# PLANT-DERIVED RECOMBINANT F1, V, AND F1-V FUSION ANTIGENS OF *YERSINIA PESTIS* ACTIVATE HUMAN CELLS OF THE INNATE AND ADAPTIVE IMMUNE SYSTEM

G. DEL PRETE, L. SANTI[1], V. ANDRIANAIVOARIMANANA[2], A. AMEDEI, O. DOMARLE[3], M.M. D'ELIOS, C.J. ARNTZEN[4], L. RAHALISON[2] and H.S. MASON[4]

*Department of Internal Medicine, University of Florence and Department of Biomedicine, Azienda Ospedaliera Universitaria Careggi, Florence, Italy; [1]Department of Biology, University of Rome "Tor Vergata", Rome, Italy; [2]Unité Peste and [3]Service d'Immunologie, Institut Pasteur de Madagascar, Madagascar; [4]Biodesign Institute at Arizona State University, Tempe, USA*

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**Plague is still endemic in different regions of the world. Current vaccines raise concern for their side effects and limited protection, highlighting the need for an efficacious and rapidly producible vaccine. F1 and V antigens of *Yersinia pestis*, and F1-V fusion protein produced in *Nicotiana benthamiana* administered to guinea pigs resulted in immunity and protection against an aerosol challenge of virulent *Y. pestis*. We examined the effects of plant-derived F1, V, and F1-V on human cells of the innate immunity. F1, V, and F1-V proteins engaged TLR2 signalling and activated IL-6 and CXCL-8 production by monocytes, without affecting the expression of TNF-α, IL-12, IL-10, IL-1β, and CXCL10. Native F1 antigen and recombinant plant-derived F1 (rF1) and rF1-V all induced similar specific T-cell responses, as shown by their recognition by T-cells from subjects who recovered from *Y. pestis* infection. Native F1 and rF1 were equally well recognized by serum antibodies of *Y. pestis*-primed donors, whereas serological reactivity to rF1-V hybrid was lower, and that to rV was virtually absent. In conclusion, plant-derived F1, V, and F1-V antigens are weakly reactogenic for human monocytes and elicit cell-mediated and humoral responses similar to those raised by *Y. pestis* infection.**

*Yersinia pestis*, the causative agent of plague, is still endemic in Africa, Asia, and the Americas (1). The bubonic form is transmitted via the bite of an infected flea, and bacteria disseminate systemically. The pneumonic form, uniformly fatal, develops from a bubonic state or by aerosol transmission. The control of plague is based on flea control, and chemoprophylaxis of exposed people. The two human vaccines formulated as killed whole cells (KWC) provide little protection against the pneumonic form, have a high incidence of side effects, and require intensive boosting to achieve protection (2). A live attenuated vaccine (EV76) has been used in humans; however, it also causes side effects (3).

Recombinant vaccines based on the fraction 1 capsular antigen (F1), the V antigen, and F1-V fusion protein have proven to be successful in animals and humans (4-5). F1, encoded on a 110-kb plasmid (pMT-1), forms a capsule conferring anti-phagocytic properties to *Y. pestis*. Patient sera show high anti-F1 antibody levels. F1 stimulates protective immunity

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*Mailing address:* Prof. Gianfranco Del Prete,
Dept. of Internal Medicine,
University of Florence,
Viale Morgagni 85,
50134 Florence, Italy
Tel: ++39 055 4378103 Fax: ++39 055-417892
e-mail: gdelprete@unifi.it



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in mice challenged with virulent bacteria (3, 6), and elicits antibody responses in vaccinated humans (3).

The V antigen, a secreted protein encoded on the 70-kb plasmid pCD1, can suppress the host immunity (7). Recombinant V (rV) antigen produced in *Escherichia coli* elicits protection in mice (8), and passive immunization with anti-V antigen sera protects against subcutaneous challenge (9). Combined formulation of F1 and V showed levels of protection in mice three times greater than the KWC vaccine (2). A genetic fusion of the two antigens (F1-V) used as vaccine also induced high titer, long-lasting protective antibodies in animals (4-5).

Recently, there has been considerable interest in using transgenic plants to generate recombinant proteins for medical and veterinary use. A variety of molecules have been expressed in plants, including human proteins or viral and bacterial antigens (10-11). The use of unique vectors (12-13) for robust expression of *Y. pestis* recombinant (r)F1, rV, and rF1-V fusion proteins in leaves of *Nicotiana benthamiana* generated *Y. pestis* antigens that protected guinea pigs against aerosol challenge with *Y. pestis* at doses 100% lethal to unvaccinated controls (14).

