INFECTION AND IMMUNITY, Dec. 1973, p. 876-881

Vol. 8, No. 6
Printed in U.S.A.

Pathogenicity and Immunogenic Efficacy of a Live Attenuated Plague Vaccine in Vervet Monkeys

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Received for publication 18 May 1973

A live attenuated *Yersinia pestis* (*Pasteurella pestis*) vaccine strain designated EV51f, which had been passaged through guinea pigs previously treated with ferrous sulfate, was shown to be pathogenic for African green vervet monkeys (*Cercopithecus aethiops pygerythrus*), but not for guinea pigs. The bacilli multiplied in the monkeys, as shown by positive blood cultures, caused an elevation of white cell counts and rectal temperatures, and resulted in death of 26% (13/50) of animals. Postmortem findings of these animals were typical of bubonic-septicemic plague. This vaccine did not cause deaths in 50 guinea pigs even in doses up to 100 million viable bacilli inoculated subcutaneously. It is suggested that the virulence of an attenuated *Y. pestis* strain which does not produce pigment on a defined medium containing hemin, but possesses all other known virulence determinants, is dependent on the availability of iron in vivo. The serological response of the monkeys as determined by the hemagglutinating and mouse protective antibodies was high one month after vaccination and also in guinea pigs, as shown by virulent challenge. This antibody level declined in monkeys over a period of nearly 6 months, and a decline in immunity was confirmed by virulent challenge which resulted in the death of 30% of vaccinated monkeys. The level of immunity in monkeys did not appear to be related to the dose of vaccine.

In recent years, plague, caused by *Yersinia pestis* (formerly *Pasteurella pestis*), has decreased as a health hazard throughout the world with the exception of a few areas such as Brazil, Bolivia, Congo, and the Republic of Vietnam. Sporadic outbreaks, however, still occur in many other areas. A need for vaccination against plague still exists in protecting laboratory staff, field personnel working in enzootic areas, and certain other high-risk groups in endemic areas under conditions such as those of war existing in Vietnam.

Experimental work on live attenuated plague vaccines using genetically related *Y. pestis* strains established the importance of virulence determinants in immunity to plague in white mice, guinea pigs, and rabbits (3, 4). The strains which were most immunogenic in both mice and guinea pigs were found to have the virulence characters of the two vaccine strains used most widely in man, i.e., EV76 (10) and Tjiwidej (18).

Although much is known about the virulence determinants and protective antigens of *Y. pestis*, it was suggested (2) that there are probably other antigenic components which are important in immunity but have not been identified. Thus, although killed vaccines may possess all the known antigenic components, live vaccines possess the potential of producing antigenic components in vivo which are not formed in vitro. This may account for the higher immunogenicity of most living attenuated *Y. pestis* strains than killed vaccines in experimental animals.

Different attenuated plague strains have different immunizing capacities associated with the method of maintaining the cultures (7, 12). A successful method which was employed to stabilize attenuated vaccines antigenically and to increase the immunogenic capacity has been

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to passage the strain through guinea pigs previously treated with ferrous sulfate (12).

The adoption of a standardized plan to evaluate any plague vaccine first on rodents, and then on nonhuman primates, and finally on human subjects (14) prompted us to investigate further a plague vaccine of the EV strain which had been stabilized antigenically by passage through iron-treated guinea pigs as recommended by Soviet workers. The vaccine, designated EV51F, was lyophilized and made available to us for experimental use in laboratory animals.

This is the first study in which one of the subspecies of *Cercopithecus pygerythrus* of the Aethiops group of the subgenus *Cercopithecus L.* (16) has been used in plague work. (This work was submitted by A. F. Hallett for partial fulfillment of the Ph.D. degree at the University of Witwatersrand, Johannesburg.)

