Direct Neutralization of Type III Effector Translocation by the Variable Region of a Monoclonal Antibody to Yersinia pestis LcrV

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Plague is an acute infection caused by the Gram-negative bacterium Yersinia pestis. Antibodies that are protective against plague target LcrV, an essential virulence protein and component of a type III secretion system of Y. pestis. Secreted LcrV localizes to the tips of type III needles on the bacterial surface, and its function is necessary for the translocation of Yersinia outer proteins (Yops) into the cytosol of host cells infected by Y. pestis. Translocated Yops counteract macrophage functions, for example, by inhibiting phagocytosis (YopE) or inducing cytotoxicity (YopI). Although LcrV is the best-characterized protective antigen of Y. pestis, the mechanism of protection by anti-LcrV antibodies is not fully understood. Antibodies bind to LcrV at needle tips, neutralize Yop translocation, and promote opsonophagocytosis of Y. pestis by macrophages in vitro. However, it is not clear if anti-LcrV antibodies neutralize Yop translocation directly or if they do so indirectly, by promoting opsonophagocytosis. To determine if the protective IgG1 monoclonal antibody (MAb) 7.3 is directly neutralizing, an IgG2a subclass variant, a deglycosylated variant, F(ab′)2, and Fab were tested for the ability to inhibit the translocation of Yops into Y. pestis-infected macrophages in vitro. Macrophage cytotoxicity and cellular fractionation assays show that the Fc of MAb 7.3 is not required for the neutralization of Yop or YopE translocation. In addition, the use of Fc receptor-deficient macrophages, and the use of cytochalasin D to inhibit actin polymerization, confirmed that opsonophagocytosis is not required for MAb 7.3 to neutralize translocation. These data indicate that the binding of the variable region of MAb 7.3 to LcrV is sufficient to directly neutralize Yop translocation.

Yersinia pestis is a Gram-negative bacterium and the agent of plague, an acute, often fatal infection that can manifest in three forms: bubonic, pneumonic, or septicemic (1, 2). Y. pestis has several characteristics that could facilitate its development as a biological weapon, resulting in its classification as a tier 1 select agent. These characteristics include the capacity for aerosol dissemination and the high fatality rate of pneumonic plague (3). In addition, Y. pestis remains a constant threat to public health because there are large enzootic reservoirs of plague in rodents in North and South America, Asia, and Africa, resulting in regular outbreaks of the disease in human populations (3).

It is important to develop new strategies to counteract Y. pestis infection. For example, there is a need for the development of immunotherapeutics to treat plague. Y. pestis secretes several proteins that have been studied as immunotherapeutic targets (2, 4, 5). The F1 protein is encoded on plasmid pMT1 and is assembled into an antiphagocytic capsule by a chaperone-usher pathway (1, 6). Mice passively immunized with an anti-F1 monoclonal antibody (MAb) (e.g., F1-04-A-G1) are protected against bubonic or pneumonic Y. pestis infection (4, 5). Anti-LcrV antibodies opsonize Yersinia by binding LcrV at the needle tip (13, 14). Protection by an anti-LcrV antibody in vivo correlates with reduced Yop translocation and cytotoxicity and increased opsonophagocytosis by macrophages in vitro (15, 16). Polyclonal F(ab′)2 to LcrV is as effective as intact IgG at inhibiting cytotoxicity in Y. pestis-infected macrophages (16). However, F(ab′)2 specific for LcrV was ineffective in promoting opsonophagocytosis (15, 16). These results suggest that the Fc region of the anti-LcrV antibody is not required for the neutralization of Yop translocation but is required for opsonophagocytosis. However, an anti-LcrV antibody did not neutralize the translocation of Yops into Y. pestis-infected macrophages that were treated with cytochalasin D (CD) to inhibit actin polymerization (15), suggesting that opsonophagocytosis neutralizes translocation indirectly, through internalization of the bacteria. Thus, the mechanism by which anti-LcrV antibodies neutralize the translocation of Yops into immune cells infected with Y. pestis remains unclear.

