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1
2 **Oral vaccination against bubonic plague using a live avirulent *Yersinia***
3 ***pseudotuberculosis*.**
4

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18
19 Running title: Live oral *Yersinia pseudotuberculosis* vaccine against plague

20
21 Key words: *Yersinia*, *pestis*, *pseudotuberculosis*, plague, vaccine, live, avirulent

22

23 **Abstract**

24 We evaluated the possibility of using *Yersinia pseudotuberculosis* as a live vaccine against plague
25 because it shares high genetic identity with *Y. pestis* while being much less virulent, genetically
26 much more stable, and deliverable orally. Forty-one *Y. pseudotuberculosis* strains were screened
27 by PCR for the absence of the High Pathogenicity Island, the superantigens YPM and the type IV
28 pilus and the presence of the pYV virulence plasmid. One strain (IP32680) fulfilled these criteria.
29 This strain was avirulent in mice upon intragastric or subcutaneous inoculation, and persisted for
30 two months in the mouse intestine without clinical signs of disease. IP32680 reached mesenteric
31 lymph nodes, spleen and liver, without causing major histological lesions and was cleared after 13
32 days. The antibodies produced in vaccinated animals recognized both *Y. pseudotuberculosis* and
33 *Y. pestis* antigens efficiently. After an sc challenge with *Y. pestis* CO92, bacteria were found in
34 low amounts in the organs and unfrequently in the blood of vaccinated animals. One oral IP32680
35 inoculation protected 75% of mice and two inoculations induced much higher antibody titers and
36 protected 88% of mice. Our results thus validate the concept that an attenuated *Y.*
37 *pseudotuberculosis* strain can be an efficient, cheap, safe and easy-to-produce live vaccine for oral
38 immunization against bubonic plague.

39 **Introduction**

40 *Yersinia pestis*, the etiologic agent of bubonic and pneumonic plague, is a Gram-negative
41 bacterium with an extraordinary pathogenicity. Transmitted by infected fleas, the bacteria cause a
42 hyperplasia of the draining lymph node (bubo), followed by septicemia and the patient's death
43 within one week if an effective antibiotherapy is not administered on time. Although the bubonic
44 form of the disease is by far the most frequent in the world (45), a primary bubonic infection
45 sometimes develop into a secondary pneumonic infection highly contagious from man to man via
46 the generation of aerosols. This form of the disease is almost systematically fatal within 3 days
47 and there is a concern that *Y. pestis* could be used this way as a weapon in bioterrorism (20).
48 Additionally and of great public-health concern was the recent identification of a *Y. pestis* strain
49 naturally resistant to eight antibiotics, including those recommended for plague treatment and
50 prophylaxis (16). The widespread diffusion of the transmissible plasmid conferring this resistance
51 suggests that other resistant strains are very likely to appear (43). Against such multidrug
52 resistance in *Y. pestis*, one of the only alternatives would be mass vaccination, but no safe and
53 efficient vaccine against plague is currently available.

54
55 The live attenuated *Y. pestis* strain EV76 efficiently protected against bubonic plague and induced
56 high titers of antibodies, but did not confer long-lasting immunity and induced mild to severe side
57 reactions (27). Furthermore, the immunogenicity and virulence of the EV76 vaccine preparations
58 used in different countries were found to be highly variable, most likely because of the genetic
59 drift of the bacteria used (27, 51). Indeed, a high genomic plasticity is observed in *Y. pestis* (5,
60 30) and results from frequent chromosomal rearrangements between the numerous copies of

61 insertion sequences (IS) present in its genome. Killed whole cell *Y. pestis* vaccines has been
62 produced previously in the USA (USP) and in Australia (CSL). They are also known to be
63 reactogenic in humans and induce a shorter and less effective protection than EV76 (27). The
64 USP killed vaccine was recently discontinued in the USA.

65 A recent effort has been made to develop new recombinant subunit vaccines against human
66 plague. Most of these subunit vaccines use *Y. pestis*-specific capsular antigen F1, and/or the low
67 calcium response antigen V (LcrV) that is present in the three pathogenic *Yersinia* species. Such
68 vaccines were found to efficiently protect mice against bubonic and pneumonic plague and are
69 well-tolerated in humans (3, 4, 18, 23, 37, 41, 47, 48). However, vaccines composed of a limited
70 number of antigens may not be able to protect against F1-negative strains (49) or strains
71 harboring LcrV variants (5). The technical expertise required for the production of recombinant
72 vaccines is also an obstacle for developing countries, and injectable vaccines always raise the
73 problem of contamination via used syringes.

74 Live vaccines offer several advantages over recombinant vaccines. Their high antigenic
75 complexity guarantees a response against a broad range of antigenic targets. In live vaccines,
76 antigens are present in their native forms with normal glycosylations, they are produced *de novo*
77 as long as the bacteria persist, which provides a prolonged stimulation of the immune system, and
78 there is no need for an adjuvant since bacterial antigens (LPS and other pathogen-associated
79 signatures) naturally stimulate the innate immune system. They generally induce both antibody
80 production and a cell-mediated response, and once developed and validated, these vaccines can be
81 produced locally, allowing more economically feasible mass production.

82 *Yersinia pseudotuberculosis* is the recent ancestor of *Y. pestis* (1) but is much less virulent
83 and usually causes enteric diseases that are rarely fatal. The two species are very close with more
84 than 95% genetic identity and virulence plasmids that share a conserved colinear backbone (8).
85 The genome of *Y. pseudotuberculosis* is however much more stable than that of *Y. pestis* because
86 it contains fewer IS copies (5, 8, 51). We report that oral inoculation with a *Y. pseudotuberculosis*
87 strain, selected for its very low virulence, induces an efficient immunity against bubonic plague
88 without causing adverse reactions. This demonstrates that a live attenuated *Y. pseudotuberculosis*
89 can be a promising vaccine against bubonic plague.

90 **Materials and methods**

91 ***Yersinia* strains and culture media.**

92 The *Y. pseudotuberculosis* strains used in this study are listed in Table 1. The fully virulent *Y.*
93 *pestis* strain CO92 whose genome has been sequenced (30) was used for animal challenge and the
94 attenuated strain EV76 (27) was used as a control vaccine. Bacteria were usually grown at 28°C
95 on Luria-Bertani agar plates supplemented with 0.2% hemin (LBH) for 48h before use. A
96 selective medium containing Irgasan (0.1 g/μl: LBHI) was defined to quantify yersiniae in ex-
97 vivo samples. For inoculation in mice, bacteria grown at 28°C for 48h on LBH plates were
98 collected and resuspended in sterile saline for immediate use. Bacterial concentrations were
99 evaluated by spectrometry at 600nm and confirmed by cfu counts on LBH plates.