Here we describe the *in vitro* effects of plant-derived *Y. pestis* rF1, rV, and rF1-V on cells of the human innate and adaptive immunity. Recognition of microbial products by Toll-like receptors (TLR) leads to initiation of adaptive immune responses (15-16). We show that plant-derived rF1, rV and rF1-V are TLR2 agonists, and they significantly increase IL-6 and, to a lesser extent, CXCL-8 production by human monocytes, without affecting TNF-α, IL-12, IL-10, IL-1β, and CXCL10 production. Moreover, rF1, rV, and rF1-V are recognized by memory T-cells and by serum antibodies of a number of patients who recently recovered from plague.

## MATERIALS AND METHODS

rF1, rV, and rF1-V fusion protein were cloned, expressed and purified from leaves of *Nicotiana benthamiana* (14). The Fl portion of rF1-V consists of 150 amino acids followed by two linker amino acids, and the 326 amino acids of the V sequence. rFl-V consists of 478 amino acids with a predicted molecular mass of 53,193 Da, whereas rF1 and rV are predicted to weigh 15,694 and 37,240 Da, respectively. ELISAs for rF1, rV, and rF1-V were performed by using the *E. coli* recombinant antigens as standards and specific polyclonal antisera (14). Endotoxin-free preparations were lyophilized until used for both human and animal studies (14). Purified native F1 antigen was prepared as reported (6, 17). F1 antigen purification was carried out by 5 precipitations with a gradient of ammonium sulphate (20 to 30%). Purified F1 antigen was lyophilized and stored at -20°C.

PBMC from 16 healthy donors were isolated by centrifugation on Ficoll-Paque. PBMC were laid on Percoll 46% v/v solution (Amersham, Sweden). Monocytes were harvested and further purified by adherence (1 h at 37°C) to plastic. Monocytes ($CD14^+$ cells were >98%) were cultured in RPMI 1640-10% FCS in the absence or presence of graded concentrations (from 0.1 to 10 μg/ml) of rF1, rV, and rF1-V. After 36 h, supernatants were collected and assayed for TNF-α, IL-12, IL-10, IL-1β, IL-6, CXCL-8 and CXCL10 content by specific ELISAs (R&D Systems). To evaluate the effects of rF1, rV, and rF1-V on the LPS-induced cytokine production, monocytes were incubated with graded concentrations of rF1, rV and rF1-V for 2 h before addition of LPS (1 μg/ml), and cytokine secretion was assessed in supernatants after an additional 36 h.

Human embryonic kidney (HEK) 293 cells were used for analysis of TLR-dependent activation. NF-κB dependent reporter gene activation was used as read out, as described (18). Briefly, the analysis of TLR activation was performed on engineered HEK 293 cell lines constitutively expressing one single TLR, as well as pNiFty (an ELAM1 reporter gene driven by NF-κB) (InvivoGen, Toulouse, France). Each HEK 293-TLR cell line was induced with rF1, rV, and rF1-V, and a known specific ligand as positive control (18). Recombinant HEK 293 cell line for the reporter gene only was the negative control. The various NF-κB activation assays were quantified on a luminometer as optical density values (OD) after 24 h stimulation.

PBMC were obtained from 20 subjects (10 males and 10 females) who experienced *Y. pestis* infection (17 bubonic and 3 pulmonary) and were followed up by the Institute Pasteur of Madagascar. The diagnosis of plague was based on F1 antigen detection (19), and direct culture on selective medium, or after a mouse inoculation of clinical specimens (20). Three donors were tested within 20-40 days from diagnosis of bubonic plague. Five patients with bubonic plague were tested between 60 and 90 days after diagnosis. Other 6 donors were sampled between 5 and 20 months after diagnosis (5 bubonic and 1 pulmonary), whereas 5 donors were tested between 26 and 41 months from plague (4 bubonic and 1 pulmonary) and one donor was sampled after 11 years from her pulmonary plague. The 52-year-old mother of a boy who contracted

bubonic plague 18 months before testing, asked to be sampled as a healthy exposed individual. PBMC were also obtained from 20 healthy donors never exposed to *Y. pestis*. PBMC were resuspended in RPMI 1640 medium-5% human serum and seeded (2 x $10^5$/0.1 ml/microwell) in the presence of medium or graded concentrations (0.1, 1 and 10 μg/ml) of purified native F1, or rF1, rV, rF1-V. After 5-day culture, 2 μCi [3H]TdR was added to each microwell, and T-cell proliferation was measured as [$^3$H]TdR uptake 20 hours later. When mitogenic index (MI) was > 2, responses were considered as positive.

