

Review

Potential human immunotherapeutics for plague

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Summary

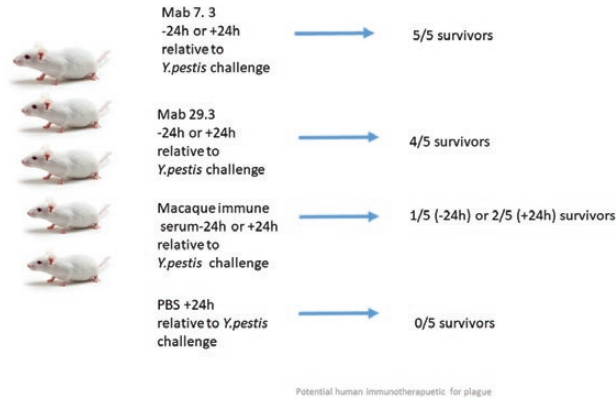
Two monoclonal antibodies directed to the V antigen of *Yersinia pestis* have been tested for protective efficacy in a murine model of bubonic plague. Mice were infected with a current clinical isolate from Madagascar, designated *Y. pestis* 10–21/S. Mab7.3, delivered to mice intra-peritoneally at either 24 h prior to, or 24 h post-infection, was fully protective, building on many studies which have demonstrated the protective efficacy of this Mab against a number of different clinical isolates of *Y. pestis*. Mab 29.3, delivered intra-peritoneally at either –24 h or +24 h, protected 4/5 mice in either condition; this has demonstrated the protective efficacy of this Mab *in vivo* for the first time. These results add to the cumulative data about Mab7.3, which is currently being humanized and highlight its potential as a human immunotherapeutic for plague, which is an enduring endemic disease in Madagascar and other regions of Africa, Asia, and South America.

Abbreviations: CFU: Colony forming units; F1: Fraction 1 antigen; Kd: Dissociation constant; LOD: Limit of detection; LD₅₀: 50% lethal dose; Mab: Monoclonal antibody; MLD: Median lethal dose; OF1: Oncins France 1 mouse; qPCR: Quantitative PCR; rV: Recombinant V antigen; SDS: Sodium dodecyl sulphate; T_{1/2}: Half-life; TTSS: Type three secretion system; V: Virulence antigen.

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Graphical Abstract



Keywords: Plague, immunotherapeutic, passive therapy, protection, clinical isolate

Introduction

Although an ancient disease, plague is still an unwelcome presence in certain regions of the world, where it exists in enzootic reservoirs and erupts from time to time. According to the World Health Organization (WHO), 3248 cases of human plague have been reported between 2010 and 2015 by 11 countries from Africa, the Americas and Asia [1]. During this period, Madagascar accounted for 74% of all cases reported worldwide with an 18% case fatality rate, making this island the most affected country in the world [1]. Despite good public health measures, human plague cases occur on a seasonal basis in the central highlands (September–April), the most active plague foci in Madagascar, with a particularly serious outbreak occurring during the 2017/18 season with 2417 cases and 209 deaths [2].

Plague pathogenesis, caused by the bacterium *Yersinia pestis*, relies on two major virulence mechanisms: the expression, triggered by the human host's body temperature of a protective proteinaceous capsule around the bacteria, which render them resistant to phagocytosis and cell-mediated killing [3]; and a type III secretion system (TTSS) which injects cytotoxic factors into host cells to promote bacterial colonization of tissues and organs [4]. Central to these mechanisms are the F1 protein in the capsule and the V antigen which orchestrates the TTSS [3, 4]. F1 and V also provide targets for vaccine and therapeutic approaches [5].

Characterization of anti-V monoclonal antibodies

Here, we focus on the protective potential of two monoclonal antibodies (Mabs) directed against the V antigen.

Both Mabs have previously been shown to have at least *in vitro* binding or neutralization activity, protecting cultured cells against the cytotoxic effect of *Yersinia pseudotuberculosis* expressing the *Y. pestis* V antigen [6].

The first Mab is 7.3, an IgG1, which has been extensively studied by ourselves and others to be protective in both bubonic and pneumonic murine models of plague, when given at a range of time-points pre- or post-infection [7–10].