100

101 **PCR.**

102 DNA was obtained by thermolysis of bacteria in a solution containing 50 mM NaOH and 0.25%
103 SDS. The primers (Proligo France) amplified the *int* gene of the High Pathogenicity Island (HPI,
104 (24), the *yopM* (33) and *yopK* (forward 5'-CTGCTCATTACTGAGAACG-3', reverse:
105 5'-CTATAAGTAGAGAGTTTTTCGG-3'), genes of the pYV virulence plasmid, the *pilR* and
106 *pilN* genes of the type IV pilus locus (10) and the *ypmA/C* or *ypmB* genes coding for YPM
107 superantigens (15). The house-keeping gene *ureB* encoding an urease subunit was used as an
108 internal positive control for the PCR reactions. PCR amplification reaction mixtures contained 1
109 unit of AmpliTaq polymerase (Applied Biosystems), 10mM dNTPs, and 10μM of each primer. A
110 PCR program working for all gene fragments was defined, involving one step at 95°C for 5 min,
111 followed by 30 cycles of amplification consisting in 3 steps each: (i) 94°C for 30 s, (ii) 55°C for

112 30 s, and (iii) 72°C for 90 s. Strains used as positive controls for the various PCR primers were:
113 IP32953 for *int*, *yopM* and *yopK*, IP33053 for *pilN* and *pilR*, IP32887 and IP31411 for *ypmA/C*.
114 These strains were taken from the National Reference Center strain collection except strain 1093
115 positive for *ypmB*, a kind gift of Prof. M. Simonet, Institut Pasteur of Lille. The amplification
116 reactions were done in a PTC-100 thermocycler (Bio-Rad).

117

118 ***In vivo* analyses.**

119 Female OF1 mice, 6 to 8 weeks old, were purchased from Charles River (L'Arbresle, France). All
120 animal care and experimentations were conducted according to the guidelines of the Institut
121 Pasteur. Mouse infections with virulent bacteria were performed in a BSL3 animal facility.
122 Inoculation of bacteria via the intragastric (ig) route (200 µl in saline) was done with a curved
123 feeding needle and subcutaneous (sc) inoculations (100 µl) were performed in the abdominal
124 skin. Mice were not fasted before intragastric inoculation. Mortality, body weight, behavior and
125 fur appearance were recorded daily for 3 weeks.

126 To follow the kinetics of bacterial dissemination *in vivo*, groups of three mice per time point were
127 humanely euthanized after one or two ig inoculations. Various organs (Peyer's patches,
128 mesenteric lymph nodes, liver, spleen), as well as feces (two fecal pellets taken in the large
129 intestine) were collected aseptically and homogenized in sterile PBS, using 3 mm glass beads and
130 an electric mixer mill (Retsch, Germany). Serial dilutions of the homogenates were plated on
131 LBH plates for counting.

132 For histological analysis, organs were fixed with buffered formaldehyde for 48h, embedded in
133 paraffin, cut in 5 micrometers sections, and stained with hematoxylin–eosin or Giemsa stain. The
134 severity of lesions caused to tissues by *Y. pseudotuberculosis* strains were observed on blinded

135 histological sections and quantified using a newly defined lesions score ranging from 0 to 10. 0:
136 no inflammation; 1: minimal inflammation (congestion, microhemorrhages); 2: less than 2
137 abscesses in a given organ; 3: from 2 to 5 microabscesses; 4: from 5 to 10 microabscesses; 5: from
138 10 to 20 microabscesses; 6: from 20 to 50 microabscesses; 7: confluent intratissular necrosis zone;
139 8: organ destruction below 50%; 9: organ destruction from 50 to 80%; 10: total destruction and
140 very abundant polymorphonuclear cells (PMN). At least 10 fields per slide were read on 2 slides
141 per mouse.

142 Vaccination using the IP32680 strain consisted of 1 intragastric inoculation with 2×10^9 cfu in
143 saline or 2 inoculations at 30 days interval. Vaccination using the *Y. pestis* EV76 strain consisted
144 of a single subcutaneous (sc) injection of 10^7 cfu as described by Flashner (14).

145 To obtain immune sera, mice were anesthetized and blood was collected from the retro-orbital
146 vein. To analyze IgA in intestinal lavages, mice were humanely euthanized and the gut section
147 extending from the stomach to the caecum was flushed with 10 ml cold PBS containing protease
148 inhibitors (*Complete*®, Roche). After centrifugation, the supernatant was filtered and frozen until
149 use.

150

151 **Immuno-assays**

152 To produce the *Y. pseudotuberculosis* or *Y. pestis* antigenic solutions used for coating in the
153 ELISA assays, either strains IP32680 or CO92 was grown at 37°C on LB Agar for 48h,
154 centrifuged, resuspended in cold PBS containing protease inhibitors and sonicated (Branson
155 sonicator). Centrifuged supernatant was sterilized by filtration (0.22 µm), and its protein content
156 was measured using a Bradford assay (Bio-Rad). ELISA plates (NUNC) were coated overnight

157 with the antigenic solution (5 µg/ml) in PBS. The recombinant LcrV fusion protein for LcrV-
158 specific ELISA was produced by fusion of the *Y. pestis* CO92 *lcrV* gene with the *Schistosoma*
159 *japonicum* Glutathione-S-Transferase (GST) gene (39). Briefly, the *lcrV* gene amplified by PCR
160 was cloned in frame with the GST gene into the pGEX B expression vector under control of the
161 lactose promoter. *E. coli* strain BL21 was transformed with the vector and expression of the
162 recombinant protein was induced by addition of IPTG (Sigma). The recombinant protein was
163 purified from the *E. coli* bacterial lysate using Glutathione-agarose beads (Sigma). Serum and
164 intestinal lavage samples were serially diluted in PBS containing 0.1% BSA for determination of
165 Ab titers by ELISA. Secondary rat antibodies directed against mouse IgG, IgG1, or IgG2a were
166 from Becton-Dickinson Pharmingen. Secondary goat antibodies directed against mouse IgG2b,
167 IgG3 or IgA were from Caltag Laboratories. All secondary antibodies were coupled to
168 horseradish peroxidase (HRPO) used with TMB substrate (OptiEIA, BD Pharmingen). Titers
169 were determined by reading the sample dilution giving half of the maximal signal on the titration
170 curve (generally OD₄₅₀ = 1). The risk that the very low IgA titers obtained could result from a
171 defect of the HRPO-coupled anti-IgA 2nd antibody was excluded by demonstrating that this
172 reagent works in an ELISA designed to quantify antigen-specific IgA in bronchoalveolar lavages
173 of *Bacillus anthracis* – vaccinated mice (data not shown; courtesy of Dr P. Goossens, Institut
174 Pasteur).