As reviewed in reference 17, several murine MAbs specific for...
LcrV have been shown to passively protect mice from bubonic or pneumonic plague (9, 18–21). The murine MAb 7.3 is potently protective; a single dose of 30 μg fully protects mice against intranasal challenge with 12.5% lethal doses (LD₅₀) of Y. pestis (22). MAB 7.3 neutralizes Yop-dependent cytotoxicity and promotes opsonophagocytosis in macrophages infected with Y. pestis in vitro (16, 23).

The protective epitope in LcrV that is recognized by MAB 7.3 is conformational and localizes to amino acids 135 to 275 (18, 24, 25). Determination of the 3-dimensional structure of LcrV (26) revealed that it has an overall dumbbell shape, with the “handle” composed of two helices (alpha 7 and alpha 12) that form a coiled-coil. The LcrV N terminus forms a globular domain at one end of the handle. A second globular domain that is formed by the region between alpha 7 and alpha 12 in LcrV is found at the other end of the handle. The protective epitope recognized by MAb 7.3 corresponds to alpha helix 7 and the globular domain between helices 7 and 12.

The goal of this study was to determine if MAb 7.3 neutralizes Yop translocation directly or indirectly, by promoting opsonophagocytosis. To achieve this goal, variants of the IgG1 MAB 7.3 were obtained, by either class switching (to IgG2a), deglycosylation, or removal of the Fc region [F(ab')₂, or Fab]. The resulting variants were tested for the ability to inhibit the translocation of Yops into macrophages infected with Y. pestis in vitro. In addition, the importance of opsonophagocytosis for MAB 7.3 to neutralize Yop translocation was tested using macrophages deficient in the Fc receptor (FcR) or macrophages treated with CD to inhibit actin polymerization.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All Y. pestis strains used lack the pigment ation locus (Δp69) and are exempt from select agent guidelines. KIM5 and KIM5opB contain the pCD1 and pPCP1 plasmids and have been described previously (27). To prepare bacteria for macrophage infection assays, Y. pestis cultures were grown in heart infusion (HI) supplement with ampicillin at 25 μg/ml with aeration overnight at 26°C. Bacteria were subcultured into HI broth containing 2.5 mM CaCl2 to an optical density at 600 nm (OD₆₀₀) of 0.1. Cultures were shaken at 37°C for 16 h before infection. To prepare bacteria for macrophage infection assays, Y. pestis in DMEM containing 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate (DMEM-10%) was incubated for 16 h before infection. Wells incubated in 3 ml of DMEM-10% without RAW 264.7 cells but otherwise treated identically were used as controls for the translocation assay. Prior to addition to the tissue culture plates, bacteria prepared as described above were dislodged into fresh tissue culture medium, either BMM Low for BMDMs or DMEM-0.2% for RAW 264.7 cells, containing the MAb, a MAb variant, or PBS as indicated in the figure legends. The tissue culture medium in the wells was aspirated; wells were washed twice with PBS; and tissue culture medium containing diluted bacteria was added to each well. After the addition of bacteria, the plate was centrifuged for 5 min at 50 × g and was then incubated at 37°C in a CO₂ incubator.

**LDH release assay.** The LDH assay measures cytotoxicity resulting from the activity of translocated Yop E has been described previously (32). Samples of culture media from wells containing BMDMs were collected at 5 h after infection. Levels of LDH were assayed by using the Cytotox 96 assay kit (Promega) according to the manufacturer’s instructions. After 30 min of incubation with the substrate, the reaction was stopped, and absorbance at 490 nm was measured using a VersaMax tunable microplate reader (Molecular Devices). The level of spontaneous LDH release was determined by assaying the supernatants of uninfected macrophages. The level of total LDH was determined by assaying supernatants from infected BMDMs that had been lysed by a freeze-thaw cycle. The percentage of LDH release was calculated according to the manufacturer’s protocol, which includes a correction for spontaneous release.