Mab 7.3 has prophylactic and therapeutic potential against *Y. pestis* causing the bubonic and pneumonic forms of plague. In addition, it is known that Mab7.3 binds to a region spanning amino acids 135–275 [9, 11] in the centre of the V antigen conformational structure [12], and this region is strongly influenced by the residue N255 [13]. Mab 7.3 has a fast association with the V antigen, high binding affinity for its target and long half-life ($t_{1/2}$) of the antigen-antibody complex (Kd of Fab for V antigen is $80 \pm 7 \text{ pM}$ with $t_{1/2}$ $533 \pm 42 \text{ min}$) [14]. However when scaled against a number of other anti-V Mabs, this binding affinity was not exceptional and so it seems that it is the location of Mab7.3's binding to the V antigen which is particularly significant in determining its protective effect [10].

It has been hypothesized that the conformational epitope in the V antigen may be accessible to Mab7.3 [15] in the homopentamer formed by the V antigen on the tip of the TTSS needle [4], thus preventing Yop translocation, or disrupting the cooperative activity of Yops B and D to achieve pore formation in host cells, a pre-requisite for translocation [15]. This conclusion is reinforced by the observation that the Fc portion of Mab7.3 is not required for its neutralizing activity [14, 15], suggesting that its neutralizing effect is due to the direct binding

of the variable portions of Mab7.3 to the V antigen. Interestingly, Mab7.3 was originally derived by immunization of mice with an SDS-denatured rV antigen; although one of a panel of 7 Mabs generated in the same way with denatured V antigen and all recognizing the region of V spanning amino acids 135–326, Mab7.3 was the only protective Mab [7].

The second Mab is 29.3, which is also an IgG1 and directed to the V antigen. Much less is known about this Mab, although evidence suggests that it binds to V at a locus near the epitope for Mab7.3 [14]. Also, although its *in vitro* binding to the V antigen is slower, of lower affinity and with shorter half-life (Kd of 399 ± 27 pM with $t_{1/2}$ of 247 ± 11 min) than that of Mab7.3, the variable regions, and not the Fc portion of this Mab have significant affinity for the V antigen [14] and it also has significant neutralizing activity *in vitro* for the cytotoxic effect of *Y. pestis* [15].

Prior to their use in this passive transfer study, the hybridoma supernates of each Mab were purified on a HiTrap Protein L column (Cytiva) to eliminate contamination from bovine immunoglobulin present in hybridoma growth media, and to achieve purified Mab's at concentrations of 1 mg/ml (Mab7.3) and 1.7 mg/ml (Mab 29.3) respectively.

The Mab's were evaluated by SDS-Page and a representative gel is shown for Mab7.3 (Fig. 1).

The limit of detection of V antigen by Mab 7.3 determined by titrating dilutions of the V antigen against a fixed concentration of Mab was 20 ng.ml^{-1} (Fig. 2).

To determine more about the prophylactic and therapeutic potential of these anti-V Mabs and building on the recent data in Amemiya *et al.* 2020 [10], we have now tested the ability of both Mab7.3 and Mab29.3 to protect a different strain of outbred mice from a current clinical isolate of *Y. pestis* from Madagascar.

OF1 female outbred mice (6–8 weeks of age) were used for this study. They were bred at the Institut Pasteur de Madagascar animal facility. Mice were randomized for allocation to treatment group with food and water *ad libitum* and acclimatized to the facility for 5 days prior to any procedures. Mice were dosed with Mab at either 24 h prior to, or 24 h post-infection with a clinical isolate of *Y. pestis*, which had been isolated in 2021 from the bubo of a confirmed plague case (F1⁺, qPCR⁺, culture⁺) in an 18-year-old male survivor, from Ankazobe District, one of the active plague foci in the Central Highlands of Madagascar. The *Y. pestis* 10/21-S strain has been genotyped and is of biovar Orientalis. Before use, the 10/21-S *Y. pestis* isolate was cultured for 2 days in Brain- Heart