175
176 **Evaluation of mouse protection against a challenge with *Y. pestis*.**

177 The fully virulent *Y. pestis* strain CO92 was grown at 28°C and a suspension in saline containing
178 1000 cfu (≥100 times the LD₅₀) was injected sc in the *linea alba* of IP32680-vaccinated and non-
179 vaccinated mice 30 days after the last vaccine dose. Animal survival was followed for 21 days. *Y.*

180 *pestis* dissemination in the body and septicaemia were assessed two days after the challenge with
181 CO₂ by homogenizing various organs and plating serial dilutions of organ samples or blood.

182

183 **Statistical analysis**

184 Spearman's rank test was used to evaluate the correlation between antibody titers against *Y. pestis*
185 and *Y. pseudotuberculosis*. Fisher's exact test was used to evaluate the protection in mice and
186 Student's t test to compare bacterial loads in challenged mice groups.

187 **Results**

188 Selection of avirulent *Y. pseudotuberculosis* strains.

189 Our criteria to select a *Y. pseudotuberculosis* strain suitable for use as a vaccine were that this
190 strain should be: (i) avirulent, (ii) able to persist for at least one week in the intestine without
191 causing severe lesions, and (iii) capable of inducing an adaptative immune response. Forty-one *Y.*
192 *pseudotuberculosis* strains of serotype II (Table 1), known to lack the HPI (12), were tested for
193 the presence of major virulence factors. Absence of the HPI was confirmed by PCR amplification
194 of the HPI-borne gene *int* (24). Absence of other known virulence factors such as the
195 superantigens YPM A/B/C (15), the type IV pilus encoded by the *Yersinia* Adhesion
196 Pathogenicity Island (YAPI, (9)) and presence of the pYV virulence plasmid was checked by
197 PCR. Strains had to possess the pYV plasmid to be selected because this element is essential for
198 the bacterial persistence in the host and encodes antigens of interest for vaccination (41). Among
199 41 strains tested by PCR (Table 1), seven (listed in Table 2) met our criteria.

200 To eliminate virulent isolates, high doses of bacteria were inoculated sc (10^9 cfu) or ig (2×10^9
201 cfu) to groups of 5 mice. Strains IP32680 and IP32721 were the only isolates completely
202 avirulent at these doses (Table 2).

203 Persistence of these two strains in the intestine of infected mice was checked at different time
204 points (days 1, 2, 3, 6, 13) after inoculation. The fully virulent *Y. pseudotuberculosis* strain
205 IP32953 (8) was used as positive control. While IP32721 was rapidly cleared from the intestinal
206 tract (data not shown), IP32680 was able to persist over the 13 day survey period in the feces of
207 infected mice (Fig. 1A) and was detectable for up to 2 months in some mice (data not shown).

208 Because of its avirulence and prolonged persistence, strain IP32680 was selected for further
209 analyses.

210

211 Infectivity of strain IP32680 for the mouse.

212 In a first set of experiments, the infectivity of strain IP32680 given orally was analyzed by
213 comparison with the highly virulent IP32953 strain that was given at the same dose. Since the
214 lethal dose 50% (LD₅₀) of IP32953 via the ig route is 1.6×10^7 cfu, a dose of 10^8 cfu was injected
215 to induce high bacterial loads and lesions in the organs without immediately killing all mice.
216 Mice that received IP32680 ig did not develop any clinical signs of infection (normal fur and
217 behavior, no body weight loss) up to day 60, as opposed to those infected with IP32953 (Fig. 2)
218 which were sick and often died.

219 To quantify tissue colonization, groups of mice infected ig with IP32680 or IP32953 were
220 sacrificed at various time points post-infection and the bacterial content of their Peyer's patches,
221 mesenteric lymph nodes, liver and spleen was measured. IP32680 was present and persisted in the
222 Peyer's patches and mesenteric lymph nodes longer than the two-week survey period (Fig. 1B),
223 showing bacterial colonization of these tissues. In these intestinal lymphoid tissues, the number of
224 IP32680 cfu at the initial phase of infection (first week) was lower than that of IP32953, though
225 the number of cfu became similar at later stages (but only survivors of IP32953 infection could
226 then be examined). In contrast, IP32680 invaded deep organs much less efficiently than IP32953
227 (Fig. 1C). Bacterial counts of both strains reached a peak between days 3 and 6, yet the number of
228 IP32680 cfu in the spleen and liver of infected animals was 100 to 1000 times lower than that of
229 IP32953. Furthermore, the spleen and liver of the animals infected with IP32680 were cleared of

230 the infection at day 15, while the spleen of the mice surviving infection with IP32953 still
231 contained live bacteria (Fig. 1C).

232 Histological analysis showed a massive necrosis at day 6 (peak of infection) in the Peyer's
233 patches of IP32953 infected mice whereas only some marginal polymorphonuclear cells were
234 present in those infected with IP32680 (Fig. 3A). Similarly, IP32953 induced numerous abscesses
235 and abundant inflammatory polymorphonuclear infiltration in the spleen and liver whereas
236 IP32680 caused only rare micro-abscesses (Fig. 3A). To quantify the extent of organ lesions,
237 samples from six mice per group were assessed using a numeric scoring system. The mean scores
238 indicated that lesions caused by IP32680 were very mild (Fig. 3B) whereas, as a comparison,
239 mouse infection with 10 times less IP32953 resulted in much higher scores in surviving animals
240 (Fig. 3B).

241 In a second set of experiments, the infectivity of IP32680 was evaluated using a vaccination
242 protocol (two ig inoculations of 2×10^9 cfu of IP32680 at a 30 day interval). As previously
243 observed, mice did not show signs of stress and did not loose weight (data not shown). Similarly,
244 upon the first inoculation, IP32680 colonized the gut and reached the deep organs (Fig. 1D). After
245 the second inoculation, colonization of the gut and Peyers' patches was similar to that observed
246 after the first inoculation. Bacteria were however recovered in lower numbers from the
247 mesenteric lymph nodes and the liver, and were absent from the spleen. Lesions generated after a
248 second inoculation of IP32680 (Fig. 3B) were significantly milder in the liver ($p=0.02$) and not
249 significantly different in the other organs tested. At least during the 60 day observation period, the
250 bacteria could be found in feces, showing that mice develop a carrier state.

