Modified Caspase-3 Assay Indicates Correlation of Caspase-3 Activity with Immunity of Nonhuman Primates to *Yersinia pestis* Infection

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Capsaicin-Rich Extract (CRE)-Based Assay Indicative of Immunity to *Yersinia pestis* Infection

Yersinia pestis, the etiologic agent of plague, has a significant potential to be utilized as a bioterror or a bioweapon (6, 7). Countermeasures, especially those that are effective against pneumonic plague, are critically needed. New candidate vaccines consisting of the F1 capsule antigen and the LcrV, or V, virulence antigen have been developed (5, 10). Both the F1 and the V antigens are highly immunogenic, and they stimulate antibodies that confer passive protection. As the efficacy of these new vaccines cannot be tested in humans, it is essential to develop in vitro surrogate assays that are valid predictors of immunity. We recently described the development of macrophage (MΦ)-based correlate assays of immunity to infection by *Y. pestis* (1). These assays included a microtiter fluorometric assay of the apoptosis-specific enzyme caspase-3 in J774.A1 MΦs, and a fluorescence-activated cell sorter (FACS)-based live-dead staining assay of terminal necrosis of human-derived HL60 cells (1). Sera from mice and nonhuman primates (NHP) vaccinated with the F1-V fusion protein vaccine (5) were evaluated for the levels at which they neutralized the cytotoxicity induced by infection of MΦs with *Y. pestis* or the *Y. pseudotuberculosis* strain Yptb (pTrcV) (1). The results of both the FACS live-dead assay and the caspase-3 assay for serum cytotoxicity neutralization activity (CNA) correlated well with immunity to plague in mice. However, the caspase-3 assay results did not correlate as well as those of the FACS assay with survival in vaccinated NHP, the species that appears to best model human responses to plague, and the FACS assay was proposed as a candidate in vivo correlate assay. Nonetheless, compared to the FACS assay, the caspase-3 assay is easier to perform and analyze, it is faster and requires less expensive equipment, and it is more amenable to large-scale testing of sera from vaccinees. In addition, NIAID- and DOD-sponsored research of new biodefense countermeasures stipulates the development of a validated in vitro correlate assay(s) of immunity (4). Such validated assays are also required, as part of the advanced development, to secure FDA licensure of the F1-V human plague vaccine; and phase 1 and 2 clinical trials of the F1-V fusion vaccine have been initiated (2). Results of prevalidation studies using the FACS and caspase-3 assays (data not shown) indicated that the latter would more readily fulfill the validation requirements that include demonstration of assay reproducibility, repeatability, and robustness (3, 8). Thus, the purposes of this study were to modify and optimize the performance of the caspase-3 procedure and to test this modified assay as a potential in vitro predictor of immunity in vaccinated NHP. Sera from a large, recently completed study done with cynomolgus macaques to test F1-V vaccine efficacy were available for use, as described below.

Numerous changes were made to the caspase-3 microtiter assay, and a complete protocol is shown in Table 1. Changes were made in the following: the cell and bacterial culture media, the final concentration of bacteria in the assay, the amounts of antibody used in pretreatment and titration steps, and the assay incubation periods. Also, greater emphasis was given to important aspects of bacterial propagation, and more details were provided for statistical analyses of data (Table 1). The modified procedure was evaluated initially by titrating the CNA of a rabbit anti-V immunoglobulin G (IgG) preparation, which was used as the positive control in the original MΦ assay (1). A good dose response was observed in twofold titration assays as determined by regression analysis. Whereas rabbit anti-V IgG diluted 1/100 protected 85% of the cells, the highest dilution of 1/1,600 protected only 3% of the MΦs against *Yersinia* infection-induced apoptosis (Fig. 1), and a 50% reciprocal neutralization titer value of 457 was determined, as described in the legend to Fig. 1.