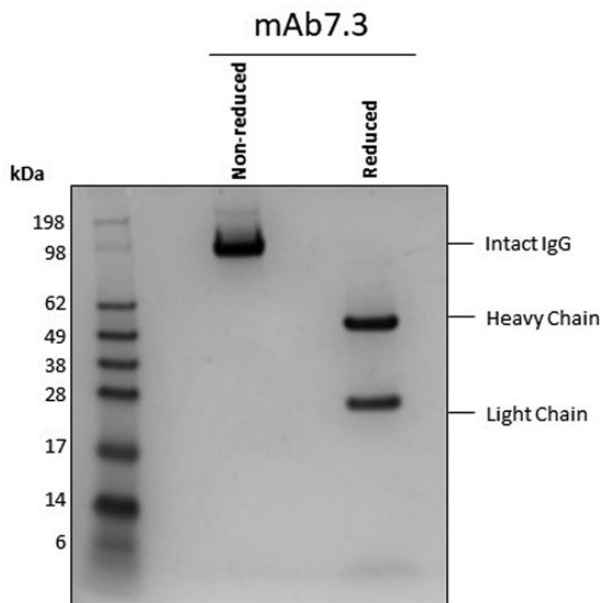


Figure 1. SDS-Page for Mab 7.3. For the nonreduced sample, Mab7.3 was diluted to $5 \mu\text{g}$ in a final volume of $15 \mu\text{l}$ using NuPage LPS 4x sample buffer (Novex, NP007) in accordance with the manufacturer's instructions. The reduced sample was prepared similarly, but with the addition instead of NuPage 10x sample reducing agent (Novex, NP004) and heating of the sample (95°C for 15 min). SDS-PAGE was performed after loading $10 \mu\text{l}$ of antibody samples and $5 \mu\text{l}$ of SeeBlue Plus2 marker (Invitrogen, LC5925) into a NuPAGE 4–12% gradient Bis-Tris gel. Electrophoresis conditions were 200 V for 20 min. The gel was stained with SimplyBlue Safe Stain (Invitrogen, LC6065) for 1 h and destained in distilled water overnight.

Infusion (BHI) broths (Oxoid Ltd., Basingstoke, England) to provide an inoculum and on Cefsulodin-Ingrasan-Novobiocin (CIN) agar plates to enumerate viable bacterial colonies. Following culture of *Y.pestis* 10/21-S in BHI at 37°C for >24 h, F1 and V expression were detected by immunoassay, whilst DNA from a 26°C-cultured extract of *Y. pestis* 10/21-S was positive for V antigen by qPCR. An *in vivo* pre-screen in naive OF1 mice had determined the median lethal dose (MLD) of the 10/21-S strain to be 65cfu (data not shown).

For the efficacy study, an estimated 30 cfu of the *Y.pestis* 10/21-S, determined by measuring the OD₆₀₀ of

the bacterial suspension, was inoculated sub-cutaneously (s.c.) in 0.1 ml at the back of the neck to all groups of mice. Mice were divided into seven groups, each comprising five mice and were dosed as follows:

Groups 1 and 2 were dosed with 50 µg Mab 7.3 at 24 h prior to (-24 h), or at 24 h post-infection (+24 h) respectively, with *Y. pestis*. Mab7.3 was diluted in PBS and delivered in 0.1 ml by the intra-peritoneal (i.p.) route. Groups 3 and 4 were dosed with 50 µg Mab 29.3 in 0.1 ml i.p. at -24 h or +24 h, respectively. Groups 5 and 6 were dosed with a 1:10 dilution in PBS of a pooled macaque immune serum, delivered in 0.1 ml i.p. as a positive