251 Antibody response elicited by IP32680 against *Y. pseudotuberculosis* and *Y. pestis*

252 The capacity of strain IP32680 to induce an antibody response after ig administration either once
253 or twice at a 30 day interval (prime/boost strategy; 2×10^9 cfu per dose) was evaluated. Blood was
254 collected 30 days after the last inoculation and serum antibody titers against *Y.*
255 *pseudotuberculosis* antigens were analyzed by ELISA. After a single inoculation of IP32680 (Fig.
256 4A), moderate titers ($< 10^3$) of IgG were detectable. The second IP32680 inoculation induced a
257 marked increase (10 times) of these antibody titers. The IgG2a and IgG2b isotypes predominated
258 after both the first and the second inoculations, suggestive of a type 1 immune response (13).
259 Immunization via the intestinal route was also expected to induce a mucosal type of immune
260 response, characterized by high amounts of IgA released in mucosal secretions and subsequently
261 by the presence of IgA in blood. Unexpectedly, *Y. pseudotuberculosis*-specific IgA production
262 was low (titers < 50) in both the intestinal secretions and the serum of mice infected once with
263 IP32680. The IgA titers increased moderately (titers ≤ 100) in the double-inoculation regimen
264 (data not shown).

265 Since the pYV-encoded LcrV antigen has previously been shown to induce an immune response
266 and a protection against plague (3, 41), we examined whether antibodies were raised against LcrV
267 in sera of mice vaccinated with IP32680 given once. IgG titers varied over a large range from
268 animal to animal, with values from more than 8000 to undetectable (data not shown). Because the
269 number of vaccinated animals dying upon plague challenge was low, it was not possible to
270 analyze statistically the relationship between the anti-LcrV titer and survival. However, the fact
271 that several mice, in which very low anti-LcrV titers were recorded, survived the *Y. pestis*
272 challenge, suggests that anti-LcrV IgG might not be an essential component of the protective
273 immune response raised, in agreement with the high number of possible antigenic targets present
274 on live bacteria.

275 The antibodies elicited by IP32680 also strongly recognized *Y. pestis* antigens (Figure 4B), and
276 titers directed against the two species were closely correlated (Spearman's Rho = 0.94; $p =$
277 0.0002), confirming the extensive antigenic identity between *Y. pestis* and *Y. pseudotuberculosis*.

278 Protection conferred by IP32680 against bubonic plague

279 Mice vaccinated orally once or twice at a 30 day interval with IP32680 were exposed to a sc
280 lethal challenge (1,500 cfu = 150 LD₅₀) with the fully virulent *Y. pestis* strain CO92 30 days after
281 the last immunization. Negative controls included groups of unvaccinated mice and positive
282 controls groups of mice vaccinated with the EV76 live vaccine (14, 27). While all unvaccinated
283 mice died, 75% of animals vaccinated with a single inoculation of IP32680 survived ($p = 0.007$).
284 The protection conferred by two inoculations of IP32680 was superior (88% survival, $p \leq 0.001$;
285 Fig. 5A), although not statistically different from the protection conferred by one inoculation. By
286 contrast, the protection conferred by the conventional EV76 vaccine strain was limited, with a
287 mortality of 50% (Fig. 5A).

288 *Y. pestis* CO92 bacteria were found in high amounts in the liver, spleen, inguinal lymph nodes
289 and blood of the unvaccinated mice at day 2 post injection (Fig. 5B) and the animals started to die
290 at day 4. In contrast, *Y. pestis* was only rarely found in the organs and blood of IP32680
291 vaccinated mice, and in most of these animals there was a complete absence of bacteria in the
292 organs (Fig. 5B). The difference in bacterial loads was statistically highly significant ($p \leq$
293 0.00003) in all organs tested.

294 Therefore, ig inoculation of IP32680 conferred a protection against plague superior to that
295 obtained with the conventional *Y. pestis* strain EV76, and two inoculations further increased this
296 protection.

297 **Discussion**

298 This study demonstrates that vaccination against bubonic plague can be obtained using an
299 attenuated *Y. pseudotuberculosis* strain given orally. A live *Y. pseudotuberculosis* could thus be
300 an interesting alternative to the live attenuated *Y. pestis* strain EV76, which causes severe side-
301 effects (27) and is genetically unstable. Vaccination with IP32680 given once or twice did not
302 cause any visible symptoms in the vaccinated animals, and protection against bubonic plague
303 induced by one (75%) or two (88%) inoculations of IP32680 (88%) was higher than that induced
304 by EV76 (50%). An avirulent *Y. pseudotuberculosis* is therefore an interesting alternative to the
305 use of a live attenuated *Y. pestis*, such as EV76, against bubonic plague.

306

307 Two ways of producing live vaccines have historically been employed: attenuation of the original
308 pathogen (for example development of the BCG tuberculosis vaccine) or selection of a natural
309 avirulent strain that is closely related to the pathogen (for example the *vaccinia* strain used in
310 Smallpox vaccines). The option of attenuating *Y. pestis* by genetic engineering has been used by
311 several laboratories, for instance by deletion of the *yopH* or *pcm* genes (6, 14), or by addition of
312 an *E. coli lpxL* gene to modify the LPS (28). All of these mutations led to a strong attenuation of
313 virulence and a good protection against bubonic plague. Still, the use of *Y. pestis* presents a
314 number of drawbacks. The major one is its genome instability (5, 30) caused by the high number
315 of IS present in its genome, which are known to favor genetic rearrangements. *Y.*
316 *pseudotuberculosis* has a much more stable genome due to its small number of IS (5, 8, 51).
317 Finally, a *Y. pseudotuberculosis* live attenuated vaccine would be less problematic than a *Y. pestis*
318 if accidentally released in the environment.

319

320 The main pitfall with a live vaccine is its ability to cause adverse reactions. IP32680 did not cause
321 any visible clinical symptoms in the vaccinated mice. According to the concept of “danger“
322 proposed by Matzinger (26), self or commensal material is tolerated because it does not cause
323 inflammation, and vaccines must be able to trigger a certain level of inflammation in order for an
324 immune response to start. The fact that IP32680 was able to colonize deep organs, to persist in
325 situ for some weeks and to induce mild lesions was most likely a key factor in the development of
326 an effective immune response. Whereas an adjuvant is added to particulate vaccines to induce this
327 inflammation, bacterial signatures like LPS are likely to play this adjuvant role in our live
328 vaccine.