To assess whether rF1, rV and rF1-V proteins might be recognized by specific antibodies present in the serum of *Y. pestis*-primed subjects, microplates were coated with each one of the recombinant proteins (rF1, rV or rF1-V) or with native F1. ELISAs were performed as reported (21). Briefly, each microwell was coated with 50 μl antigen (3.3 μg/ml final concentration). After overnight incubation at 4°C, 50 μl of a 1/100 dilution of sera were added, followed by anti-human γ-chain peroxidase conjugate. The enzymatic activity was determined with ortho-phenylene diamine and $H_2O_2$ and OD measured at 492 nm. The cut-off value was determined by testing sera from negative controls. A serum with OD > 0.350 was considered as positive.

Results are expressed by means ± SD. Cytokine levels in supernatants of cultures stimulated with medium alone or plant-derived rF1, rV, and rF1-V proteins were compared using the paired Student's *t* test using Microsoft Excel (Redmond, WA). A *p* value less than 0.05 was considered to be significant.

## RESULTS

After 36 h incubation with medium alone, monocyte cytokine production was low, with the exception of CXCL-8 and IL-6 that were consistently detectable in supernatants from all donors (Table I). Graded concentrations (0.1 to 10 μg/ml) of rF1, rV and rF1-V were added in parallel cultures of monocytes from the same donors and cytokine production was assessed after 36 h. No significant and consistent change was observed in the levels of secreted TNF-α, IL-12, IL-10, IL-1β, or CXCL10 at any concentration of rF1, rV or rF1-V tested. In contrast, 10 μg/ml rF1, rV or rF1-V resulted in a consistent and significant increase of IL-6 production ($p < 0.0001$). Such an increase was less significant ($p < 0.05$) at 1 μg/ml of each protein, but it was undetectable at 0.1 μg/ml. Likewise, 0.1 and 1.0 μg/ml rF1, rV and rF1-V induced no or little increase of CXCL-8, whereas 10 μg/ml of either rF1 or rV significantly increased CXCL-8 secretion ($p < 0.002$). Up-regulation of CXCL-8 induced by 10 μg/ml rF1-V was lower and not significant ($p = 0.66$) in comparison with paired CXCL-8 values measured in cultures stimulated with medium (Table I).

Since earlier studies reported that V antigen inhibited the LPS-induced TNF-α and IL-12 expression in murine macrophages (22-23), rF1, rV, and rF1-V were tested for their effects on the LPS-induced cytokine production by human monocytes. At any rF1, rV, and rF1-V concentration, no significant change was observed in the LPS-induced TNF-α and IL-12 secretion, nor in other cytokines measured in this study (data not shown). Likewise, addition of rF1, rV or rF1-V to the culture did not affect human T-cell proliferation induced by antigen (tetanus toxoid or PPD) or alloantigen stimulation, nor induced proliferation in T-cell derived from donors never exposed to *Y. pestis* (data not shown). These data suggest that plant-derived rF1, rV and rF1-V are mildly reactogenic *in vitro* on human monocytes, and do not affect the Ag-induced activation of human T cells.

To define whether a given TLR could interact with plant-derived rF1, rV and rF1-V, we used HEK 293 cells transfected with plasmids encoding distinct human TLRs (18). Activation was observed only in cells expressing TLR2 (Table II). Dose-response experiments showed the activation of NF-κB by rF1, rV, and rF1-V in a range from 0.1 to 10 μg/ml (Fig. 1), which was weaker than that observed with the positive control PAM2.

Twenty subjects who had previously experienced *Y. pestis* infection provided their informed consent to donate 10 ml blood. A cohort of 20 healthy subjects who had never come into contact with *Y. pestis* served as negative controls. PBMC from donors (# 1, # 2, # 3) tested within 40 days from diagnosis failed to respond to any of the *Y. pestis* antigens (F1, rF1, rV, or rF1-V) (Fig. 2). Of the 5 tested between 60-90 days after diagnosis, 3 subjects (# 5, # 6, # 7) showed T-cell response to F1, rF1 or rF1-V at 10 μg/ml, but not to lower antigen dose (data not shown). T-cells from subjects # 6 and # 7 also showed a moderate response to rV antigen. A significant T-cell proliferation was detected in 4 (# 10, # 12, # 13, # 14) out of the 6 donors tested between 5 and