MATERIALS AND METHODS

Animals. African green vervet monkeys were captured wild in South Africa and then conditioned for approximately 3 months in large outdoor cages, which housed about 25 monkeys each. Healthy monkeys in good condition were selected from these and put in separate cages in the laboratory. The cages measured 75 by 77 by 105 cm with a false back which could be drawn forward to pinion the monkeys against the front, to facilitate handling the animals. The diet consisted of brown bread, sweet potatoes, bananas and oranges given twice daily. Water was always available in a trough.

Guinea pigs were noninbred, conventional animals from a colony maintained for various purposes. The animals were fed on standard mouse biscuits daily and water was supplied by a drop bottle.

Vaccine. Details of the vaccine preparation have been previously described (14). Serial dilutions for inoculation were made in normal saline, and total bacterial counts were done in a counting chamber after dilution in 1.5% formal saline. Viable counts were done by inoculation of 0.2 ml of each serially diluted suspension onto each of five blood agar plates and then spreading them with metal spreaders. These were then incubated at 28 C for 72 h before counting the colonies. The vaccine and virulent challenge cultures were checked for purity and confirmed biochemically to be *Y. pestis* after inoculation.

Challenge organisms. The virulent *Y. pestis* strain F357 used for challenge of vaccinated and control animals was isolated in Lesotho on 11 July 1968 from a flea, *Xenopsylla* sp., during a plague epidemic. After isolation, this strain was maintained in deep nutrient agar stab culture.

Vaccination procedure. Fifty African green vervet monkeys weighing 4 to 6 kg were put into individual cages and divided into five groups of 10. Both sexes were fairly evenly distributed. No more than five to six animals were kept in a room, to minimize cross-infection with microorganisms unrelated to *Y. pestis*. To minimize rough handling and frightening of the animals, all handling was done after intramuscular injection of phencyclidine hydrochloride (0.05 ml/kg bodyweight) which resulted in full anesthesia. In this manner all animals were weighed, their rectal temperatures were taken, and they were bled for white cell counts and serological tests.

Each of five vials of the lyophilized EV51f vaccine was reconstituted with 10 ml of sterile normal saline and pooled in a 100-ml Erlenmeyer flask. Tenfold dilutions were made in normal saline, and total counts were then done. Five dilutions were selected for inoculation on the basis of an estimated viable count (30% of total count) and these were injected in 0.5-ml amounts per monkey subcutaneously (s.c.) on the shaved inner thigh of the right hind leg of 10 monkeys for each dilution. At the same time five groups of 10 guinea pigs were inoculated s.c. with the identical doses, and viable (plate) counts were done. The doses of viable bacilli inoculated, determined by the plate counts, varied from 10^2 to 10^8 organisms (Table 1).

The monkeys were inspected daily and examinations were done initially on alternate days to determine reactions at the site of inoculation, size of the inguinal and popliteal lymph nodes, and rectal temperature. Where an animal had a temperature of 40 C or higher, blood was taken for white cell count, serology, and quantitative blood cultures. After 2 weeks, these examinations were reduced to twice weekly. The guinea pigs were only examined for reactions at the site of inoculation and enlarged regional lymph nodes.

Virulent challenge procedure. Accommodation for the virulent challenge of vaccinated monkeys was limited, and therefore all but 10 were sacrificed on day 30 after vaccination. The 10 animals (1 to 3 from each vaccine dosage group) were housed together with 2 unvaccinated controls in one large room. They were then challenged s.c. in the right thigh on day 170 with 8,770 (as determined by viable counts) virulent *Y. pestis* strain F357. Regular examinations were carried out during which rectal temperatures were taken and blood was collected for white cell counts, serology, and quantitative cultures. Seven days before challenge the monkeys were bled for hemagglutination (HA) and passive mouse protection indices (MPI) tests. The 50 guinea pigs were challenged s.c. in the right hind leg with 1.6×10^5 viable *Y. pestis* strain F357 on day 28 after vaccination.

