then started and continued for about 12 days. The stools were collected in periods of 3 days and lumped together for extraction of the iron and measurement of the activity. Finally, after the last 3-day period, the patient was dewormed and quantitative worm counts made on the stools for the next 5 days. Where possible, ova concentration methods were employed after the fifth day to check the efficiency of deworming.

The radioactivity of the blood was determined with an end-window Geiger counter after the sample had been ashed and electroplated on to copper disks, following essentially the method described by Vosburgh, Flexner, and Cowie (*6*). The stools were dried with concentrated HNO_3 on a water bath, ashed, extracted with HCl, and the activity of the liquid measured with a Veall type (*7*) liquid counter. A reference solution of Fe^{59} was also made from each batch used for intravenous injection. Then if

Cs = counts/min in the liquid counter of the stool ash extract,
Cb = average counts/min ml blood over the period during which the stools were collected,
Se = counts/min ml of the reference solution electroplated,
Sf = counts/min in the liquid counter of some of the same reference solution,
Vs = volume of stool ash extract in ml,

the blood loss is given by

$$(Cs \times Se \times Vs)/(Cb \times Sf).$$

Our results, as summarized in Table 1, appear to support Wells' conception of a considerable variation in the blood loss per worm. The daily loss of 10–20 ml (i.e., 5–10 mg iron) would seem to account for the hemoglobin values obtained. They are definitely low for Angola Africans, who have a high iron intake (*8*). The observed hookworm loads combined with a normal iron intake should, therefore, produce a marked anemia.

We feel for the present unable to explain the high blood loss of patient A, harboring such a small worm load, unless there are one or two profusely bleeding injuries left by the worms. To what degree blood loss is due to these bleeding injuries, or to the consumption of blood by the worm, remains to be determined in future experiments.

TABLE 1. Hemoglobin values; blood loss during subsequent 3-day periods; average blood loss per day; number of worms recovered from the stools after deworming; and blood loss per worm per day for the 3 patients examined, and one control.

Patient	Hb (g %)	Blood loss (ml)					Hookworms	Blood loss per worm per day (ml)
		1st period	2nd period	3rd period	4th period	Av/day		
M	10.7	33.5	26.8	25.1	28.7	9.5	368	0.026
A	13.1	48.6	30.8	47.9	—	14.1	63	.22
C	13.1	71.2	60.0	42.8	51.6	18.8	354	.053
Control	—	2.3	2.3	2.0	—	0.7	—	—

On the basis of our results, it is not necessary to argue that the anemia is caused by the effect of toxins (*9*). However, our results have been obtained on patients with light loads in the age group from 18 to 30 yr, and various factors such as adaptation and immunity might counteract the full effect of hookworm toxins and so affect our conclusions. There is, therefore, a need for further studies on younger patients and those with heavier hookworm loads.

References

1. LEICHTENSTERN, O. *Deut. med. Wochschr.* **12**, 173 (1886).
2. WELLS, H. S. *J. Parasitol* **17**, 167 (1931).
3. WHIPPLE, G. H. *J. Exptl. Med.* **11**, 331 (1909).
4. LANE, C. *Trop. Diseases Bull.* **34**, 1 (1937).
5. HAHN, P. F., and OFFUTT, E. P. *Science* **110**, 711 (1949).
6. VOSBURGH, G. J., FLEXNER, L. B., and COWIE, D. B. *J. Biol. Chem.* **175**, 391 (1948).
7. VEALL, N. *Brit. J. Radiol.* **21**, 347 (1948).
8. GERRITSEN, TH., and WALKER, A. R. P. *S. African Med. J.* **27**, 577 (1953).
9. MANSON-BAHR, P. *Manson's Tropical Diseases*, Baltimore: Williams & Wilkins; London and Toronto: Cassell and Co., 1945, p. 764.

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Virulence in *Pasteurella pestis*[1]

E. Englesberg, T. H. Chen, J. B. Levy, L. E. Foster, and K. F. Meyer

George Williams Hooper Foundation, University of California, San Francisco

Studies on the nature of *Pasteurella pestis* infection have suggested that at least two factors contribute to the virulence of this bacterium: (*a*) the envelope substance (Fraction I) produced by this organism (*1–3*), which has been shown to protect it from phagocytosis (*4*) and probably blocks the action of antibodies on the cell (*4*), by virtue of the freely soluble nature of this substance (*3*); and (*b*) toxin production (*1, 2, 5, 6*), which no doubt is responsible for death from this disease (*4*). Since avirulent *P. pestis* have been found which produce both envelope and toxin, the possibility was suggested that virulence in this organism may be based upon the quantity of these substances produced. In order to investigate this possibility, a comparison of several virulent and avirulent strains was undertaken.

In initial experiments, various virulent and avirulent strains were tested for their ability to produce toxin and envelope in a casein hydrolysate mineral glucose medium at 37° C (*3*). A few virulent and avirulent strains, however, did not grow in this medium at this temperature. In the case of those strains that did grow, cell yields were so diverse that accurate estimates of toxin and envelope production were impos-

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TABLE 1. Comparison of the quantity of toxin and envelope antigen (Fraction I) produced by several virulent and avirulent strains of *P. pestis*.