**Table I.** *Cytokine production by human adherent monocytes in the presence of plant-derived recombinant F1, V or F1-V proteins.*

| Cytokine | Cytokine production (pg/ml) in the presence of | | | |
|---|---|---|---|---|
| | Medium | rF1 (10 μg/ml) | rV (10 μg/ml) | rF1-V (10 μg/ml) |
| TNF-α | 38 ± 64 | 40 ± 43 | 66 ± 57 | 49 ± 41 |
| IL-12 | 14 ± 11 | 15 ± 11 | 14 ± 12 | 15 ± 13 |
| IL-10 | 16 ± 12 | 18 ± 11 | 31 ± 21 | 22 ± 17 |
| IL-1β | 23 ± 20 | 24 ±27 | 27 ± 31 | 24 ±23 |
| IL-6 | 63 ±18 [a] | 442 ± 136 [b] | 378 ± 181 [c] | 403 ± 114 [d] |
| CXCL-8 | 2,204 ± 1,796 [e] | 2,712 ± 1,832 [f] | 2,786 ± 1,908 [g] | 2,584 ± 1,918 |
| CXCL10 | 21 ± 14 | 23 ± 13 | 26 ± 19 | 23 ± 18 |

*Adherent monocytes from PBMC of 16 healthy donors were incubated for 36 h with medium or with graded concentrations of rF1, rV, and rF1-V proteins. Culture supernatants were collected and assayed for their cytokine content by appropriate ELISA assays. Results represent mean values ± SD of triplicate cultures.*
*[a] vs [b], [a] vs [c], and [a] vs [d]: $p < 0.0001$*
*[e] vs [f], and [e] vs [g]: $p < 0.002$*

20 months from diagnosis. Finally, of the 6 donors tested after ≥26 months from diagnosis, only one (# 19) showed poor T-cell response to F1, rF1 and rF1-V, but not to rV antigen. A significant T-cell proliferation to F1 (MI: 16), rF1 (MI: 3.9) and rF1-V (MI: 4.7), but not to rV, was observed in the healthy donor who had *Y. pestis* contact 18 months before, when her son (# 13) developed bubonic plague (data not shown). None of the 20 healthy negative controls showed an MI higher than 1.5, even at the highest antigen dose, indicating that the antigen preparations used in our assays were devoid of contaminants with T-cell stimulatory activity or cross-reactivity with antigens of common pathogens.

In summary, in a time period between 2 and 20 months from diagnosis, 7 out of 11 patients who survived plague and one healthy exposed subject had, in their peripheral blood, a number of T cells specific for *Y. pestis* antigens suitable for detection by proliferation assay. In contrast, no T-cell response was detectable in donors tested within the first month after diagnosis or in the majority (5 out of 6) of donors tested later than 26 months from the disease. Even in the 7 responders to rF1, T-cell proliferation to rV antigen was usually limited. A specific T-cell response to rF1-V was observed in 9 of the donors and in the healthy exposed contact, showing that the fusion protein structure does not hamper the recognition of the relevant T-cell epitopes.

Sera from negative controls did not react with F1, rF1, rV or F1-V, whereas all 7 samples from donors who experienced *Y. pestis* infection showed F1-specific IgG response. The same sera also reacted to plant-derived rF1 with OD slightly higher than those observed with F1 at the same serum dilutions (Table III). Though the F1 sequence was present in rF1-V, the reactivity of anti-F1 positive sera to rF1-V-coated wells was lower than that to rF1, being over the cut-off in only 4 out of 7 seropositive samples (# 5, # 7, # 13, and control E). These data suggest that the rF1-V structure is not optimal for binding of specific antibodies raised by natural exposure to *Y. pestis*, or that a number of serological F1 epitopes in rF1-V are not suitable for antibody binding. The

**Table II.** *Activation by rF1, rV and rF1-V proteins of NF-κB in HEK 293 cells transfected with plasmid encoding distinct human TLRs.*