**Translocation assay.** The translocation of Yop E into RAW 264.7 cells was measured by using detergent solubility and immunoblotting as described previously (33). After a 2-h incubation, the 6-well dishes were placed on ice and were washed twice with 3 ml of ice-cold Hanks balanced salt solution (HBSS). Fifty microliters of Triton X-100 detergent solution (HBSS). Fifty microliters of Triton X-100 detergent solution containing 1% Triton X-100, 1 mM NaCl, 10 mM Tris [pH 7.5], 1% Triton X-100, 1 mM Na₃VO₄, 1 mM NaCl, and 10 mM NaF, and an EDTA-protease inhibitor cocktail (Roche) was added to each well, and the plate was incubated for 15 min on ice with occasional rocking. The contents of the wells were scraped into microcentrifuge tubes and were centrifuged for 10 min at 12,000 × g and 4°C. The supernatants were transferred to new tubes. Protein samples were boiled for 5 min in Laemmli sample buffer containing 0.1% dithiothreitol prior to electrophoresis. The proteins were separated on an SDS–10% polyacrylamide gel via electrophoresis and were transferred to nitrocellulose membranes for immunoblot analysis. The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 1% bovine serum albumin. Membranes were incubated with anti-Yop E MAbs 202
and 149 at a final concentration of 6 μg/ml in TBST, followed by a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Jackson) diluted 1:50,000 in TBST. Band signals on the membranes were detected by chemiluminescence (PerkinElmer). To quantify band signals on the immunoblots, blocking was performed with 1% casein, secondary anti-mouse antibodies conjugated with IR800 were used, and signals were detected by an infrared imaging system (Odyssey; Li-Cor). The band intensities were calculated by using the software provided by the Odyssey system, and the values were plotted as arbitrary units. Statistical analysis of data. Statistical analysis of LDH release data was performed with Prism (GraphPad) software, version 4.0. The tests used are indicated in the figure legends. A P value of <0.05 was considered significant.

RESULTS

IgG1 and IgG2a subclasses of anti-LcrV MAb 7.3 inhibit cytotoxicity equally in Y. pestis-infected macrophages. In cases where interaction with FcR is important for the neutralization of a bacterial virulence factor by a MAb, the subclass can influence the neutralizing activity (31). This results from differential Fc-FcR interactions; for example, IgG1 binds to the low-affinity receptor FcγRIII, while IgG2a binds the high-affinity receptor FcγRI (34). To determine if the IgG1 subclass of MAb 7.3 was important for the neutralization of Yop translocation by this MAb, a class switch variant belonging to IgG2a (7.3-IgG2a) was obtained. Y. pestis KIM5 opsonized with MAB 7.3 or 7.3-IgG2a was used to infect BMDMs in tissue culture wells, and the levels of cytotoxicity resulting from YopJ translocation were measured by an LDH release assay. The neutralizing activity of an additional protective MAb, 29.3, was characterized in parallel. As negative controls, the bacteria either were left nonopsonized or were opsonized with the nonprotective anti-LcrV MAB 5.28. As shown in Fig. 1, MAbs 7.3 and 7.3-IgG2a inhibited cytotoxicity equally, indicating that the subclass does not influence the neutralizing activity of this MAb. Additionally, MAB 29.3, but not MAB 5.28, had significant neutralizing activity.

Anti-LcrV MAB 7.3 inhibits cytotoxicity in Y. pestis-infected macrophages in the absence of Fc-FcR interaction. To determine if FcRs for IgG are important for the neutralization of Yop translocation by MAB 7.3, the abilities of the MAb to inhibit Yop translocation in BMDMs from wild-type and FcγR−/− mice were compared. The γ chain is required for signal transduction by all activating FcRs in mice (34). The cytotoxicity resulting from Y. pestis infection was equally inhibited by MAB 7.3 in BMDMs from wild-type mice and in BMDMs from FcγR−/− mice (Fig. 2).