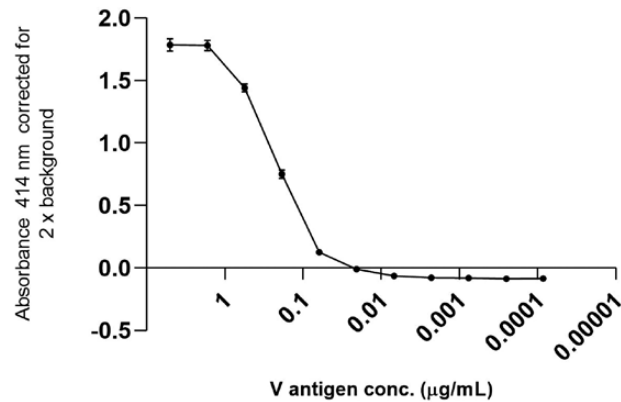


Figure 2. Limit of detection of V antigen by Mab 7.3. V antigen was coated to a microtitre plate in the dilution range 5 µg/ml to 85 pg/ml in triplicate (0.1 ml/well) and then probed with a fixed concentration (800 ng/ml) of biotinylated Mab 7.3. After washing in PBS/Tween 20 and blocking with 2% milk powder in PBS (Blotto), binding was detected with streptavidin peroxidase (Pierce High Sensitivity Streptavidin-HRP, 21132) at 1:10,000 in Blotto and developed with 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; SeraCare, 5120-0041) and the signal was read at OD₄₁₄. The LOD was 20 ng/ml.

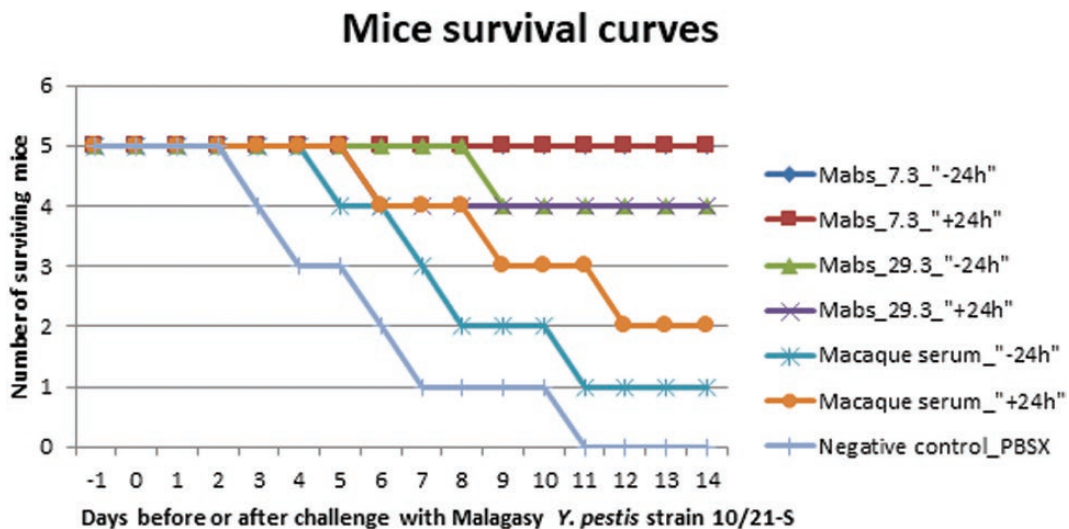


Figure 3. Survival of mice dosed with Mab 7.3. or Mab 29.3 or macaque immune serum pre- or post-infection with 95 cfu *Y. pestis* 10/21-S.

control, at -24 h or +24 h, respectively. Group 7 mice served as negative controls and were dosed with 0.1 ml PBS by the i.p. route, at +24 h. The *Y. pestis* inoculum was enumerated retrospectively, from duplicate cultures on CIN-agar and determined to be 95 cfu/0.1 ml. Mice were observed for 14 days post-infection (p.i.) and were culled if they reached the humane end point beforehand. Survival curves are shown in Fig. 3.

All of the negative control mice had succumbed to the bacterial infection by day 11. Mice receiving the pooled immune macaque serum at -24 h or +24 h had survival rates of 1/5 and 2/5, respectively, by day 14. In contrast, 4/5 mice receiving Mab 29.3 at either -24 h or +24 h, survived until the end of the observation period (day 14). However 5/5 mice administered Mab 7.3 at either -24 h or +24 h, survived to day 14 (Fig. 3; Table 2). Statistical analysis of the survival curves showed that Mabs 7.3 and 29.3 conferred significant survival benefit, compared with the negative control group ($P = 0.0002$, $P = 0.007$, respectively by Log-rank Mantel-Cox test). There were no significant differences in the survival curves resulting from Mab7.3 versus Mab29.3, when either Mab was given at -24 h or +24 h. There were no significant differences in the survival curves resulting from pre- or post-infection delivery of the macaque serum, but the macaque serum did not provide any survival benefit over untreated mice ($P = 0.06$); Mab 7.3 given at +24 h conferred significant benefit over the macaque serum at +24 h ($P = 0.05$).