| TLR trasfected | Ligand | NF-kB activation (mean OD ± SD after 24 h stimulation) |
|---|---|---|
| hTLR2 | Nil | 0.10 ± 0.02 |
| | PAM2 | 0.95 ± 0.10 |
| | rF1 | 0.51 ± 0.09 |
| | rV | 0.62 ± 0.08 |
| | rF1-V | 0.54 ± 0.11 |
| hTLR3 | Nil | 0.13 ± 0.01 |
| | Poly (I:C) | 0.98 ± 0.09 |
| | rF1 | 0.12 ± 0.02 |
| | rV | 0.14 ± 0.03 |
| | rF1-V | 0.11 ± 0.02 |
| hTLR4 | Nil | 0.09 ± 0.01 |
| | LPS | 0.87 ± 0.10 |
| | rF1 | 0.14 ±0.02 |
| | rV | 0.13 ± 0.03 |
| | rF1-V | 0.14 ± 0.02 |
| hTLR5 | Nil | 0.15 ± 0.01 |
| | Flagellin | 1.02 ± 0.11 |
| | rF1 | 0.12 ± 0.02 |
| | rV | 0.14 ±0.02 |
| | rF1-V | 0.11 ± 0.02 |
| hTLR7 | Nil | 0.08 ±0.01 |
| | R848 | 0.81 ± 0.06 |
| | rF1 | 0.11 ± 0.01 |
| | rV | 0.13 ± 0.02 |
| | rF1-V | 0.10 ± 0.02 |
| hTLR8 | Nil | 0.12 ± 0.01 |
| | R848 | 0.96 ± 0.11 |
| | rF1 | 0.14 ± 0.02 |
| | rV | 0.11 ± 0.01 |
| | rF1-V | 0.12 ± 0.01 |
| hTLR9 | Nil | 0.14 ± 0.02 |
| | ODN 1826 | 0.91 ± 0.10 |
| | rF1 | 0.15 ± 0.03 |
| | rV | 0.12 ± 0.03 |
| | rF1-V | 0.11 ± 0.02 |

*Parallel cultures of HEK 293 cells were co-trasfected with expression plasmid encoding one single human TLR for each culture and an NF-κB-dependent luciferase reporter construct. HEK hTLR-transfected lines were stimulated with rF1, rV or rF1-V(10 μg/ml), or with appropriate concentrations of the specific TLR positive control ligands (10 ng/ml $PAM_2$-Cys-SKKK for TLR2, 100 ng/ml poly I:C for TLR3, 100 ng/ml LPS K12 for TLR4, 1 μg/ml flagellin for TLR5, 10 μg/ml R848 for TLR7 and TLR8, and 10 μg/ml ODN 1826 for TLR9). A HEK 293 cell line recombinant for the reporter gene only was used as a negative control. The NF-kB activation in each line was quantified on a luminometer as optical density (OD) values after 24 h stimulation. Results represent mean OD values obtained in a representative out of three consecutive experiments.*

contribution of the V antigen to the recognition by specific antibodies was low, as demonstrated by the limited reactivity to rV antigen of sera from donors who contracted *Y. pestis* infection (Table III). Such a lack of reactivity to rV antigen was confirmed in a series of other 14 subjects who were anti-F1 seropositive (data not shown).

## DISCUSSION

Plague is still an endemic disease in different regions of the world. Discovery of antibiotic-resistant strains, and concern about *Y. pestis* as an agent of biological warfare has highlighted the need for a safe, efficacious, and rapidly producible vaccine. Subunit vaccines are the most attractive option, in particular rF1, rV, and rF1-V, already tested in mice in terms of immunogenicity and protection (6, 9, 24-25). Guinea pigs have also been used in protection studies using rF1 and rV produced in *E. coli* (2). The efficacy of the rF1 and rV to elicit immune responses in healthy volunteers in a phase-I trial has been demonstrated (26). The efficacy of plant-produced rF1, rV, and rF1-V was demonstrated in guinea pigs (14). Antigens produced in plants retain antigenicity and immunogenicity when tested in different animals and in humans (11, 27-28).

In mice, rV expressed in *E. coli* inhibited the LPS-induced macrophage production of TNF-α, CXCL10 and IL-12, and treatment of murine macrophages for 2 h with rV resulted in IL-10

**Table III.** *Serological response to recombinant F1, V or F1-V proteins in subjects who experienced Y. pestis infection*

| Serum sample | Optical density in microwells coated with | | | |
|---|---|---|---|---|
| | Native F1 | rF1 | F1-V hybrid | rV |
| Diluent | 0.058 | 0.057 | 0.068 | 0.065 |
| Negative control A | 0.156 | 0.118 | 0.156 | 0.124 |
| Negative control B | 0.221 | 0.109 | 0.145 | 0.111 |
| Negative control C | 0.213 | 0.079 | 0.092 | 0.079 |
| Negative control D | 0.098 | 0.139 | 0.118 | 0.116 |
| Donor # 5 | 1.693 | 1.714 | 0.876 | 0.151 |
| Donor # 6 | 0.863 | 0.911 | 0.224 | 0.203 |
| Donor # 7 | 1.527 | 1.821 | 0.462 | 0.268 |
| Donor # 10 | 1.120 | 1.225 | 0.210 | 0.067 |
| Donor # 12 | 1.342 | 1.389 | 0.239 | 0.097 |
| Donor # 13 | 0.917 | 1.032 | 0.397 | 0.116 |
| Positive control E | 1.386 | 2.071 | 0.944 | 0.151 |