Autopsies, serology, and white cell counts. After vaccination or virulent challenge the test animals were kept under observation daily for at least 14 days. Autopsies were carried out on the animals that died. The macroscopic appearances were noted, and stained smears were examined of the heart blood, liver, and spleen. Lymph nodes, lungs, kidneys, liver, spleen, and heart blood were cultured. Tissues for histological examination were fixed in 10% neutral formal saline and stained with hematoxylin and eosin.

Blood for serology, white cell counts, and quantitative bacterial counts was collected aseptically by venipuncture from the hind leg of *Cercopithecus*. The sera were tested for HA antibodies to the fraction I antigen of *Y. pestis* (8) by use of the microtechnique

(6), and for passive mouse protection antibodies (15). MPI below 10 and particularly below 5 are usually found in convalescent plague patients (13) and susceptible nonhuman primates which resist challenge infection (14). Normal serum indices are usually 15 or greater.

RESULTS

Response of monkeys to the EV51f vaccine. Local lesions at the inoculation sites were not observed, but the inguinal lymph nodes became enlarged and firm within 2 to 3 days and persisted for up to 25 days. Rises in temperature ranging from 0.2 C to 2.7 C were recorded for all animals, and marked increases in white cell counts were recorded in all but one of 16 with temperatures of 40.0 C or higher. By day 28 the white cell counts had returned to normal in most animals. Positive quantitative blood cultures were obtained from day 4 in 13 monkeys, and 5 of these survived (Table 1).

Positive plague HA results were obtained in 13 of 16 monkeys tested 5 to 18 days after vaccination but 3 which had rectal temperatures over 40 C had negative titers. By day 28, all but 2 of 38 surviving monkeys had HA titers between 1:8 and 1:4,096.

Thirteen (26%) of 50 monkeys died within 28 days after vaccination (Table 1). The postmortem appearances were as follows: blood-stained fluid in both pleural cavities in all; gross, nonpurulent congestion of the lungs in all; small amounts of blood-stained pericardial fluid in most. The spleens were somewhat flabby and congested, whereas the livers appeared normal, except 2 which showed a mottled appearance. The spleen was grossly enlarged in one. Widespread adrenal hemorrhage was seen in the majority, whereas the kidneys were pale. Glandular enlargement was mainly restricted to the regional lymph glands. One showed hemorrhage into a large right popliteal gland.

Cultures on blood agar of heart blood, lung, spleen, liver, kidney, and inguinal glands yielded confluent growths of *Y. pestis* from all animals which died.

Histological appearance of the tissues was as follows. Lymph nodes: inflammatory reaction was only slight, but clusters of *Y. pestis* were typical. Spleen: engorgement with red blood cells was distinctive, with a proliferation of monocytes. Some plasma was present in the sinuses with clumps of *Y. pestis* commonly seen. Classical granulomas (1), described in Ethiopian and Kenyan subspecies of *Cercopithecus* which succumbed 15 days or longer after vaccination with EV51f and which were also observed by Meyer (14), were not seen in 2 animals. Liver: marked congestion and foci of polymorphonuclear leukocytes and lymphocytes with clusters of *Y. pestis* were observed. There was desquamation of the epithelium in bile ducts and portal spaces. Microorganisms were seen in all liver sections but not in numbers as large as in the spleen and lymph nodes. Kidneys: congestion of the glomeruli and degeneration of the convoluted tubular epithelium were constantly present. Monocytes containing *Y. pestis* were seen in the capillaries and small numbers of organisms were present in the larger vessels. Lungs: congestion and desquamated cells were present in the alveolar spaces of all sections. The alveolar walls were thickened,

TABLE 1. *Pathological findings in 50 Cercopithecus aethiops, inoculated with graduated doses of attenuated Y. pestis strain EV51f*