Analysis of blood and tissues from surviving mice

Analysis of spleens and blood collected at the end of the observation period showed no recovery of *Y. pestis* from mice treated with Mab7.3 at -24 h and from only 1/5 mice treated with Mab 7.3 at +24 h (Table 1).

Remarkably, 4/5 mice pretreated with Mab 7.3 had developed IgG to F1 antigen, suggesting that they had been exposed to F1-expressing, replicating bacteria. In contrast, 0/5 mice post-treated with Mab7.3 had developed IgG to F1, suggesting that p.i. dosing with Mab 7.3 had prevented bacterial replication to the extent that F1 expression did not occur *in vivo* to a level to induce an antibody response.

The effect of pre- or post-dosing with Mab 29.3 on these parameters was less polarized: in each group, 2/5 mice were positive for IgG to F1 antigen and 1/4 mice surviving at day 14 p.i. was positive for *Y. pestis* in the pre-treatment group and 0/4 in the post-treatment group.

Pre-treatment with macaque immune serum resulted in 1 survivor at day 14 p.i.; this mouse was positive for plague and all the mice in this group had developed IgG to F1. The 2 surviving mice in the post-treatment with macaque immune serum group were negative for *Y. pestis* and negative for IgG to F1. There were no survivors in the negative control group; all were positive for *Y. pestis* and 3/5 had developed IgG to F1.

Discussion

Immunotherapy with Mab's is being widely used in clinical medicine [16, 17], particularly for conditions such as rheumatoid arthritis and cancer [18] and more recently has been adopted as an adjunct to treating infectious disease in, for example, the successful use of the anti-IL6R therapy Tocilizumab in Covid-19 infection [19]. Here, we have explored Mabs to plague as potential immunotherapeutics.

This small study adds to the burden of proof of the efficacy of the anti-V Mab 7.3 as a potential therapeutic for plague. This Mab has now been independently tested in a number of laboratories and found to be consistently

Table 1. Summary of results

Group and time of dosing relative to <i>Y. pestis</i> infection	N mice	N surviving mice (D14)	N surviving mice with <i>Y. pestis</i> ¹	N positive anti-F1 IgG serology ²
Mab 7.3 -24 h	5	5	0	4
Mab 7.3 +24 h	5	5	1	0
Mab 29.3 -24 h	5	4	1	2
Mab 29.3 +24 h	5	4	0	2
Macaque serum -24 h	5	1	1	5
Macaque serum +24 h	5	2	0	0
PBS +24 h	5	0	0	3

¹Identified by culture of spleens and blood on CIN media and on BHI broth [22]. API20E biochemical strips were used to identify *Y. pestis*.

²Whole blood and sera were assayed for IgG to F1 antigen as previously described [23]. The threshold of positivity was set at an OD of 0.150 when using serum and at OD 0.050 when using whole blood.

Table 2. The reported protective efficacy of Mab7.3 in murine bubonic and pneumonic plague models

Mab 7.3 dosage	Pre-infection delivery; mouse strain	Post-infection delivery; mouse strain	Pneumonic /bubonic; median lethal dose/LD ₅₀ ; Y.p. strain	Survival	Reference
350 µg	-24 h; Balb/c		Bubonic; 12 MLD; GB	6/6	[7]
35 µg	-24 h; Balb/c		Bubonic; 9.6MLD; GB	5/6	[8]
35 µg	-24 h; Balb/c		Bubonic; 96MLD; GB	3/6	[8]
35 µg	-4 h; Balb/c		Bubonic; 46MLD; GB	7/10	[8]
35 µg	-4 h; Balb/c		Pneumonic; 88MLD; GB	10/10	[8]
35 µg	-24 h; Balb/c		Pneumonic; 88MLD; GB	8/10	[8]
35 µg		+24 h; Balb/c	Bubonic; 46MLD; GB	7/10	[8]
35 µg		+48 h; Balb/c	Bubonic; 46MLD; GB	3/10	[8]
35 µg		+96 h; Balb/c	Bubonic; 46MLD; GB	0/10	[8]
30 µg		+24 h; C57Bl6	Pneumonic (Intranasal); 12LD ₅₀ ; CO92	10/10	[9]
3 µg		+24 h; C57Bl6	Pneumonic (Intranasal); 12LD ₅₀ ; CO92	0/10	[9]
50 µg	-24 h; Balb/c		Bubonic; 21LD ₅₀ ; CO92	8/8	[10]
100 µg	-24 h; Swiss-Webster		Bubonic; 38LD ₅₀ ; CO92	6/6	[10]
50 µg	-24 h OF1		Bubonic; 1.5MLD; 10/21-S	5/5	This paper
50 µg		+24 h OF1	Bubonic; 1.5MLD; 10/21-S	5/5	This paper