*Serum samples were diluted 1/100 and incubated overnight at ±4°C in microwell plates coated with native Y. pestis F1 antigen or rF1, rV and rF1-V. Positive control E was a reference serum from a donor who had recovered from plague 8 months before the experimental session, which was used in the laboratory to assess the inter-assay variations. Specific IgG antibodies were measured by ELISA assays, as reported in Materials and Methods. Results represent mean values of duplicate determinations.*

expression (22-23). In our experiments with human monocytes, rF1, rV and rF1-V failed to affect LPS-induced cytokine production, nor up-regulated IL-10. Likewise, rF1, rV and rF1-V showed no effect on antigen-induced T-cell proliferation, suggesting that human T-cell responses are not hampered by plant-derived rF1, rV or rF1-V. A concern for using plant-derived rF1, rV and rF1-V as oral vaccines in humans is the activation of pro-inflammatory cytokines in the gut. Being TLR2 agonists, it would not be surprising if administration of rF1, rV and rF1-V results in a proinflammatory response. However, when tested on normal monocytes, no significant and consistent change was observed in the levels of TNF-α, IL-12, IL-10, IL-1β, and CXCL10, at any of the concentrations of rF1, rV and rF1-V tested. Rather, the addition of 10 μg/ml rF1, rV or rF1-V resulted in a consistent and significant increase of IL-6 production, but such an increase was poor or moderate at 0.1 μg/ml or 1 μg/ml of each protein. Likewise, low dose rF1, rV and rF1-V induced no or little increase of CXCL-8, whereas 10 μg/ml of either rF1 or rV significantly increased CXCL-8 secretion. Interestingly, up-regulation of CXCL-8 induced by rF1-V was lower and not significant ($p = 0.66$) in comparison with CXCL-8 values in supernatants of monocytes stimulated with medium alone. The effects of rF1-V on the cytokine production by human monocytes fully overlapped those induced by the two components of the hybrid. The data suggest that plant-derived rF1, rV and rF1-V are poorly reactogenic on human monocytes.

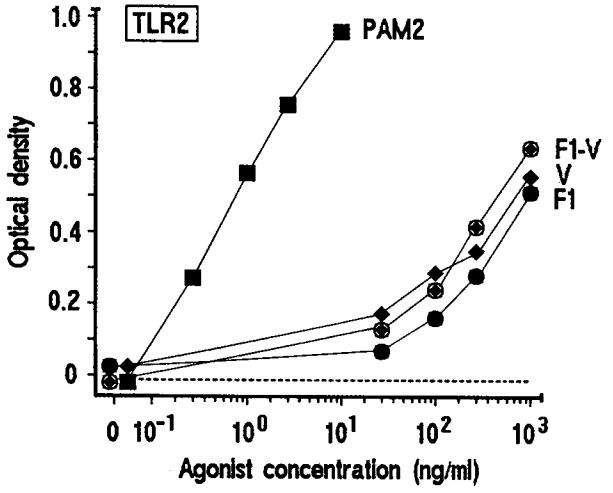


**Fig. 1.** *Activation of NF-κB in HEK 293 cells transfected with plasmid encoding human TLR2. Parallel culture samples of hTLR2-transfected HEK 293 cells were stimulated with graded concentrations of rF1, rV, or rF1-V proteins (from 0.01 to 10 μg/ml), or with graded concentrations of the specific hTLR2 positive control ligand PAM2 (from 0.1 to 10 ng/ml). A HEK 293 cell line recombinant for the reporter gene only was used as a negative control (dotted line). The NF-kB activation in each sample was quantified as optical density (OD) values after 24 hour stimulation. Results of a representative out of three consecutive experiments are reported.*

No induction of pro-inflammatory cytokines, low induction of CXCL-8 and up-regulation of IL-6 represent important features of rF1-V in view of its use as a candidate vaccine for oral immunization, since neither inflammation nor neutrophilia is expected in the gut, while up-regulation of IL-6 may be regarded as a promise of prolonged plasma cell survival and antibody response upon vaccination. Recent data indicate that human secondary lymphoid tissue contains long-lived IgA and IgG secreting plasma cells that depend on IL-6 and intact microenvironment architecture for survival and Ig secretion (29). On the other hand, IL-6, in combination with TGF-ß, is required for initiation of the differentiation of Th17 cells, a distinct $CD4^+$ T-cell lineage, whose role in autoimmunity and chronic inflammatory bowel diseases is still matter of investigation.