IgG molecules are glycosylated primarily at Asn-297 of the CH2 domain within the Fc region. This modification is essential for the FcR binding of IgG (34). A preparation of MAB 7.3 that was deglycosylated by treatment with endoglycosidase S was compared with the native MAB over a concentration range for the ability to inhibit cytotoxicity in Y. pestis-infected BMDMs. Deglycosylated MAB 7.3 had slightly lower neutralizing activity than native MAB 7.3, especially at lower MAB concentrations, but this difference was not significant (Fig. 3).

To directly examine the importance of the Fc region of MAB 7.3 for the neutralization of Yop translocation, macrophages were infected with Y. pestis in the presence of MAB 7.3 F(ab′)2, prepared by digestion of the MAB with pepsin. Like deglycosylated MAB 7.3, MAB 7.3 F(ab′)2 had slightly lower neutralizing activity than native MAB 7.3, especially at lower MAB concentrations, but this difference was not significant (Fig. 3). These results indicate that interaction with FcRs is not required for MAB 7.3 to neutralize the translocation of YopJ into macrophages infected with Y. pestis.

MAB 7.3 F(ab′)2 inhibits the translocation of YopE into Y. pestis-infected macrophages. The cytotoxicity assay measures YopJ translocation indirectly. To extend the results presented above to another effector, YopE, and to measure translocation more directly, we used subcellular fractionation by detergent sol-
7.3, deglycosylated MAb 7.3, and MAb 7.3 F(ab’)_2 that was not opsonized. The differences between the values obtained with MAb represents the average level of LDH release from BMDMs infected with KIM5 that was not opsonized. The differences between the values obtained with MAb 7.3, deglycosylated MAb 7.3, and MAb 7.3 F(ab’)_2 were not significant at any concentration (P > 0.05) as determined by 2-way analysis of variance.

The results presented above indicate that binding of the Fab and F(ab’)_2 to LcrV is sufficient to neutralize Yop translocation. To determine if actin polymerization is required for the neutralization of Yop translocation by a Fab, RAW 264.7 cells were infected with Y. pestis in the presence or absence of CD or Fab. The results of a detergent solubility assay show that CD treatment alone (Fig. 6A, lane 5, and B), these data indicate that actin polymerization is not required for the inhibition of Yop translocation by a Fab to LcrV, and they provide additional evidence that neutralization occurs directly rather than as a result of opsonophagocytosis.

**DISCUSSION**

LcrV can be considered a prototype for the class of proteins that are found at the tips of T3SSs in Gram-negative pathogens (35). Tip proteins orthologous to LcrV in other bacterial species include *Pseudomonas* PcrV, *Shigella* IpaD, and *Salmonella* SipD (36). Anti-tip protein antibodies have been shown to inhibit the function of the corresponding T3SS for LcrV (37), PcrV (38), IpaD (39, 40), or SipD (41). A better understanding of how antibodies to LcrV
neutralize Yop translocation thus has important implications for understanding how antibodies inhibit other tip proteins.

Three lines of evidence obtained here indicate that MAb 7.3 neutralizes Yop translocation directly, while opsonophagocytosis via FcRs is dispensable for this activity. First, changing the subclass of MAB 7.3 from IgG1 to IgG2a did not affect neutralizing activity significantly (Fig. 1). Second, preventing the interaction of the MAB with FcRs by deglycosylation of the Fc or removal of the Fc to generate F(ab’)'2 did not significantly reduce neutralizing activity (Fig. 3 and 4). In addition, monovalent Fab with one variable region was neutralizing (Fig. 5). Third, preventing opsonophagocytosis by use of FcR-deficient macrophages or by use of CD to inhibit phagocytosis did not reduce neutralizing activity (Fig. 2 and 6).