329
330 Immunity against bubonic plague has previously been obtained in the mouse by sc inoculation of
331 live *Yersinia enterocolitica* serotype O3 (2), which infects humans but is not very virulent in the
332 mouse. *Y. pseudotuberculosis* is genetically closer to *Y. pestis* than *Y. enterocolitica* (1, 8),
333 therefore, the immune response raised against *Y. pseudotuberculosis* is expected to be more
334 efficient against *Y. pestis* than that induced by *Y. enterocolitica*. Vaccination using live virulent *Y.*
335 *pseudotuberculosis* has been reported in the ‘50s by Wake (42) who vaccinated mice with a strain
336 of *Y. pseudotuberculosis* (10^2 cfu) delivered via the sc route. The weaker protection obtained
337 against bubonic plague (50%) was possibly due to the low dose of bacteria given, imposed by the
338 virulence of the strain. Very recently, efficient protection against bubonic plague was also
339 obtained using a *dam* mutant of *Y. pseudotuberculosis* IP32953 (clone C18) delivered orally (40).
340 The isolate used was cured of the virulence plasmid pYV because the *dam* mutation resulted in a
341 high rate of loss of the pYV plasmid, indicating that protective immunity can be raised against
342 chromosome-encoded targets. Their observation that the pYV plasmid is not required is

343 intriguing because pYV-encoded YopE and YopH toxins are required for survival in the intestinal
344 tissue (25). However it is possible that the HPI present in the *dam*-mutated pYV-cured C18 clone
345 (and not in IP32680) -or other virulence factors to be identified- confers this ability by a different
346 mechanism. Previously, Simonet et al. had reported that intravenous injection of a *Y.*
347 *pseudotuberculosis* strain, attenuated by loss of pYV, provided a 50% protection against bubonic
348 plague (36).

349

350 Attenuated live bacterial vaccines mimic natural infection and are expected to provide sustained
351 exposure to target antigens, resulting in a stronger immune response. The vaccinia vaccine against
352 smallpox and the BCG against tuberculosis are the most famous of attenuated vaccines, and other
353 such vaccines recently evaluated in humans include *Salmonella*, *Shigella*, *Vibrio cholerae* and
354 *Listeria* (22). As compared to subunit vaccines of limited antigenic complexity like those using
355 the LcrV and F1 antigens, a live *Yersinia* is expected to elicit an immune response against a wide
356 array of antigens, thus conferring protection against various natural or genetically modified
357 variants of *Y. pestis*. For example the LcrV antigen contains a region whose hypervariability may
358 allow it to escape the immune response (35). *Y. pestis* strains lacking the F1 antigen have a
359 similar virulence as F1-positive strains (44) and would escape an anti-F1 immune recognition.
360 The risk that a mutated strain bypasses the immunity stimulated by a live vaccine is, on the
361 contrary, very unlikely due to the high number of alternative targets. Protection induced by
362 IP32680 can be ascribed to the efficient recognition of *Y. pestis* antigens, in agreement with
363 genetic identity between *Y. pestis* and its ancestor.

364

365 An attractive rationale for choosing oral inoculation is the avoidance of syringes which are a
366 major source of disease transmission, a problem frequently mentioned by WHO in its vaccination
367 guidelines (46). Oral vaccination with *Y. pseudotuberculosis* takes advantage of its capacity to
368 colonize and persist in the gut for at least a few weeks, exerting a prolonged stimulation of the
369 immune system. This local stimulation of mucosal immunity was expected to result in a
370 protection against infection starting at other mucosal surfaces (19), and thus to potentially protect
371 against pneumonic plague. However, our preliminary experiments of *Y. pestis* infection by
372 aerosols in mice inoculated twice with IP32680 revealed a poor protection against pneumonic
373 plague. Only 30% of the animals survived a nose-only exposure to aerosolized CO92 (8000 cfu
374 found in lungs 1 h after exposure; data not shown). The low IgA production observed in
375 vaccinated mice may contribute to explain the low mucosal protection against pneumonic plague.
376 Little information regarding IgA production in response to *Y. pseudotuberculosis* infection in the
377 mouse is available. Whereas mouse immunization using an oral *Salmonella* –F1/V vaccine (29) as
378 well as several active "probiotic" or commensal bacteria succeeded in inducing IgA production in
379 the gut (11), intestinal infection with virulent *Y. enterocolitica* O:8 failed to induce IgA
380 production in either BALB/C or C57BL/6 mice (7). This low IgA response to live yersiniae could
381 result from the fact that pathogenic yersiniae use their type three secretion system (TTSS) to
382 induce apoptosis of antigen-presenting cells in Peyer's Patches, as reported for macrophages
383 (reviewed by Zhang; (50). Whereas the fast elimination of IP32680 from the spleen after the
384 second inoculation may be ascribed to a successful systemic immune response, the low IgA
385 response in the gut may explain how the bacteria persist for several weeks in the gut as detected in
386 feces, resulting in a "carrier" state. It also suggests that the immune response induced by IP32680
387 did not occur in the Peyer's Patches, and thus was mainly systemic and not mucosal.

388
389 The serum immunoglobulin isotype profile (dominance of IgG2a & b) found in mice vaccinated
390 with IP32680 suggest that the immune response was oriented toward the Th1 type (13). This type
391 of response is characterized by the production of cytokines such as IFN γ and TNF α which support
392 phagocyte activation, an event favorable to the capture and destruction of bacteria. Peyer's Patches
393 rather favor the development of a Th2 or regulatory T cells of response (21). Lymphocytes and
394 antibodies most likely work together to eliminate bacteria by providing help to phagocytes (38)
395 because transfer of either lymphocytes (31) or antibodies (17) from immunized mice can protect
396 against pneumonic plague. The protection against bubonic but not pneumonic plague reported
397 here may therefore reflect the presence of IgG and the lack of IgA to collaborate with cells (32).

398
399 This work demonstrates the potential of using a live attenuated strain of *Y. pseudotuberculosis* as
400 an oral vaccine against bubonic plague. Bubonic plague is by far the most frequent form of the
401 disease and naturally occurring pneumonic plague usually starts from a primary bubonic plague
402 case (34, 45). Vaccination against bubonic plague should thus reduce the frequency of both forms
403 of the disease in endemic countries. Strain IP32680 cannot be used for human vaccination
404 because the causes of its attenuation are not known and the possibility of its reversion to full
405 pathogenicity cannot be excluded. Therefore, development of live attenuated strains of *Y.*
406 *pseudotuberculosis* harboring defined, irreversible mutations is necessary. Also, addition of *Y.*
407 *pestis*-specific antigens to the *Y. pseudotuberculosis* vaccinal strain could improve its efficiency
408 against plague in both its bubonic and pneumonic forms. The most obvious antigen in that regard
409 is F1, which is the main component of the pseudo-capsule and therefore the most abundant
410 antigen in *Y. pestis* in vivo. Indeed, the *caf-1* operon that encodes for F1 has been previously

411 transferred efficiently in a *Salmonella* strain (29). Such a genetically-engineered oral *Y.*
412 *pseudotuberculosis* plague vaccine would represents a promising approach for mass vaccination
413 in endemic countries.

