suspension were plated out and counted.

but those which had received iron in addition were all moribund, as were all the M3 mice (the population of M3 did not appear to be influenced by ferrous sulphate).

The results compiled in Table IV suggest that the activity of ferrous sulphate is due to enhancement of the growth of M7 *in vivo* to a level approaching that reached by M3.

Ferric chloride was also active, but salts of other heavy metals, close to iron in the periodic table, were without effect on the virulence of M7 (Table V). The activity appeared to be specific to iron compounds.

TABLE V.—*Effects of Salts of Related Heavy Metals on the Lethality of M7 for Mice*

Salt of heavy metal.	Deaths per 10 mice.
None	3
$MnSO_4.4H_2O$	4
$CoSO_4.5H_2O$	1
$NiCl_2.6H_2O$	2
$CuSO_4.5H_2O$	4
$ZnSO_4.7H_2O$	4
$FeSO_4.7H_2O$	10

Dose of M7 organisms, $1 \cdot 3 \times 10^4$. Dose of heavy metal salt, 0·4 mg. (without apparent effect on uninfected animals).

The virulence of non-pigmented mutants of other strains, and the effects of iron thereon

As previously described (Jackson and Burrows, 1956) the majority of the strains of *P. pestis* tested, after prolonged storage, yielded mixtures of P and NP colonies on pigmentation medium. The NP organisms were presumed to have arisen by mutation from P. Preliminary tests of virulence, using cultures grown from these colonies, suggested that all NP mutants of virulent P strains resembled M7 in having reduced virulence, restored in the presence of iron.

In order to substantiate this result with authentic NP mutants, cultures were prepared from pigmented colonies, and from non-pigmented secondary outgrowths of the same colonies. Mice were infected with these strains in the presence or absence of 0·4 mg. of $FeSO_4.7H_2O$. With avirulent strains and their mutants a dose of 10^7 organisms was used; this was reduced to 10^3 organisms for virulent strains and their mutants. The important vaccine strain, EV76 (Girard and Robic, 1936) was included and also the strain Java. These strains appear to be pure NP. The results are summarised in Table VI.

Notable features of these results are a high correlation between loss of ability to pigment and reduction of virulence in mutants derived from virulent strains: that ferrous sulphate restores the full virulence of such mutants, but does not enhance the virulence of avirulent pigmented strains or of NP mutants derived from them, and that ferrous sulphate renders EV76 fully virulent.

In the case of NP mutants of virulent strains, ferrous sulphate, in addition to restoring full lethality, increased the bacterial population at death, as judged by microscopic examination of smears. The augmented population appeared to resemble that typical of virulent plague infection in all cases except Yokohama NP (*vide infra*).

The exceptional behaviour of L37LP (low pigmentation, but readily distinguished from NP) is discussed below.

TABLE VI.—*Effect of Ferrous Sulphate on the Lethality for Mice of Pigmented Strains and their Non-pigmented Mutants*

	Strain.	Dose.	Deaths per 10 mice. Pigmented strain. Without iron.	Pigmented strain. With iron.	Non-pigmented mutant. Without iron.	Non-pigmented mutant. With iron.
Virulent strains	M3	10^3	10	10	4*	10*
	F9581	10^3	10	10	1	10
	I–72	10^3	10	10	0	10
	139L	10^3	10	10	3	10
	New Mexico 499559	10^3	10	10	1	10
	Shasta	10^3	10	10	2	10
	WCDCPp5A2258	10^3	10	10	2	10
	WCDCPp6	10^3	10	10	0	10
	WCDCPp54	10^3	10	10	0	10
	Yokohama	10^3	10	10	0	10†
	L37	10^3	10	10	5	10
	L37	10^3	10	10	10‡	10
Avirulent strains	A1122	10^7	0	0	0	0
	14	10^7	0	1	1	0
	Soemedang	10^7	0	1	0	0
	Tjiwidej (TS)	10^7	0	0	0	0
Non-pigmented strains	EV76§	10^7	—	—	10	10
		10^3	—	—	0	10
	Java	10^7	—	—	0	1

* This strain is M7.

† Yokohama NP in the presence of iron does not reach such high populations *in vivo* as do other NP strains (see text).

‡ This strain is L37LP (see text).

§ Similar results were obtained with either uracil-dependent or uracil-independent strains (Burrows and Bacon, 1956*b*).