Group[a]	Dose of viable bacilli	Mean prevaccination temp (C)	Mean postvaccination temp (C)	Mean prevaccination white cell count	Mean postvaccination white cell count	No. of animals with positive blood cultures/no. of animals tested	No. of deaths	Reciprocal of geometric mean HA titer (GMT) on day 28 after vaccination
I	10^8	38.8	40.2	10,940 (5 animals)	28,780 (5 animals)	1/4	4 (on days 4, 5, 6, 6)	512
II	10^6	38.2	39.9	7,750 (2 animals)	10,350 (2 animals)	1/1	2 (on days 5, 11)	548
III	10^5	38.8	40.0	8,475 (4 animals)	41,400 (4 animals	5/7	4 (on days 6, 7, 11, 22)	445
IV	10^3	38.6	40.0	9,960 (5 animals)	22,020 (5 animals)	4/5	2 (on days 8, 28)	477
V	10^2	38.8	40.0	6,900 (1 animal)	17,000 (1 animal)	2/3	1 (on day 9)	206

[a] Group I: 10 animals numbered 737 to 746. Group II: 10 animals numbered 747 to 756. Group III: 10 animals numbered 757 to 766. Group IV: 10 animals numbered 767 to 776. Group V: 10 animals numbered 777 to 786.

containing masses of organisms and a dense infiltration of polymorphonuclear leukocytes.

Response of guinea pigs to the EV51f vaccine. All the guinea pigs survived vaccination without any abnormalities being observed.

Response of vaccinated and control monkeys to virulent challenge. Three of 10 vaccinated and both unvaccinated control monkeys succumbed to a challenge dose of 8,770 virulent *Y. pestis* 163 days after vaccination (Table 2). The rectal temperatures of the 3 vaccinated monkeys which died, 1 vaccinated survivor, and 1 unvaccinated control were elevated above 40.0 C. The temperatures of the other 6 vaccinated survivors and the other unvaccinated control were not raised. The white cell counts were raised in those which succumbed. One vaccinated animal yielded a positive blood culture before death, the other 2 were negative.

The autopsy appearances of the unvaccinated control animals were typical of plague. Those of the vaccinated animals which died after virulent challenge were similar to the unvaccinated controls, but the pathological changes were not as severe. There were no local lesions at the site of inoculation, and the inguinal glands were only slightly swollen. The livers were slightly enlarged and mottled, and the spleens were flabby but normal in size. The cortices of the kidneys were very pale and, the adrenals were hemorrhagic. Small amounts of blood-stained pleural fluid were observed, and the hearts were dilated.

When the 7 vaccinated survivors of the virulent challenge were sacrificed after 134 days, all the organs were normal except for one with an enlarged spleen.

The HA titers of the 3 animals which succumbed had declined from 1:256 (3 animals) (geometric mean HA titer [GMT] = 1:256) obtained on day 28 after vaccination, to values of 1:32, 1:64, and 1:128 (GMT = 1:64) 135 days later (Table 3). In comparison, the titers of the 7 survivors were negative (1 animal), 1:512 (2 animals), 1:1,024 (2 animals), and 1:4,096 (2 animals) (GMT = 1:477) 28 days after vaccination, and these declined to negative (1 animal), 1:128 (4 animals), and 1:256 (2 animals) (GMT 1:79) 163 days after vaccination (Table 2).

The mouse protection indices were low on day 28 after vaccination, 8 of 10 MPI values being below 10 (Table 3). This is indicative of a high level of immunity. After 163 days, only 2 of 10 values were below 10 and the others varied between 10 and 17. Seven days before the virulent challenge dose the mean MPI of the survivors was 11 and that of those which succumbed to subsequent challenge was 16.