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417 *pseudotuberculosis* strains.

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571
572

573 **Legends to figures**

574
575 Figure 1: *Y. pseudotuberculosis* IP32680 colonizes the digestive tract and transiently penetrates
576 into deep organs.

577 Strains IP32680 or IP32953 (10^8 cfu) were inoculated ig to groups of three mice that were
578 sacrificed at different time points. Bacteria recovered from either feces (A), the intestinal tract (B)
579 or deep organs (C) were counted. Given are the cfu per 2 fecal pellets (feces), per g (liver), per
580 whole organ (spleen, mesenteric lymph nodes) or per 2 Peyers' patches. In a second set of
581 experiments (vaccination protocol), mice received twice 2×10^9 bacteria ig at a 30 day interval
582 (arrows) and bacterial counts were performed similarly (D). Shown are the means of Log cfu +
583 SEM from three mice per point of one representative experiment out of two. nd: not detected.

584
585 Figure 2: Evolution of mouse weight after ig inoculation of yersiniae.

586 Strains IP32680 or IP32953 (10^8 cfu) or saline (control group) were inoculated ig to groups of 8
587 mice and their individual weight was measured daily. Shown are means \pm SD of 8 mice per group
588 for IP32680 and saline. Because IP32953 killed some mice, the mean is not given and only three
589 curves from individual animals are shown as examples, with death indicated by crosses. One
590 representative experiment out of two.

591
592 Figure 3: Intragastric inoculation of IP32680 causes minor lesions in mouse organs.

593 A. Groups of 6 mice from 2 pooled experiments were treated as described in legend of figure 1
594 and organs taken at day 6 (maximal bacterial load) were stained with haematoxylin-eosin and
595 May-Grünwald-Giemsa. Shown are representative pictures of Peyers' patches, liver and spleen
596 of animals inoculated with IP32953 or IP32680. Arrowheads show necroses and arrows show
597 abscesses. L: gut lumen, WP: white pulp, RP: red pulp. Scale bar : 100 μ m.

598 B. Tissue lesion scores recorded at day 6 after first or second oral inoculations (vaccination
599 protocol) of IP32680 to 6 mice (mean \pm SD). As a comparison, scores observed for mice
600 having survived a single IP32953 inoculation (3 out of 6) are given.

601
602 Figure 4: Humoral immune response of IP32680-inoculated mice against *Y. pseudotuberculosis*
603 and *Y. pestis*

604 A: Serum antibody titers against IP32680 in mice inoculated ig once or twice at a 30 day interval
605 with 2×10^9 cfu. Blood samples were collected 30 days after each inoculation. A filtered
606 sonicate of the IP32680 strain was used for coating in ELISA plates. Shown are mean Ig titers
607 \pm SD from 12 mice per group, pooled from 2 independant experiments.

608 B : Correlation between the IgG titers against IP32680 and CO92 extracts in the sera of mice
609 inoculated once or twice with IP32680. Shown are individual titers from 8 mice per group.
610 The linear regression analysis line is drawn and the Rho value obtained using Spearman's rank
611 test is given ($p = 0.0002$).

612
613 Figure 5: Protection against bubonic plague conferred by oral vaccination with IP32680.

614 A: Groups of 8 mice received either saline (unvaccinated), 1 sc inoculation of 10^7 cfu of EV76
615 (positive control group), or 1 or 2 ig inoculations of 2×10^9 cfu of IP32680 at a 30 day interval.
616 All mice were challenged sc with a lethal dose of 10^3 cfu of *Y. pestis* CO92 at d30 (mice with
617 EV76 or IP32680 given once) or d60 (mice with IP32680 given twice) and mortality was
618 followed thereafter daily for 3 weeks. ***: $p \leq 0.005$, **: $p \leq 0.01$, ns: not significant. One
619 representative experiment out of 2.

620 B: Enumeration of CO92 bacteria in organs of challenged mice. Organs and blood from
621 unvaccinated mice and from mice having received the IP32680 strain twice were taken to
622 determine the bacterial load two days after a *Y. pestis* challenge performed as in A. Given are
623 the cfu per g (liver), or per whole organ (spleen, 2 inguinal lymph nodes) or per ml of blood.
624 Results from 2 independant experiments were pooled and shown are the means of Log cfu \pm
625 SEM of a total of 16 vaccinated mice and 21 unvaccinated mice. ND: not detected. ****: $p \leq$
626 0.00003.

627

Table 1
 SCREENING BY PCR OF SEROTYPE II *Y. PSEUDOTUBERCULOSIS* STRAINS
 EVALUATED FOR USE AS VACCINE FOR THE PRESENCE OF VIRULENCE
 DETERMINANTS.

Strain	Origin	pYV		Type IV pilus		HPI	Superantigen	
		<i>yopK</i>	<i>yopM</i>	<i>pilN</i>	<i>pilR</i>	<i>int</i>	<i>ypmA/C</i>	<i>ypmB</i>
IP32554	Human blood	-	-	-	-	-	-	-
IP32555	Human blood	-	-	+	+	-	-	-
IP32576	Human blood	-	-	-	-	-	-	-
IP32584	Pig feces	-	-	-	-	-	-	-
IP32585	Antelope LN	-	-	-	-	-	-	-
IP32589	Human LN	-	-	-	-	-	-	-
IP32596	Rabbit	-	-	+	+	-	-	-
IP32598	Human blood	-	-	-	-	-	-	-
IP32621	ND	+	+	-	-	-	-	-
IP32628	Veterinary	-	-	+	+	-	-	-
IP32642	Bird feces	+	+	+	+	-	-	-
IP32643	Parrot	-	-	-	-	-	-	-
IP32661	Veterinary	-	-	-	-	-	-	-
IP32672	Hare	+	+	+	+	-	-	-
IP32678	Hare	-	-	-	-	-	-	-
IP32679	Hare	-	-	-	-	-	-	-
IP32680	Hare	+	+	-	-	-	-	-
IP32681	Hare	-	-	+	+	-	-	-
IP32692	Hare	+	+	+	+	-	-	-
IP32721	Hare	+	+	-	-	-	-	-
IP32828	Human feces	+	+	+	+	-	-	-
IP32860	Human blood	+	+	-	-	-	-	-
IP32870	Hare liver	+	+	+	+	-	-	-
IP32873	Hare liver	+	+	+	+	-	-	-
IP32874	Hare liver	+	+	+	+	-	-	-
IP32876	Hare blood	+	+	+	+	-	-	-
IP32892	Monkey	-	-	-	-	-	-	-
IP32896	Human	+	+	-	-	-	-	-
IP32908	Bird liver	+	+	-	-	-	-	-
IP32913	Monkey lung	-	-	+	+	-	-	-
IP32926	Hare spleen	+	+	+	+	-	-	-
IP32934	Hare liver	+	+	+	+	-	-	-
IP32969	Monkey liver	-	-	-	-	-	-	-
IP33006	Human blood	-	-	-	-	-	-	-
IP33047	Hare	-	-	-	-	-	-	-
IP33054	Human blood	-	-	-	-	-	-	-
IP33067	Human LN	+	+	+	+	-	-	-
IP33098	Human Feces	+	+	-	-	-	-	-
IP33286	Human blood	+	+	+	+	-	-	-
IP33293	Human Feces	-	-	-	-	-	-	-
IP33306	Hare	-	-	-	-	-	-	-