The effect of iron was tested by using a dose of 0·4 mg. $FeSO_4.7H_2O$ in 0·1 ml. of arachis oil.

DISCUSSION

For full virulence it has been established that a strain of *P. pestis* must have all the following capabilities: (1) To elaborate "envelope" antigen (Schütze, 1932), (2) to be highly toxigenic (Englesberg, Chen, Levy, Foster and Meyer, 1954), (3) to develop resistance to phagocytosis in the absence of visible capsulation (Burrows and Bacon, 1956*a*), (4) to synthesise antigens V and W (Burrows and Bacon, 1956*b*), (5) to synthesise purines (Burrows and Bacon, 1954). To this list may be added the ability to produce pigmented colonies on a defined medium containing haemin. This ability appears to reflect a mechanism enabling the fully virulent strain to derive adequate supplies of iron to permit the development of large populations *in vivo*.

It may be tentatively suggested that P strains are able to derive iron from some compound, present in the mouse, which cannot be metabolised by NP strains. The connection between this strain difference and the difference in haemin absorption on a special medium at 28° is at present not understood. We presume that the amount of iron available to M7 in normal mice is always small, but somewhat variable. In some mice, it may be just sufficient to permit

multiplication of the organisms to a lethal level, while in others this level (which is probably much less than the enormous number ultimately reached with highly virulent strains) may be unattainable. This factor, and not the size of the dose, would determine the incidence of deaths, thus affording an explanation of the absence of proportionality between dose and lethal effect with M7. The injection of iron compounds would permit the bacterial population always to exceed the lethal level.

It has proved difficult to make decisive comparisons of iron metabolism between P and NP strains. Experiments with M3 and M7 as representative strains suggest that their growth requirements for iron *in vitro* are quantitatively similar.

The growth of M3 and M7 at 37° in serum from normal mice has been compared, but without significant results, since the bacterial population of both seems to be determined largely by the extent of haemolysis occurring during collection of the serum. Although serum which appeared visually to be free from haemolysis could be collected by very careful methods, no specimen gave a negative benzidine test (King, 1947). Work on this problem is continuing.

A conspicuous anomaly is the behaviour of the mutant L37LP, which, although fully virulent, cannot be assigned to the P group. Further exploration has not, so far, revealed any other strain resembling L37LP.

The appearance of all NP strains is similar after five days' incubation at 28° on pigmentation medium, but further incubation leads in most cases to slight absorption of haemin. This occurs to a different extent with different strains, so that after 10 days most NP strains can be distinguished from each other. L37LP absorbs haemin more strongly than L37NP or any of the others; at the other end of the scale, Yokohama NP appears to be quite incapable of haemin absorption.

In apparent correlation with these observations, L37LP is evidently not limited by iron deficiency *in vivo*, the population reached without added iron being similar to that with typical virulent strains, while on the other hand, with added iron Yokohama NP does not reach such a high population *in vivo* as other NP strains. (The population reached is sufficient to account for the consistent lethality of Yokohama NP in the presence of iron.)

It is possible that the strain of reduced virulence B741–10–1, isolated from an old laboratory culture by Chen and Meyer (1955) may correspond to the NP type described here.

Attention is drawn to the similarity of the virulence-enhancing effects of iron and of cortisone (Payne *et al.*, 1955) on strain EV76. Both greatly enhance the virulence of this strain, but neither has any apparent effect on the lethality of the avirulent strains TS and A1122.

SUMMARY

M3, a highly virulent strain of *P. pestis*, forms dark brown colonies by absorption of haemin from a suitable medium. M7, a non-pigmented mutant strain cultivated from a non-pigmented secondary colony of M3, has a peculiar pattern of reduced virulence for mice, associated with failure to proliferate freely *in vivo*.

The lethality of M7, and its extent of growth *in vivo*, are augmented to those of M3 if sub-lethal amounts of iron compounds are injected with the organisms. These effects are specific to iron compounds.

In general, non-pigmented mutants of virulent pigmented strains behave like M7 *in vivo*. Non-pigmented mutants of avirulent pigmented strains remain avirulent, even in the presence of iron compounds.

It is postulated that M7 is unable to metabolise some iron compound, present in the mouse, which is utilised by M3. The connection between ability to form pigmented colonies *in vitro* and superior iron metabolism *in vivo* is not fully understood.

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