Response of vaccinated guinea pigs to virulent challenge. All guinea pigs vaccinated with 10^5 EV51f organisms or more survived a virulent challenge of 1.6×10^5 F357 *Y. pestis* (Table 4). Three of 10 guinea pigs vaccinated with 10^3 EV51f succumbed to the challenge dose 37 and 39 (2 animals) days after vaccination, but only coliform organisms were isolated from the tissues. Since 1,000 EV51f should protect guinea pigs (14), these late deaths were unexpected. Three of 10 guinea pigs vaccinated with 100 EV51f died on day 15 after challenge, and virulent *Y. pestis* (confirmed in mice) was isolated from the heart blood and spleen from one. The results of the unvaccinated controls are also presented in Table 4. The high rate of

TABLE 2. *Response to virulent challenge of monkeys vaccinated with 8,770 live attenuated Y. pestis strain EV51f*

Vaccine dose	Animal no.	Reciprocal of HA titer 28 days after vaccination	Reciprocal of HA titer 163 days after vaccination (7 days before challenge)	Rectal temp (4 days after challenge) (C)	Day of death after challenge	White cell count	Blood culture (colonies/ml)
10^8	737	256	32	40.0	8	23,000 (day 4)	Negative (day 4)
	740	1,024	128	38.7			
10^6	748	1,024	256	39.2			
	750	256	128	40.9	12	21,900 (day 8)	150 (day 8)
	756	4,096	256	38.8			
10^5	758	512	128	39.2			
10^3	767	256	64	40.2	10	13,500 (day 4)	Negative (day 4)
	769	512	128	39.1			
10^2	783	4,096	128	39.4			
	784	Negative	Negative	40.2			
Nil	Control 1		8	40.5	6	31,300 (day 4)	200 (day 4)
Nil	Control 2		Negative	39.0	7		

TABLE 3. *Mouse protection indices of monkeys vaccinated with live attenuated Y. pestis strain EV51f*

Vaccine dose	Animal no.	MPI 28 days after vaccination	MPI 163 days after vaccination	MPI 134 days after virulent challenge
10^8	737	3	17	Succumbed
10^8	740		17	9
10^6	748	6	5	Not done
10^6	750	3	17	Succumbed
10^6	756	1	5	6
10^5	758	1	13	5
10^3	767	3	15	Succumbed
10^3	769	2	14	1
10^2	783	3	10	00
10^2	784	11	14	2

TABLE 4. *Resistance to virulent Y. pestis of guinea pigs after S.C. inoculation of live attenuated Y. pestis strain EV51f and of unvaccinated controls*

Viable EV51f dose	Virulent challenge dose	Results of challenge infection (survivors/total)	Days of death after challenge	Culture (heart blood and spleen)
10^8	1.6×10^5	10/10		
10^6	1.6×10^5	10/10		
10^5	1.6×10^5	10/10		
10^3	1.6×10^5	7/10	37, 39, 39	Coliform organisms
10^2	1.6×10^5	7/10	15	*E. coli*
			15	*Proteus* sp.
			15	*Y. pestis*
0	800	0/3	6, 7, 12	*Y. pestis*
0	80	0/3	10, 11, 12	*Y. pestis*
0	8	1/3	8, 8	*Y. pestis*

protection conferred by the EV51f vaccine on guinea pigs is in agreement with previous results (14).

DISCUSSION

The EV76 *Y. pestis* from Madagascar which was passaged through guinea pigs previously inoculated with nontoxic doses of iron salts and known as EV51f was shown in this study to be highly pathogenic for African green vervet monkeys (*Cercopithecus aethiops pygerythrus*) from South Africa. Meyer (14) had shown that the EV51f vaccine strain was also pathogenic for the Ethiopian and Kenya races of this monkey species, but not for macaques (*Macaca mulatta*). In the present study, doses up to 100 million viable organisms inoculated s.c. caused a total of 13 deaths (26%) in 50 monkeys. The pathological changes observed in animals which died after vaccination were on the whole characteristic of a virulent *Y. pestis* infection. These vaccination fatalities did not have any dosage-lethality relationship, since even doses as low as 100 bacilli were able to invade, multiply, and cause death in these animals. This can be explained by an individual genetic high susceptibility of the animals concerned. The vaccine was not lethal to 50 guinea pigs inoculated simultaneously with the same doses.