Table 2

COMPARISON OF VIRULENCE IN THE MOUSE OF THE 7 SELECTED
YERSINIA PSEUDOTUBERCULOSIS STRAINS AND THE VIRULENT STRAIN
 IP32953

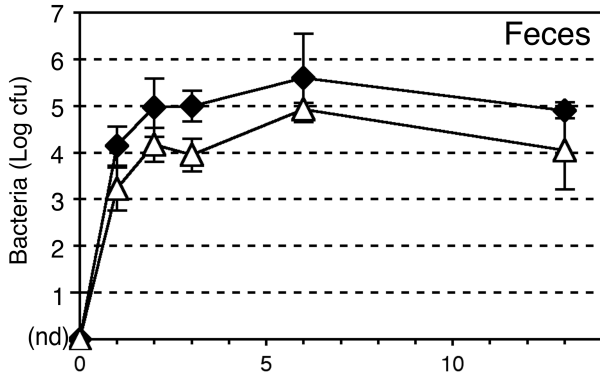
Strains	Serotype	Mortality* after sc inoculation**	Mortality* after ig administration***
IP32953	I	8/10	9/10
IP32896	II	2/5	2/5
IP33098	II	0/5	5/5
IP32908	II	1/5	3/5
IP32621	II	2/5	0/5
IP32860	II	0/5	2/5
IP32721	II	0/10	0/10
IP32680	II	0/10	0/30

* Mortality (dead mice/total) was followed daily for 3 weeks. For strains killing no mice in both conditions, experiments were reproduced to confirm the absence of virulence and results were pooled.

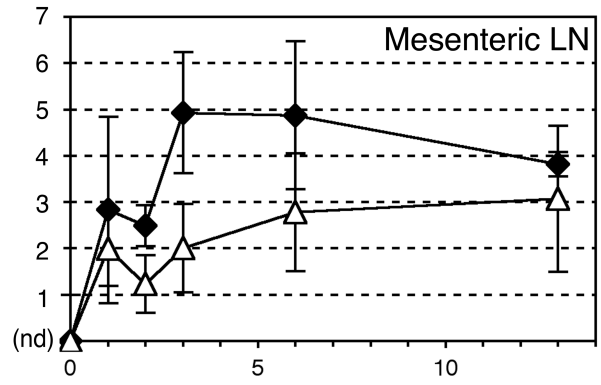
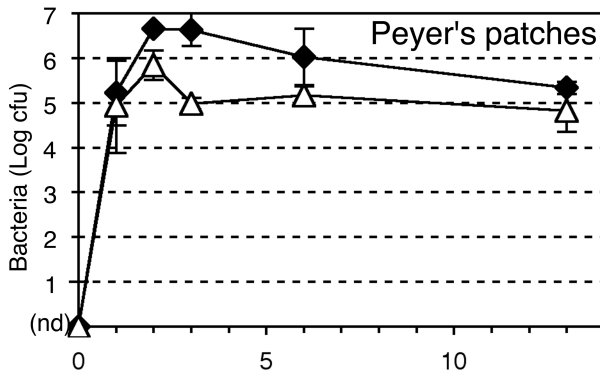
** 10^9 cfu were inoculated subcutaneously in ventral skin

*** 2×10^9 cfu were given intragastrically using a feeding needle

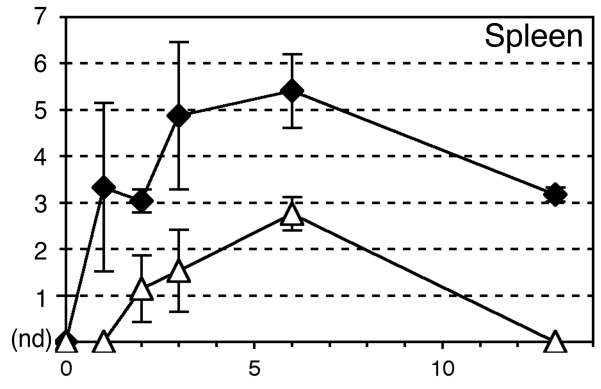
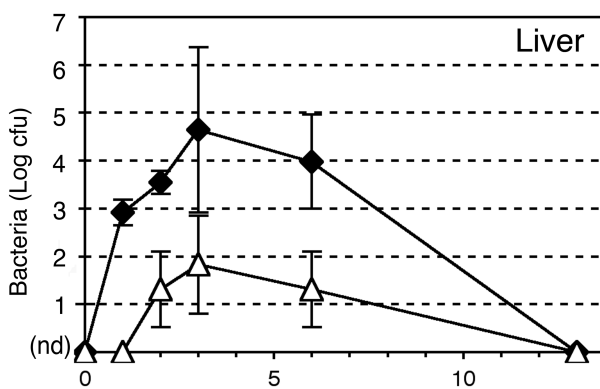
A



