Sequential Proteolytic Processing of the Capsular Caf1 Antigen of Yersinia pestis for Major Histocompatibility Complex Class II-restricted Presentation to T Lymphocytes*

We studied the mechanisms of antigen presentation of CD4 T cell epitopes of the capsular Caf1 antigen of Yersinia pestis using murine bone marrow macrophages as antigen presenting cells and T cell hybridomas specific for major histocompatibility complex (MHC) class II-restricted epitopes distributed throughout the Caf1 sequence. The data revealed diversity in the pathways used and the degrees of antigen processing required depending on the structural context of epitopes within the Caf1 molecule. Two epitopes in the carboxyl-terminal globular domain were presented by newly synthesized MHC class II after low pH-dependent lysosomal processing, whereas an epitope located in a flexible amino-terminal strand was presented by mature MHC class II independent of low pH and with no detectable requirement for proteolytic processing. A fourth epitope located between the two regions of Caf1 showed intermediate behavior. The data are consistent with progressive unfolding and cleavage of rCaf1 from the amino terminus as it traverses the endosomal pathway, the availability of epitopes determining which pool of MHC class II is preferentially loaded.

Caf1 capsular protein is a component of second generation plague vaccines and an understanding of the mechanisms and pathways of MHC class II-restricted presentation of multiple epitopes from this candidate vaccine antigen should inform the choice of delivery systems and adjuvants that target vaccines successfully to appropriate intracellular locations to induce protective immune responses against as wide a T cell repertoire as possible.

Yersinia pestis is the causative agent of plague, probably the most devastating disease in human history (1). Reports of recent outbreaks in India (2) and continuing isolated cases in the United States (3) demonstrate that plague has not yet been eradicated and that the need for an effective vaccine remains. The bubonic form of the disease is transmitted by fleas from the reservoir of rats and domestic cats in some cases (4). Bubonic plague can develop into a highly infectious pneumonic form that is transmitted from person to person by aerosols produced during coughing (5).

Laboratory mice have provided useful models to elucidate the mechanisms of virulence of yersiniae (6) as well as regimes for vaccination against Y. pestis (7). In addition to the Ysc-Yop type III secretion system common to several pathogenic yersiniae (8), Y. pestis bears a unique 100-kb plasmid pFra encoding the capsular Caf1 protein, expression of which is thought to mediate resistance to phagocytosis (9). The mechanism of polymerization of Caf1 by Y. pestis under the influence of the chaperone CafM and subsequent export via the usher CafA has been elucidated recently (10). An additional component of the Caf operon, CafR, regulates temperature-dependent expression of the Caf1 capsule upon infection of the mammary host (11).

Immunization against Caf1 alone (12–17) or in combination with the Ysc-Yop LcrV plasmid-encoded V antigen protects mice against bubonic (18–21) and pneumonic (22–24) forms of plague and has been used in human trials (25). Recombinant Caf1 (rCaf1)2 forms polymers in a similar manner to the native Caf1 encapsulating Y. pestis, and monomerization by denaturation dramatically reduces the protective efficacy, without a detectable effect on the anti-Caf1 antibody response induced (26). Passive immunization with monoclonal IgG antibodies specific for Caf1 is also protective (27,28), suggesting that opsonization is a major mechanism to overcome the resistance of Y. pestis to phagocytosis conferred by the Caf1 capsule. Protective IgG antibody responses are presumed to be T cell-dependent, and the additional role of T cells in amplifying the inflammatory response in plague has recently been investigated (29,30). However, T cell responses to Caf1 have not been studied in any detail (31).

It has been suggested that vaccine delivery to induce protective immune responses is critically influenced by antigen structure as well as the role of adjuvants in targeting protein antigens for optimal antigen processing and presentation to T cells (32, 33). However, few candidate vaccine antigens have been sub-
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| T cell hybridoma | MHC restriction | Synthetic peptide | T hybrid response | 20 residue synthetic peptide |
|------------------|-----------------|-------------------|------------------|-----------------------------|
| P4H1             | A^b             | 1–19              | +                | ADLTASTTATATLVEPARI          |
| P2H2             | A^b             | 10–29             | +/−              | TATLVEPARIYLVKGAPI          |
| H6B              | A^a             | 50–69             | +                | GYKTGTTSSTSVNFDMDP          |
| P1H6             | A^b             | 130–149           | +                | GKLAAKTYDAVTVSNQ           |

TABLE 1
Specificity of T cell hybridomas

A single peptide indicates no response to flanking overlapping peptides. +/− indicates a partial response compared with +. MHC restriction was determined using single MHC allele transfected fibroblasts, and hybridomas from H-2^a mice were assumed to be A^a-restricted. P1H6 also fully recognizes a recombinant Caf1 molecule lacking amino acids 148–149 (data not shown).