A high rate of protection was conferred on guinea pigs to challenge with virulent *Y. pestis* on day 28 after vaccination. Even a dose as low as 100 viable EV51f organisms protected at least 7/10 guinea pigs, and the three highest vaccine doses conferred complete protection. In *C. aethiops pygerythrus*, there did not appear to be a relationship between the rate of protection and dose of the vaccine, as 3 of 10 monkeys succumbed to a virulent s.c. challenge infection after vaccination with 100 million, 1 million, or 1,000 viable EV51f organisms.

There was a marked decline in the titers of both HA and MPI antibodies between day 28 and 163 after vaccination. However, at the time of virulent challenge all but one monkey had significant hemagglutinin titers, and 2 had MPI values below 10, indicative of a high level of immunity. There was no apparent correlation between the HA titer and protection against virulent challenge, as a titer of 1:128 failed to protect, whereas one animal, which had failed to convert to a positive HA titer, survived. The three which succumbed had MPI values of 15 or over, but one survivor had a value of 17.

The pathogenicity of the EV51f strain and protection conferred against virulent *Y. pestis* shows marked differences in vervets, macaques, and guinea pigs. It is known that P^- (pigment factor negative) derivatives of virulent *Y. pestis* strains show the high virulence of their P^+ parents in mice which had been injected with small, nontoxic amounts of iron salts (11). The injected iron not only reduces the mean lethal dose (LD_{50}) value to that of virulent strains, but also permits growth in vivo to the large populations characteristic of virulent infections. Burrows (2) concluded that injected iron provides an essential element not sufficiently available to P^- bacilli in vivo. As the attenuated EV76 *Y. pestis* strain is fully virulent in mice previously inoculated with ferrous sulfate (11), the passage of this strain through iron-treated guinea pigs would, therefore, select a population of bacilli lacking only the virulence determinant for which iron compensates (P). Differences in the pathogenicity of the EV51f strain in different animal species would therefore most likely be a reflection of the availability of iron. In African green vervet monkeys, this availability is apparently such that low doses of these bacilli are able to

multiply and invade and cause death of a certain proportion (26% in our study) of individuals, whereas the others become ill, as reflected by a rise in temperature and white cell count. Multiplication of the EV51f bacilli in the monkeys was confirmed by positive blood cultures 4 to 11 days after s.c. inoculation of vaccine doses as low as 10^2 bacilli. This would explain why all doses cause similar HA and MPI serological responses and similar rates of protection against virulent challenge 6 months after vaccination. It is likely that the availability of iron in guinea pigs is less than in vervet monkeys but sufficient for multiplication of the organisms to be immunogenic, yet not sufficient for the bacilli to reach a lethal level in the animal, which is estimated to be 1.6×10^{11} virulent organisms (9). A similar relationship between virulence and availability of iron also appears to exist in other species of the genus *Yersinia* (19).

The finding that the EV51f strain is pathogenic in certain nonhuman primates and not in others has important consequences for the evaluation of plague vaccines. It has been stated (5) that a demonstration of non-pathogenicity of a bacterial strain toward a presumed sensitive animal species need not assure its safety for man. This followed the finding that a number of *Y. pestis* isolates from Brazil had attenuated virulence in guinea pigs but not in white mice. The host specificity of these isolates was found to be determined by a nutritional requirement identified as asparagine. It has long been realized that the response of man to plague vaccines is not the same as that of guinea pigs and white mice, and it was suggested by Otten (17) that the monkey appeared to be the most suitable animal for their investigation, but he also recognized that different monkey species vary in their susceptibility to plague. The results of the present work agree with those of Meyer (14) and other workers in that the response of guinea pigs and African green vervet monkeys to live attenuated plague vaccines was shown to be very different, and it probably differs in both from the response in man.

ACKNOWLEDGMENTS

We wish to thank J. H. S. Gear, Director of the South African Institute for Medical Research, for providing laboratory facilities and also for permission to publish this paper. Our thanks also go to H. J. Koornhof, head of the department of Microbiology, S.A.I.M.R., for his advice and guidance.

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