B

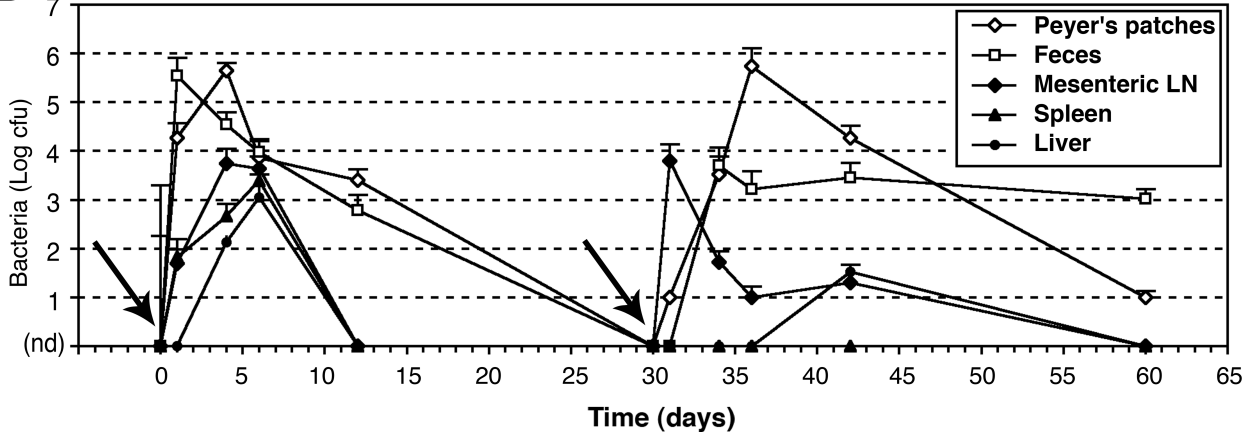


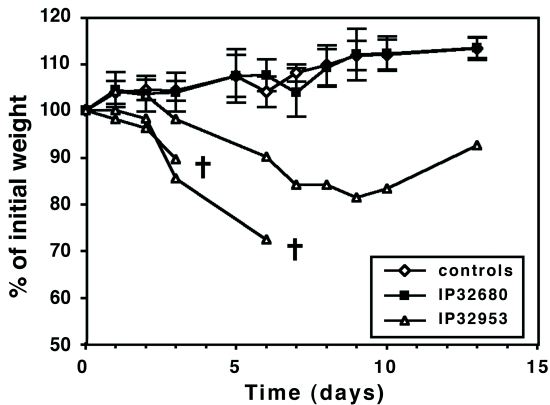
C



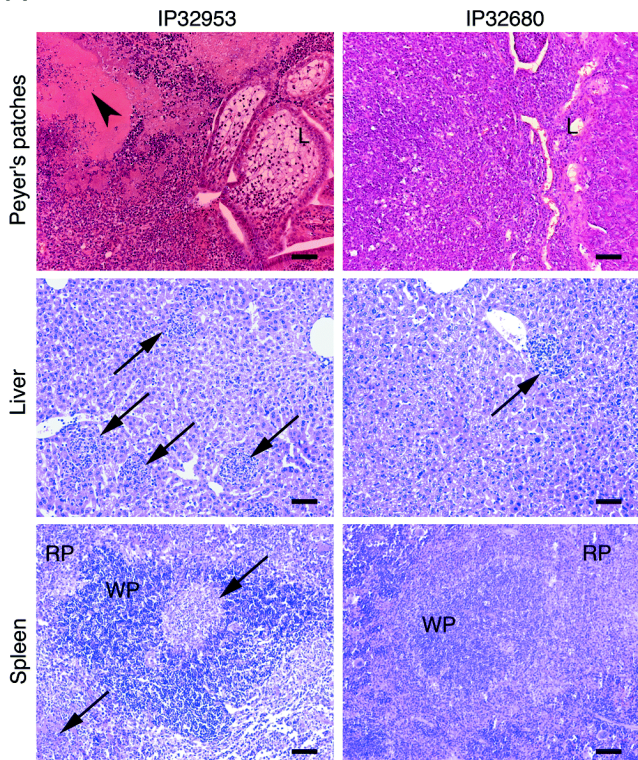
Time (days)

D





A



B

Lesion scores in target organs

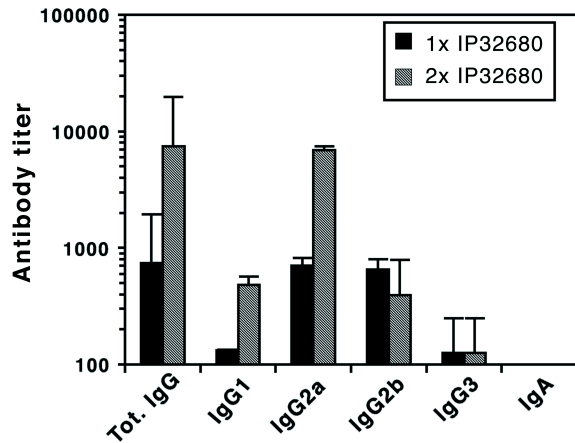
	IP32953 10^8 cfu (a)		IP32680 10^9 cfu (b)	
	-		1st inoculation	2nd inoculation
Liver	≥ 6		2.1 ± 1.3	1.0 ± 1.4
spleen	≥ 4		2.1 ± 1.2	1.6 ± 1.5
Peyer's P.	≥ 4		1.5 ± 1.3	1.0 ± 1.7
MLN	≥ 7		ND	0.7 ± 1

(a): scores from 3 survivors out of 6 mice inoculated once

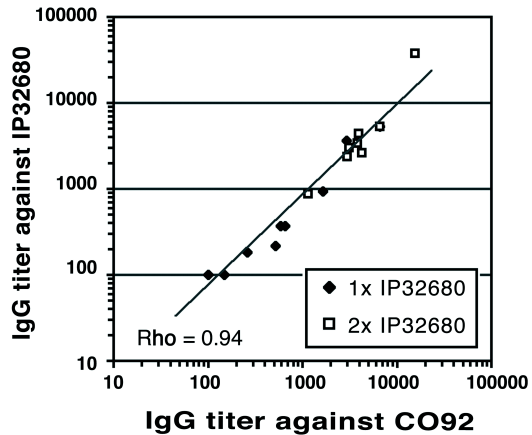
(b): mean scores \pm SEM of 6 mice

Figure 4

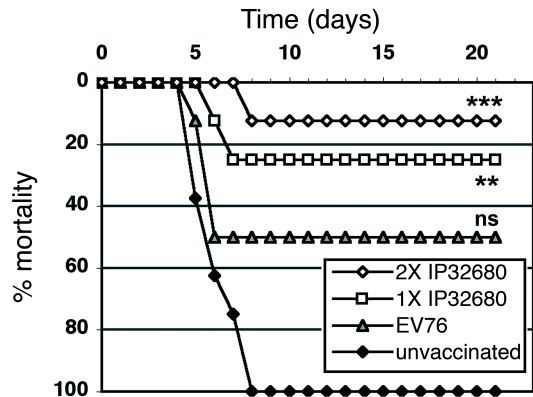
A



B



A



B