EXPERIMENTAL PROCEDURES

Antigens and Cells—Recombinant Caf1 protein (rCaf1) of Y. pestis was cloned and expressed in Escherichia coli and purified as described previously (26). Denatured rCaf1 was prepared by boiling rCaf1 for 5 min, cooling, and using immediately as described previously (26). Synthetic peptides of 20 amino acids in length based on the published Caf1 sequence (accession number Q65AJ6) were synthesized by Dr J. Gray, Institute of Cell and Molecular Biosciences, University of Newcastle, UK. Peptides were shown to be non-mitogenic and non-toxic to proliferating T cells. Culture media, metabolic inhibitors, and other chemicals were purchased from Sigma (Poole, Dorset, UK). All cells were grown in culture medium (RPMI 1640 medium containing 3 mM l-glutamine, 50 μM 2-mercaptoethanol, 10% v/v fetal bovine serum, and 30 μg/ml gentamicin, Sigma).

In preliminary experiments proliferation responses of popliteal lymph node cells from rCaf1-immunized H-2^d and H-2^b mice to overlapping synthetic peptides representing the complete Caf1 sequence identified four T cell epitopes, two for each haplotype. Lymph node cells from mice immunized with each of these four peptides also responded to the relevant peptide as well as to rCaf1 indicating that the four epitopes were immuno-dominant. We derived T cell lines from the lymph nodes of BALB/c (H-2^d) and BALB.B (H-2^b) mice (Harlan UK Ltd., Oxon, UK) immunized with 25 μg of rCaf1 antigen in Titermax adjuvant (Sigma). rCaf1-specific T cell lines were used to generate T cell hybridomas by polyethylene glycol fusion of T cell lines with BW5147 (TCRαβ) cells (a gift from Dr. P. Marrack, Denver, CO). The majority of T cell hybridomas were shown to express CD4, CD3e, and TCRαβ by flow cytometry and responded to rCaf1 as well as to synthetic peptides that included one of the 4 epitopes identified in lymph node assays (Table 1). The MHC restriction phenotype of H-2^d-derived T cell hybridomas was determined by synthetic peptide presentation by L cells transfected with either Aαββ or Eαββ (a gift from Dr. R. Germain, National Institutes of Health, Bethesda, MD), and the H-2^b-derived T cell hybridomas were assumed to be A^b-restricted.

Bone marrow macrophages were used as antigen presenting cells and generated as described previously (36) by culture of femoral bone marrow cells for 6 days in bacteriological Petri dishes in the culture medium above supplemented with 5% horse serum, 1 mM sodium pyruvate, 10 mM HEPES buffer (Sigma), and 10% of a culture supernatant from the L929 cell line as a source of macrophage-colony-stimulating factor. Cells were shown to be least 90% macrophages by flow cytometry using fluorescein isothiocyanate-conjugated anti-F4/80 antibodies (Serotec, Oxford, UK). Macrophages were activated by treatment overnight with 1 ng/ml IFNγ (R&D Systems, Abingdon, UK) before use in antigen presentation assays.

Antigen Presentation Assay—Macrophages (4 × 10^4/well) were allowed to adhere to flat-bottom 96-well microtiter plates for 2 h at 37 °C in a humidified CO2 incubator. Macrophages were pretreated or not with inhibitors of antigen presentation (Sigma) before intact or denatured rCaf1 or synthetic peptide was added for up to 5 h. Macrophages were washed to remove free antigen and inhibitors, fixed in 1% paraformaldehyde for 4 min and then fixation was stopped with 0.06% Gly-Gly followed by washing three times. The viability of macrophages after inhibitor and antigen treatment was confirmed by light microscopy. In some experiments macrophages were fixed prior to addition of antigen.