The V-antigen (LcrV) was described as the major virulence marker of *Y. pestis* (30). In mice, V antigen is an immunomodulator (TNF-α and IFN-γ down-regulation and IL-10 induction) both *in vivo* and *in vitro* (31-32). Such effects would depend on TLR2 stimulation (33). Evidence of the interaction of V antigen with human TLR2 has been provided (34-35). Overheim et al. (35) showed that LcrV derived from *Y. pestis* and purified from *E. coli* induced IL-10 from murine macrophages and human monocytes. It also suppressed induction of TNF from murine macrophages. However, experiments with highly purified V molecule indicated that only a very high-molecular-weight multimer or aggregate had stimulatory activity (33). In our study, the agonistic interaction of rV with TLR2 was further confirmed. Also rF1 and rF1-V were TLR2 agonists in the same range of relatively high protein concentration required for V protein. Although rV was slightly more reactogenic on human monocytes than rF1, no up-regulation of IL-10, nor down-regulation of pro-inflammatory cytokines were detected. Whether the lack of rV-induced IL-10 increase depends on the source of responder cells (murine vs human) or on recombinant V proteins (bacterial vs plant), remains unclear. If high-molecular-weight multimer of V protein is required for efficient IL-10 stimulation (34), it is possible that plant-derived rF1, rV and rF1-V fail to assemble multimers or aggregates and therefore fail to efficiently stimulate IL-10. In any case, the lack of IL-10 induction in human cells by plant-derived rV and rF1-V represents a positive feature of these candidates for oral vaccination.

Plant-derived rF1, rV and rF1-V are recognized by T cells from *Y. pestis*-primed individuals. In the three subjects tested within 20-40 days from disease, no proliferation of T cells was detectable, though they had already converted to seropositive for anti-F1 antibodies. Likewise, apart from one donor who showed a poor T-cell response to F1, rF1 and rF1-V, but not to rV antigen, other 5 donors tested after 26 months or more from diagnosis showed no proliferation of their circulating T cells to native or recombinant *Y. pestis* antigens. Of another 11 *Y. pestis*-primed donors tested between 2 and 20 months from diagnosis, 7 showed T-cell proliferation to either native F1 or rF1 and rF1-V at the highest concentration (10 μg/ml). In 6 of these 11 donors, T-cell proliferation to rV antigen was also detectable, though lower than to native F1, rF1 or rF1-V. A significant T-cell proliferation to native F1, rF1, and