Strains	Envelope antigen (μg/mg dry wt.)	Toxicity (No. LD_{50}/mg dry wt.)
Virulent		
B741	150	29
195/P	140	20
New Mexico	140	32
Yreka	138	45
Shasta	137	42
Alexander	127	23
Avirulent		
B1456#4	121	25
A1122	110	9
B868#8	104	22
B2764#6	94	23
E.V.76	62	33
F7793#10	52	13
14	30	30
TRU	25	0
Harbin	10	0

sible. To avoid these difficulties, the various strains were subsequently cultured on hormone agar (*7*), which produced excellent growth of all strains tested.

The following procedure was employed. Strains were cultured on hormone agar for 72 hr at 37° C. The cells were washed off with saline and precipitated with 2 vol of acetone at −70° C. The cells were then collected by centrifugation and dried by several washings with acetone. The residual acetone was removed by placing the cells in a desiccator over concentrated H_2SO_4 and evacuating. Weighed samples of the various dried powders were extracted with saline, the cells were spun down, and precipitin tests using anti-Fraction I serum and intravenous mouse toxicity tests were performed on the supernatant fluid (*3*).

The virulence of the strains employed was tested by subcutaneous injection in mice. All strains that killed 50 percent of the mice with less than 300 organisms were termed virulent, whereas those that failed to kill when 1×10^4 organisms were administered were considered avirulent.

The following virulent strains were tested: Yreka, B741, Alexander, Shasta, and New Mexico, all of which were isolated from cases of plague on the West Coast, and strain 195/P, which was obtained from the Haffkine Institute, Bombay. Avirulent strains employed were A1122, E.V.76, B2764#6, B1456#4, B868#8, F7793#10, 14, TRU, and Harbin.

The results (Table 1) demonstrate a positive correlation between virulence and quantity of envelope produced, thus suggesting that envelope production must reach a given level before it is effective in combating the host defenses. Although the virulent strains do produce more toxin on the average, it is clear that high toxicity alone is not sufficient to render an organism virulent. This is shown quite clearly by avirulent strains E.V.76 and 14, both of which produce more toxin than three of the virulent strains. It is interesting to note that, although E.V.76 is able to multiply in mice when inoculated intravenously, producing necrosis in the liver and spleen before being eliminated and causing death as a result of an active plague infection when administered in large doses of about 1×10^5 organisms, strain 14 shows no multiplication and produces no pathological signs (*8*). The difference in behavior of these two organisms *in vivo* may very well be explained by the lower envelope production on the part of strain 14. Similarly, the relative avirulence of E.V.76 may be interpreted on the basis of its comparatively lower envelope production as compared with that of virulent strains. The fact that A1122 produces more envelope substance than E.V.76, yet fails to produce pathological signs in mice (*8*), may be attributed to the relative atoxicity of the former.

The similarity between the avirulent B1456#4 and the virulent Alexander strains with respect to quantity of toxin and envelope substance produced would seem to indicate that, although high levels of envelope and toxin production appear to be essential for virulence, these are not the only factors involved. Since virulence is a measure of the ability of a bacterium to grow and cause disease in a particular host, all aspects of energy metabolism and assimilation, for example, which may conceivably have no direct bearing on toxin and envelope production *per se*, may be involved here. The apparent correlation between virulence in *P. pestis* and catalase production (*9*) should be noted.

Experiments to be reported in detail elsewhere have demonstrated that the purified envelope substance and lyophilized lysates of avirulent strains (*3*) were as effective in immunizing mice as the corresponding antigens isolated from virulent strains. (In the pneumococcus, avirulent strains have been isolated which produce small amounts of capsular material and yet are able to immunize against virulent strains [*10, 11*]). Furthermore, recent studies indicate that *P. pestis* toxin produced from E.V.76 (*5*) is similar in physiological effect to that produced from virulent strains (*12*). These results eliminate the possibility of a "mouse virulence antigen"—that is, one present solely in virulent *P. pestis*—and thus add further weight to the observed quantitative relationship between envelope and toxin production and virulence.

References

1. E. E. Baker, *et al. Proc. Soc. Exptl. Biol. Med.* **64**, 139 (1947).
2. E. E. Baker, *et al. J. Immunol.* **68**, 131 (1952).
3. E. Englesberg and J. B. Levy. *J. Bacteriol.* In press.
4. K. F. Meyer. *J. Immunol.* **64**, 139 (1950).
5. E. Englesberg and J. B. Levy. *J. Bacteriol.* In press.
6. E. Meyer, K. F. Meyer, and L. Pillemer. Unpublished.
7. M. Levine, and H. W. Schoenlein. *Monograph on Systemic Bacteriology.* Vol. II. (The Williams and Wilkins Co., Baltimore, Md., 1930.)
8. D. L. Walker, *et al. J. Immunol.* **70**, 245 (1953).
9. M. Rockenmacher. *Proc. Soc. Exptl. Biol. Med.* **71**, 99 (1949).
10. G. Middlebrook. *Bull. N.Y. Acad. Med.* **26**, 498 (1950).
11. C. M. MacLeod and M. R. Krauss. *J. Exptl. Med.* **86**, 439 (1947).
12. M. Schar and K. F. Meyer. Unpublished.

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