T cell hybridoma cells (4 × 10^5/well) were added to the fixed antigen-pulsed macrophages and plates were incubated for 24 h before collecting culture supernatants. Fixed non-antigen-pulsed macrophages were used as a negative control. The response of T cell hybridomas was determined as the amount of interleukin-2 released in a subsequent bioassay measuring proliferation of CTLL-2 cells (4 × 10^5/well) in the presence of T cell hybridomas culture supernatants diluted 1:2 and cultured for 24 h at 37 °C in triplicate wells of flat-bottomed 96-well microtiter plates. 38.4 KBq of [3H]thymidine (TRA310, specific activity: 74 GBq/mmol; Amersham Biosciences International plc, Buckinghamshire, UK) was added for the final 16 h before harvesting with water onto glass fiber membranes. Radioactivity was quantitated using direct beta counting (Matrix 9600, Packard Instrument Co., Meridan, CT) and results plotted as mean cpm of triplicate wells ± S.D. Experiments were repeated at least twice, and the data for representative experiments are shown.

RESULTS

We studied the mechanisms of antigen presentation of the candidate plague vaccine antigen rCaf1 using murine bone marrow macrophages as antigen presenting cells and T cell hybridomas specific for four MHC class II-restricted epitopes...
distributed throughout the Caf1 sequence (Table 1). Three epitopes were localized to the globular region and the fourth to the amino-terminal donor strand involved in Caf1 polymerization (Fig. 1). In kinetic experiments, responses to all four epitopes processed from intact rCaf1 increased progressively throughout a 5-h assay period (Fig. 2, a–d). Presentation of the epitopes from rCaf1 denatured by heating to 100 °C was more rapid, closely matching the response to synthetic peptides (Fig. 2, a–d). The two epitopes in the amino-terminal region of Caf1 were presented faster than the two in the carboxyl-terminal region.

Fixation of macrophages with paraformaldehyde before, but not after, incubation with intact rCaf1 prevented presentation of all four epitopes over a range of antigen doses (Fig. 3 a–d), suggesting a dependence on uptake and proteolytic processing of intact rCaf1. Prefixation had a differential effect on presentation of epitopes from denatured rCaf1, the epitope in the amino-terminal donor strand being unaffected by prefixation and epitope 48–61 being less affected than epitopes 123–136 and 134–147 in the carboxyl-terminal half of Caf1 (Fig. 3, a–d). Peptide presentation was independent of fixation for all four epitopes consistent with previous reports that peptides can load MHC class II on the surface of fixed cells for presentation to T cells (37).

Exogenous protein antigens are thought to be presented largely by newly synthesized MHC class II molecules following proteolytic degradation in acidic endosomal compartments by the so-called classical MHC class II presentation pathway (32, 33). However, a number of epitopes have been shown to bind mature MHC class II recycled into early endosomes from the cell surface independent of acidic endosomal compartments (33).

We investigated the source of MHC class II molecules engaged in presentation of epitopes from rCaf1. Newly synthesized MHC class II molecules can be depleted from endosomal compartments by treatment of cells with brefeldin A, which disrupts Golgi transport (38) or the protein synthesis inhibitor cycloheximide (39). For intact rCaf1, presentation of the two carboxyl-terminal epitopes was sensitive to both inhibitors whereas epitope 48–61 was partially sensitive. However, brefeldin A (Fig. 4) and cycloheximide (Fig. 5) treatment had no effect on presentation of epitope 7–20 located in the amino-terminal donor strand of Caf1. Presentation of denatured rCaf1 was less affected by the inhibitors, although the same pattern of increasing sensitivity was observed as the location of epitopes approached the carboxyl terminus of Caf1 (Figs. 4 and 5). Peptide presentation was unaffected by either inhibitor. The data suggest that Caf1 epitopes in the globular carboxyl-terminal half of Caf1 are presented by newly synthesized MHC class II molecules whereas epitopes in the more flexible amino-terminal half are more likely to load mature MHC class II molecules recycled from the cell surface.

We treated macrophages with ammonium chloride to raise endosomal pH, which reduces the efficiency of lysosomal proteolysis and/or peptide loading of newly synthesized MHC class II molecules in acidic endosomal compartments (40). Ammonium chloride had no effect on presentation of 7–20 from intact rCaf1, whereas raising endosomal pH dramatically reduced the response to the remaining three epitopes compared with the peptide
controls (Fig. 6). Presentation of denatured rCaf1 also showed a progressive increase in sensitivity to ammonium chloride as the location of epitopes approached the carboxyl terminus (Fig. 6).