efficacious in murine studies (summarized in Table 2) in protecting against a number of clinical isolates of *Y. Pestis*, e.g. the GB isolate from a fatal laboratory accident in the UK in the 1960's [cited in 20], the CO92 isolate from the USA [21], and now the 10/21-S isolate from Madagascar. Additionally, Mab 7.3 has been fully characterized in terms of its amino acid sequence (unpublished data), it is likely binding site for the V protein, its ability to neutralize the cytotoxic effect of the V antigen expressed *in vitro* and its protective effects *in vivo* in models of both pneumonic and bubonic plague. This Mab is currently being humanized for eventual clinical use.

Here, we have shown for the first time that Mab 29.3 can also protect mice *in vivo* against injected infection with the 10/21-S isolate of *Y. pestis*, adding to the previously published *in vitro* neutralization data for this Mab. Although not as solidly protective as Mab7.3, the level of protection observed (80%) for Mab 29.3 remains high, particularly since Mab therapy for the serious infection of plague, would likely be part of a layered approach in which the post-exposure therapy of individuals might also include vaccination and/or antibiotic therapy. Additionally, here we have tested only the 50 µg dose level of Mab29.3; by increasing the dose administered it may well be possible to improve efficacy.

A single bolus of passively transferred antibody (monoclonal or polyclonal) will have a limited half-life *in vivo* which, exacerbated by lesser neutralizing activity, is why we have seen breakthrough in this study in the protective effect conferred by the immune macaque

serum used for reference here. *In vitro* data gained recently indicate that this pooled, polyclonal macaque reference serum [6] has become poorly neutralizing for the V antigen over some years of storage (unpublished data).

The F1 and V antigens are only produced by *Y. pestis* when the bacteria enter the host and expression is triggered at 37°C. F1 secreted by the bacteria forms a dense proteinaceous capsule around the bacteria, while the V antigen is intracellular and is secreted through the bacterial injectisome into host cells. It is not certain how long this process takes *in vivo*, perhaps 24 h or more and the level of expression will be challenge dose-related. Since none of the mice given Mab7.3 at 24 h p.i. had developed an anti-F1 response, this suggests that delivery of the Mab at this time may have effectively limited host cell invasion and replication of the bacterial challenge so that F1 was not expressed *in vivo*, at least not to a sufficient level to induce an IgG response. In contrast, 4/5 of the mice dosed with Mab 7.3 at -24 h had developed an anti-F1 titre, suggesting that although all of these mice survived, delivery of the Mab this much in advance did not limit bacterial replication such that the mice were exposed to F1-expressing bacteria.

The data reported here for Mab7.3 and 29.3 reinforce the importance of antibody responses to V as a key protective mechanism in plague. As well as their obvious immunotherapeutic potential, these Mabs also constitute a reference reagent to underpin the development and evaluation of existing and new vaccines against new isolates of *Y. pestis*.

Clearly, more work will need to be done in non-human primates and clinical safety trials, to determine a translational dosing regimen in man for Mab 7.3, but the accumulated murine data to date provides a sound basis for this.

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Author contributions

Conceptualization (E.D.W.); Funding acquisition (N.A., S.K.); Investigation (K.M.M., V.A., L.N.R.); Methodology (N.J.W., S.G.L.); Project administration (M.R.); Writing (V.A., K.M.M.); Review and editing (E.D.W.); Resources (M.R., S.G.L., E.D.W.).

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Conflict of interest

None of the authors declares a conflict of interest.

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