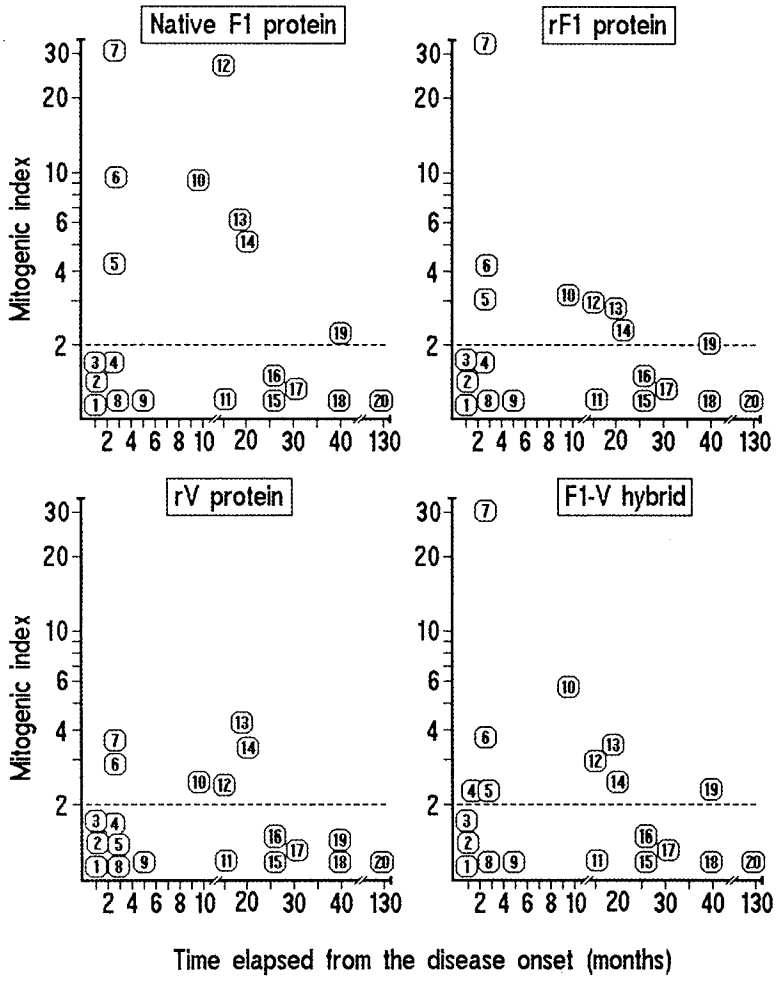


**Fig. 2.** *Proliferative response to Y. pestis native F1, rF1, rV and rF1-V antigens by peripheral blood T cells of Y. pestis-primed individuals. Twenty subjects, who experienced Y. pestis infection, were sampled at different times after diagnosis of bubonic or pulmonary plague. PBMC (2 x $10^5$/0.1 ml) from each donor were cultured in complete medium-5% fresh human serum and seeded in microwells in the presence of 0.1 ml medium or 10 μg/ml purified native F1 antigen, plant-derived rF1, rV or rF1-V. The proliferative response of T cells was assessed by measurement of $^3$H-TdR uptake after 5-day culture. Single donors are indicated by numbers inside symbols. Results represent mean values of triplicate microcultures. Dotted lines indicate a value of 5 SD over the mean of control cultures.*

rF1-V, but not to rV antigen, was observed also in the healthy exposed donor. These data suggest that in the peripheral blood of subjects who experienced plague, the proportion of T cells specific for native F1 or rF1, rV, and rF1-V was quite low, and needed at least one month to become detectable in proliferation assays. The need of relatively high antigen concentration to achieve significant mitogenic indexes argues in favor of this explanation. Data also suggest that in *Y. pestis*-primed donors, the presence of circulating *Y. pestis* antigen-specific T cells is relatively short-term, vanishing after 2 years. Since the analysis of responsiveness by T cells derived from other sources, such as lymph nodes, was not feasible, the question of why T-cell memory for *Y. pestis* antigens is short-lasting in comparison to that against other pathogens remains unclear.

T-cell responsiveness to V antigen was never

dissociated from that against F1, but T-cell proliferation to V was consistently lower than to F1. The simplest explanation is that during *Y. pestis* infection, priming of T-cell response to V antigen was less powerful than to F1. This would have resulted in lower proportions of recirculating V-specific memory T cells and hence lower *in vitro* proliferative responses. Since many pathogens evade the host immune response by variation of surface antigens, it is possible that, as observed for *Y. enterocolitica.* (36), V antigen polymorphism of *Y. pestis* bacteria that had infected our donors might account for their relatively poor T-cell and antibody reactivity to plant-derived rV.

ELISA with native F1 or rF1 showed anti-F1 IgG antibodies in *Y. pestis*-primed individuals. As for T-cell response, antibody response to F1 was detectable in subjects tested within 20 months from the disease, vanishing thereafter. In the same sera, reactivity to rF1 was slightly, but consistently, higher than to native F1, suggesting that rF1 is suitable for diagnostic procedures. In comparison with rF1, rF1-V was not equally well recognized by sera of *Y. pestis*-primed subjects. The lower efficiency of rF1-V for antibody binding may be due to poor recognition of its V portion and to the reduction of B-cell epitopes in the F1 portion due to the fusion protein conformation.

*Y. pestis* rF1, rV and rF1-V generated in *N. benthamiana* show a number of positive features: i) poor reactogenicity on human innate immunity without induction of pro-inflammatory cytokines nor of IL-10, ii) low CXCL-8 up-regulation, iii) induction of IL-6, a promise of prolonged antibody response, and iv) interaction with T-cell receptor of specific T cells similar to the short-term memory T-cells from *Y. pestis*-primed individuals. These aspects, together with the possibility to elicit antibody responses resembling those of *Y. pestis*-infected subjects, the high protection of guinea pigs from the pulmonary disease (14), and demonstration of oral immunogenicity of tomato-derived rF1V (37) suggest a strong potential for these plant-derived antigens as oral vaccine for the prevention of plague.

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