To determine the requirement for lysosomal proteolysis, we investigated which enzyme families were involved in Caf1 processing by treating macrophages with broad spectrum enzyme inhibitors. (2S,3S)-trans-Epoxy succinyl-l-leucylamido-3-methylbutane ethyl ester (E-64d), phenylmethylsulfonyl fluoride, and pepstatin A inactivate most but not all cysteine (41), serine (42), and aspartic proteinases (43), respectively. Processing of intact rCaf1 for presentation of 7–20 was resistant to all three inhibitors (Fig. 7, a–c). Presentation of 48–61 required serine proteinase activity (Fig. 7c) but was unaffected by inhibitors of cysteine and aspartic proteases (Fig. 7, a and b), whereas T cell responses to epitopes 123–136 and 134–147 were prevented by all three inhibitors (Fig. 7, a–c). The data suggest that epitopes located within the carboxyl-terminal half of Caf1 showed a dependence on multiple cleavage steps by lysosomal cysteine and aspartic proteases for presentation to T cells. In contrast, presentation of 48–61 in the amino-terminal half of Caf1 only required serine proteinase activity and 7–20 located in the domain-swap region showed no dependence on proteolytic cleavage of Caf1 by the three major enzyme families. The cysteine proteinase inhibitor appeared to enhance presentation of 48–61, suggesting cysteine proteinase activity contributed to degradation of the epitope. When rCaf1 was denatured, presentation of three of the epitopes was completely resistant to the enzyme inhibitors, although 123–136 retained some dependence of the activity of all three enzyme families (Fig. 7, a–c), probably related to the degree of unfolding during denaturation.

**DISCUSSION**

The development of second generation plague vaccines is based on the combination of two immunogenic virulence determinants of *Y. pestis*, the Caf1 capsular protein, and the type II secretion system-encoded V antigen (7, 44). Our understanding of the role of Caf1 and V antigens in immunity to *Y. pestis* and their
evaluation as vaccine candidates has largely been gained from the study of mouse infection models. However, the mechanisms and pathways of MHC class II-restricted presentation of multiple epitopes from antigens of yersiniae in particular, or bacterial antigens in general, have not been studied in any detail (reviewed in Ref. 33) and it is widely assumed that CD4 T cell responses result exclusively from targeting antigens to acidic compartments of antigen presenting cells for lysosomal processing and loading of peptides on newly synthesized MHC class II (45, 46). Here we describe an investigation of the mechanisms of presentation of four CD4 T cell epitopes of the capsular Caf1 antigen of Y. pestis by macrophages that reveal diversity in the pathways used and the degrees of antigen processing required depending on the context of epitopes within the Caf1 structure. It was of particular interest to study the behavior of an epitope located within the exposed amino-terminal donor strand involved in Caf1 polymerization, in comparison with the remaining epitopes within the globular region of Caf1 (Fig. 1).

Experiments to determine the source of MHC class II molecules used for presentation of epitopes from intact rCaf1 indicated that the two epitopes within the carboxyl-terminal globular region were presented by newly synthesized MHC class II characteristic of the classical MHC class II-restricted presentation pathway. However, T cell responses to the epitope within the amino-terminal donor strand were dependent on mature MHC class II molecules, suggesting presentation by MHC class II recycled from the cell surface. The remaining epitope located between these two structurally distinct regions of Caf1 showed intermediate behavior consistent with availability of this epitope during uptake and processing for presentation by both pools of MHC class II. A similar pattern was seen with regard to requirements for an acidic intracellular compartment as well as lysosomal processing. A stepwise increase in dependence on an acidic environment and on the activity of serine and then cysteine and aspartic proteinases was observed as the location of epitopes approached the carboxyl terminus of Caf1. Presentation of the donor strand epitope 7–20 was independent of low pH and processing by the three major enzyme
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![Graph](Image)

**Figure 7.** Enzyme families required for presentation of intact and denatured rCaf1. Macrophages were pretreated with the enzyme inhibitors (+INH) 2.5 μM E-64d (a), 350 μM pepstatin A (b), or 4 mM phenylmethylsulfonyl fluoride (PMSF) (c), or were untreated, for 3 h before addition of intact rCaf1 (rCaf1) or denatured rCaf1 (denat rCaf1) for a further 5 h and fixation. T cell hybridomas specific for the four Caf1 epitopes (a–d) were added and responses assayed as described in the legend to Fig. 2. Peptides were used as controls for the inhibitors at these concentrations and had little or no effect on T cell hybridoma responses (data not shown). Each bar shows results for a single antigen dose of 3 μM, and the results were essentially the same over the antigen dose range 0.75–3 μM.