Sera from a recent study of the efficacy of the F1-V fusion vaccine in cynomolgus macaques were tested with the caspase-3 assay; the results of that study will be reported separately. That study, which involved four cohorts of 16 animals each, assessed the association between vaccine dose and protection against lethal aerosol challenge with the
virulent \textit{Y. pestis} strain C092 (cohorts 2 to 4). It also analyzed the long-term immunity of the vaccine (cohort 1). The NHP in all four groups were vaccinated three times with F1-V by the intramuscular route on days 0, 56, and 182 and were challenged by aerosol exposure to lethal doses of strain C092 at 1 month (cohorts 2 to 4) or 1 year (day 547; cohort 1) after the third vaccine dose. Sera were collected at various times over the course of the prechallenge vaccination

| Assay stage     | Step | Description*                                      | Comment |
|-----------------|------|---------------------------------------------------|---------|
| Cell and bacterial culture | 1    | Seed J774.A1 M$$\Phi$$s into 96-well trays (200 $$\mu$$l/well) of a 5 \times 10^5 cell/ml suspension. | Use freshly prepared EMEM with Earle’s salts (Invitrogen) supplemented with 2 mM nonessential amino acids (Sigma), 2 mM L-glutamine (HyClone), and 10% FBS (Gibco). The J774.A1 M$$\Phi$$s performed optimally in the assay when used at a passage less than 25; cells passaged at <22 were used. |
| Preparation of bacterial inoculum | 2    | Inoculate an overnight culture of \textit{Versinia pseudotuberculosis} strain Yptb pTrcV (1) in BHI broth supplemented with ampicillin, kanamycin, EDTA, and MgCl$_2$, as described previously (1). | Use fresh growth from a BHI agar slant consisting of BHI agar with ampicillin and kanamycin. |
|                | 3    | Dilute the overnight culture 1/20 into 25 ml of BHI broth and incubate the flask at 37°C for 2 h with shaking at 225 to 250 rpm. | Vigorous growth of the bacteria during the 2-h preculture step is important; cultures that do not increase at an OD$_{620}$ of at least six- to eightfold (3–4 doublings) are often not sufficiently cytotoxic. |
| Pretreatment | 4a   | After the culture has incubated for 2 h, transfer the culture to a 50-ml centrifuge tube and centrifuge the culture for 10 min at 2,600 rpm to sediment the bacteria. | |
|                | 4b   | After centrifugation is complete, resuspend the pellet in 3 ml of EMEM-FBS and determine the bacterial concn (with, e.g., an Ultrospec UV spectrophotometer; Amersham Pharmacia) at OD$_{620}$, Dilute and adjust the bacterial inoculum in warm EMEM-FBS to an OD$_{620}$ of 0.25 in the required vol (see step 6). | |
| Incubation and development | 5    | Prepare the J774 tray. Just before treating the bacteria with anti-V Ab/serum, remove the medium and immediately add prewarmed EMEM-FBS to the 96-well trays (200 $$\mu$$l of EMEM-FBS in uninfected control wells and 100 $$\mu$$l in the well to be infected). | |
|                | 6a   | Pretreat the bacteria and add the sample to the tray. For each sample, combine 0.5 ml of adjusted bacteria in a microtube with Ab/serum (in amts of 10–40 $$\mu$$l) diluted to twice that of the final desired dilution in the tray. Use medium instead of Ab for the negative control sample. For titration experiments, the Ab/serum is prediluted in EMEM-FBS in a microtiter tray, and 20 $$\mu$$l of each is added to a tube of bacteria. | |
|                | 6b   | Incubate the microtubes at 38°C for 5 min with shaking (1, 9) and add 100 $$\mu$$l of the pretreated bacteria to the wells that are to be infected. Test all samples in quadruplicate. | |
|                | 7    | Centrifuge the tray for 5 min at 800 rpm in a centrifuge that accommodates 96-well-plate carriers. | |
|                | 8    | Incubate the tray for 2 h at 37°C in 5% CO$_2$. Dilute gentamicin (Invitrogen) into EMEM-FBS and add 20 $$\mu$$l of the dilution to each well, for a final concn of 50 $$\mu$$g/ml gentamicin. | |
|                | 9    | Incubate the tray once more for 1.5 h, remove the supernatants from the wells, and wash the samples once, gently, in 10–20 mM PBS without magnesium or calcium. | |
|                | 10   | Remove the medium and measure caspase-3 levels as described in the EnzChek caspase-3 II kit instructions (Molecular Probes). After adding the lysis buffer, place the tray in a $-70^\circ$C freezer until the medium is frozen, and then thaw the medium completely by placing the tray in a 37°C incubator. Centrifug e the tray and transfer the supernatants to a black, clear bottom tray (Costar). Add an equal volume of reaction solution and, after incubating the sample for 30 min, read the fluorescence with, e.g., Softmax Pro software on a Gemini or M2e spectrometer (Molecular Devices) with settings of 485 nm em and 530 nm ex. Export the data to an Excel spreadsheet for regression analyses (see text). | |