Studies showing that anti-LcrV antibodies can inhibit the translocation of Yops into HeLa cells infected with Y. pestis (19) could also be taken as evidence that interaction with FcRs is dispensable for neutralizing activity. However, HeLa cells can express FcyIII (42), and blocking of FcRs on HeLa cells infected with Y. pestis has been reported to reduce the neutralizing activity of an anti-LcrV antibody (15).

Weeks et al. showed that polyclonal anti-LcrV F(ab’)'2 prevented cytotoxicity in Y. pestis-infected macrophages (16), findings that support the direct neutralization mechanism. However, the study of Cowan et al. reported that polyclonal anti-LcrV did not inhibit the translocation of Yops into Y. pestis-infected macrophages treated with CD (15), a finding that is inconsistent with direct neutralization. Weeks et al. and Cowan et al. both used J774A.1 cells, but different assays (cytotoxicity versus detergent solubility) and different preparations of polyclonal anti-LcrV, suggesting that one of the latter two variables resulted in different findings. Experiments that measure the translocation of Yops into Y. pestis-infected macrophages in the presence of CD need to be carefully controlled and quantified, because CD treatment by itself can reduce Yop translocation (43) (Fig. 6).

An important question that remains is how anti-LcrV antibodies directly neutralize translocation. One possibility is that antibody binding induces a conformational change in LcrV. From structural analysis and modeling of LcrV and the T3SS needle, it has been suggested that LcrV forms a homopentamer complex at the tip (44, 45). Binding of neutralizing antibodies could change the conformation of this complex, thereby inactivating the key function of LcrV in translocation. Interestingly, models of the tip complex indicate that the LcrV central globular domain that contains neutralizing epitopes is oriented away from the needle, potentially allowing access to antibodies. Determination of the structure of LcrV bound to MAb 7.3 Fab to allow comparison with the...
unbound monomer might provide evidence for a conformational change. A second possibility is that neutralizing antibodies prevent LcrV from interacting with another Yersinia protein required for translocation. LcrV is proposed to act as a platform for the insertion of YopD and YopD into the plasma membrane to form the translocon (12). Anti-LcrV antibodies reduce the amounts of translocator proteins that are inserted into the membranes of red blood cells infected with Yersinia (46). Therefore, neutralizing antibodies could prevent the interaction of LcrV with YopD or YopB. Neutralizing antibodies could also prevent LcrV from interacting with an unidentified host cell receptor.

A previous study reported that to be fully protective, anti-LcrV MAb s might also provide new opportunities for dispensable for neutralizing activity was not protective (20). However, it will be important to test more directly the importance of Fc for protection against plague by an anti-LcrV MAb in vivo.

A protective MAb specific for Pseudomonas aeruginosa PcrV, Mab166, recognizes a conformational epitope between amino acids 144 and 257 (38, 47). This corresponds to the needle-distal globular domain and is analogous to the domain recognized by protective anti-LcrV antibodies. Interestingly, Mab166 (F(ab′)2) has been shown to be protective against P. aeruginosa in animal infection models (36, 47), indicating that Fc receptor function is dispensable for protection against P. aeruginosa. If Mab 7.3 Fc is dispensable for neutralizing activity in vivo, this could lead to the development of novel immunotherapeutics for plague, such as synthetic variants of protective antibodies, including single-chain variable fragments. Detailed understanding of the LcrV–Mab 7.3 binding relationship might also provide new opportunities for antiplaque drug discovery.

ACKNOWLEDGMENTS

We thank Matthew Scharff, Ray Dattwyler, Maria Gomes-Solecki, Stylianos Bournazos, and Jeffrey Ravetch for providing reagents, Galina Romanov for preparing macrophage cultures, and Matthew Scharff and Hana Fukuto for reviewing the manuscript. We also thank the Hybridoma Facility of the Albert Einstein College of Medicine and Susan Bulh for generating the isotype-switched IgG2a MAb 7.3.

This research was supported by awards from the NIAID (RO1AI099222 and U54AI057158-15).
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