families studied, whereas the two epitopes clustered in the carboxyl-terminal globular region of Caf1 required low pH and all three enzyme families. The epitope 48–61 positioned between these showed an intermediate pattern of processing requiring the activity of serine but not cysteine and aspartic proteinases. The data indicate a strong correlation between the requirement of a particular epitope for lysosomal processing and its availability for loading on newly synthesized MHC class II. The corollary is that epitopes presented by mature MHC class II were not dependent on the activity of lysosomal processing. It is known that the donor strand remains flexible in monomeric Caf1 but is constrained by its interaction with a cleft in an adjacent Caf1 molecule in the polymeric form (10). Thus, the data presented here are consistent with dissociation of polymeric rCaf1 during endosomal transport in macrophages so that the donor strand becomes accessible for presentation by mature MHC class II without proteolytic processing. The pattern of presentation of the remaining epitopes suggests that rCaf1 continues to unfold sequentially, with an increasing requirement for cleavage of Caf1, first by serine proteinases followed by typical cysteine and aspartic lysosomal enzymes to progressively expose epitopes in the globular domain.

Presentation of the four epitopes from intact rCaf1 was sensitive to prior fixation of antigen presenting cells suggesting a requirement for internalization by macrophages. Optimal T cell responses required extended exposure of antigen presenting cells to rCaf1 compared with heat denatured rCaf1 or synthetic peptides containing the epitopes. A consensus figure of 2–3 h uptake and processing for optimal T cell responses has also emerged for a number of epitopes from a variety of antigens studied previously (reviewed in Ref. 33). We have additional data to show that presentation of Caf1 by macrophages requires clathrin but is independent of actin, suggesting uptake by receptor-mediated endocytosis.3 As the Caf1 capsule mediates resistance of *Y. pestis* to phagocytosis (9), it is likely that rCaf1 binds to macrophage surfaces poorly in the absence of opsonic antibody so that uptake of the intact (polymeric) rCaf1 is the rate-limiting step in antigen presentation accounting for the slow kinetics observed here.

Heat denaturation of rCaf1 caused a shift toward presentation by mature MHC class II and toward independence of low pH and proteolytic processing, most marked for the two epitopes in the amino-terminal half of rCaf1. The same pattern was reflected in presentation of denatured but not intact rCaf1 by prefixed macrophages. The data provide clear evidence that epitopes within unfolded regions of proteins are presented by surface MHC class II molecules without the need for uptake and processing, as suggested previously (33) and supported by studies of epitopes in different structural contexts in a viral glycoprotein (47). However, the two epitopes in the carboxyl-terminal half of rCaf1 were not presented optimally from denatured rCaf1 by prefixed macrophages, suggesting heat treatment did not completely denature the globular domain of rCaf1.

Our preliminary experiments as well as the specificity of the T cell hybridomas reported here mapped four immunodominant epitopes for two MHC haplotypes of inbred mice (H-2b and H-2d), distributed throughout the Caf1 sequence. Two of the epitopes clustered near the carboxyl terminus and a preliminary study has shown that synthetic peptides from this region induce both helper T cells and IgG antibody responses in outbred mice (31). In addition, we have pilot data to show that the dominant T cell response in lymph nodes from rCaf1-immunized HLA-DR1 transgenic mice is specific for an epitope within amino acids 120–139 in this same region of Caf1.4suggesting the carboxyl terminus of Caf1 is the focus of epitopes that bind multiple MHC class II alleles of mice and man.

3 J. A. Musson, N. Walker, D. M. Altmann, E. D. Williamson, and J. H. Robinson, unpublished observations.
4 J. A. Musson, M. Morton, N. Walker, E. D. Williamson, and J. H. Robinson, unpublished observations.
Overall, the data presented in this report are consistent with a model of sequential unfolding of Caf1 during progress through the endosomal pathway of antigen presenting cells. The differential dependence on pH and proteolytic processing of Caf1 epitopes suggests that adjuvants that target this candidate vaccine antigen to lysosomal compartments may lead to the induction of T cells specific for an incomplete repertoire of available protective CD4 T cell epitopes.

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