*Abbreviations: EMEM, Eagle’s minimal essential medium; FBS, fetal bovine serum; BHI, brain heart infusion; OD$_{620}$, optical density measured at 620 nm; Ab, antibody; Ab/serum, antibody or serum; em, emission; ex, excitation.

The complete medium, containing the components listed here, is referred to herein as EMEM-FBS.
period for in vitro evaluation. Further details of the vaccination doses and schedule are described in the figure legends and by Adamovicz et al. (manuscript in preparation).

Figure 2 illustrates the caspase-3 results of tests done with sera collected on five separate days from three representative F1-V-vaccinated NHP of cohort 1. These results, as well as those of assays of additional sets of sera and those including sera collected on days 0, 14, 56, 70, 182, 196, and 469 (cohort 4 only) of the vaccination period, confirmed a finding reported previously for the live-dead assays (1). For all four animal groups, the day 70 and day 196 sera, which were collected 2 weeks after the second and third doses of vaccine, respectively, exhibited the greatest extent of MΦ protection compared to that associated with sera collected at other times during vaccination (Fig. 2 and data not shown).

The relationship between the caspase-3 levels of MΦs infected with serum-pretreated Yersinia and the survival of the vaccinated NHP was then evaluated by logistic regression analysis of the pooled cohort data. As illustrated in Fig. 3, the extent to which caspase-3 levels decreased was significantly associated with survival for both the day 70 and the day 196 results (\(P = 0.0038\) and \(P = 0.0024\), respectively). The serum CNA was also highly correlated with the anti-V and anti-F1 enzyme-linked immunosorbent assay titers for both the day 70 and the day 196 sera (\(P \leq 0.001\), data not shown). The significance was observed when the caspase-3 levels measured in MΦs infected with the pretreated bacteria were compared to either the experimental negative control level (infected, nontreated MΦs; \(P = 0.0020\) to 0.0001; data not shown) or to the caspase-3 levels measured in infected MΦs pretreated with day 0 (preimmune) sera from each animal (\(P < 0.0001\)) (Fig. 3).

![FIG. 1. Titration of the CNA of the rabbit anti-V IgG positive control. Twofold dilutions of the IgG were assayed in the caspase-3 microtiter fluorescence assay. Caspase-3 levels represent a marker for MΦ cytotoxicity (apoptotic cell death) associated with the Yersinia infection. The data shown (dashed line with symbols) are the mean percentages of decrease in caspase-3 values for each dilution tested (in quadruplicate). These values were analyzed by four-parameter nonlinear regression (solid line) using GraphPad Prism5 software. The dilution interpolated to represent 50% neutralizing activity in this analysis was 1/457 (log 2.66); \(r^2\) value, 0.9998.](http://cvi.asm.org/)

![FIG. 2. Neutralization of Yersinia-mediated J774.A1 macrophage cytotoxicity by immune sera from F1-V-vaccinated cynomolgus macaques, as detected in the caspase-3 fluorescence assay. The caspase-3 data are shown as mean relative fluorescence units (RFU) (+ standard deviations) of four replicates per serum sample (1). Tests were done with sera from three representative F1-V-vaccinated NHP of cohort 1 of this vaccine trial, and the results shown are those using sera collected on vaccination days 0, 56, 70, 182, and 196. Data are grouped by the day of serum collection. The three controls were (left to right) uninfected MΦs, infected untreated MΦs, and MΦs infected with bacteria pretreated with the positive control rabbit anti-V IgG. NS, nonsurvivor; S, survivor. *, \(P \leq 0.0001\), compared to the mean value for the day 0 serum-treated samples (analysis of variance and t test).](http://cvi.asm.org/)
Thus, the results of the modified caspase-3 microtiter assay for serum-mediated CNA were highly correlated with survival status of F1-V-vaccinated NHP after they were challenged with virulent *Y. pestis*.

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