Differential Processing of CD4 T-cell Epitopes from the Protective Antigen of Bacillus anthracis

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We have mapped CD4+ T-cell epitopes located in three domains of the recombinant protective antigen of Bacillus anthracis. Mouse T-cell hybridomas specific for Bacillus anthracis contributed to a clearer picture of the location of binding sites for the host cell PA receptor and the other toxin subunits in relation to the four domains of the PA molecule. In addition to a role in virulence, PA is the major antigenic component of the current anthrax vaccine, and subunit vaccines based on truncated recombinant PA molecules are under development. However, the relationship between the assembly and function of PA as a toxin-translocating pore and the mechanisms of antigen processing and presentation of PA to CD4+ T-cells has not been elucidated.

Peptide epitopes recognized by CD4+ T-cells are liberated from protein antigens following some form of antigen processing before they are accessible to bind MHC class II molecules usually in acidic endocytic compartments of antigen presenting cells (APC) such as macrophages, dendritic cells, and B cells. Following uptake by APC, processing is thought to be initiated during transport through the endosomal system by unfolding of antigens induced by low pH, oxidation, disulphide reduction, or limited cleavage by lysosomal enzymes such as asparaginyl endopeptidase. Some or all of these steps may be essential to "unlock" or progressively expose protein antigens to further proteolytic degradation by lysosomal hydrolases, including cathepsins of the cysteine and aspartyl enzyme families. Serine proteinases have been implicated in antigen processing in the extracellular space for presentation of peptide epitopes on the surface of dendritic cells, but the role of serine proteinases in intracellular processing has not been studied in any detail.

The availability of a crystal structure for PA has contributed to a clearer picture of the location of binding sites for the host cell PA receptor and the other toxin subunits in relation to the four domains of the PA molecule. In addition to a role in virulence, PA is the major antigenic component of the current anthrax vaccine, and subunit vaccines based on truncated recombinant PA molecules are under development. However, the relationship between the assembly and function of PA as a toxin-translocating pore and the mechanisms of antigen processing and presentation of PA to CD4+ T-cells has not been elucidated.

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The protective antigen of Bacillus anthracis (PA) binds to a host cell-surface receptor before cleavage and heptamerization to form a transmembrane pore that delivers two further toxin subunits, edema and lethal factors, into the host cell cytosol.
Experimental Procedures

Recombinant Protective Antigen and Synthetic Peptides—Recombinant PA protein (rPA) of B. anthracis was cloned and expressed in Escherichia coli and purified as previously described (16). Synthetic peptides representing 20 amino acid stretches of the PA sequence were synthesized by the Molecular Biology Unit, Newcastle University, Newcastle upon Tyne, UK. The diagrams of PA structure shown in Fig. 1 were created by DS ViewerPro 5.0 software (Accelrys Inc., San Diego, CA) using the Protein Data Bank file Accl obtained from the NCBI website www.ncbi.nlm.nih.gov/entrez/query.fcgi.

Cells and Chemicals—Culture media, chemicals, and metabolic inhibitors (Table I) were purchased from Sigma. All cells were grown in culture medium (RPMI 1640 medium supplemented with 5 mM D-glutamine, 50 μM 2-mercaptoethanol, 10% fetal bovine serum, and 30 μg/ml gentamycin). T-cell hybridomas were generated by polyethylene-glycol fusion of BW5147 (TCRαβ) cells (provided by Dr. P. Marrack, Departments of Microbiology and Immunology, and Medicine, University of Colorado Health Sciences Center, Denver) with CD4+ T-cell lines from rPA-immunized BALB/c (H2d haplotype) and BALB.K (H2k) mice. The specificity of T-cell hybridomas was determined from their response to overlapping synthetic peptides representing the complete PA sequence. T-cell hybridomas, each specific for one of the 5 major epitopes distributed in domains 1, 3, and 4 of PA, recognized rPA as well as synthetic peptides including the sequence of the relevant epitope (Table II) when presented by histocompatible APC.

Bone marrow-derived macrophages were used as the source of APC. Macrophages were grown from femoral bone marrow cells of BALB/c and BALB.K mice by culture for 6 days in culture medium supplemented with 5% of a supernatant from the L929 cell line as a source of macrophage colony stimulating factor, as described previously (41). Macrophages were activated by treatment overnight with 1 μg/ml interferon-γ (R&D Systems, Abingdon, UK) before use in antigen presentation assays.

Antigen Presentation Assay—Macrophages (4 × 10⁶/well) were allowed to adhere to flat-bottom 96-well microtiter plates for 1 h at 37 °C in a humidified CO₂ incubator. Macrophages were pretreated with metabolic inhibitors (Table I) for 30 min before adding antigen in the form of rPA or synthetic peptides for up to 5 h, and were then washed to remove inhibitors, fixed in 1% paraformaldehyde for 4 min, and fixation was stopped with 0.05% Gly-Gly followed by washing three times. The viability of macrophages was confirmed by light microscopy. Fixation was stopped with 0.05% Gly-Gly followed by washing three times. The viability of macrophages was confirmed by light microscopy. T-cell hybridomas were added to the fixed antigen-presenting cells at a ratio of 1:10 and the plates were incubated at 37 °C in a humidified CO₂ incubator. Macrophages were treated with metabolic inhibitors (Table I) for 30 min before adding antigen in the form of rPA or synthetic peptides for up to 5 h, and were then washed to remove inhibitors, fixed in 1% paraformaldehyde for 4 min, and fixation was stopped with 0.05% Gly-Gly followed by washing three times. The viability of macrophages was confirmed by light microscopy in all experiments showing the inhibitors were not toxic for cells in the dose ranges used.

T-cell hybridomas (4 × 10⁴/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 controls. The response of T-cell hybridomas was measured as the amount of interleukin-2 released in a subsequent bioassay as proliferation of CTL-L1-2 cells (3 × 10⁵/well) in the presence of T-cell hybridoma culture supernatants diluted 1:2 and cultured for 24 h at 37 °C in triplicate wells of flat-bottomed 96-well microtiter plates. The wells containing CTL-L1-2 cells were pulse-labeled with 38.4 KBq of ³H-thymidine (TRA310, specific activity 74 GBq/mmol; Amersham Biosciences) for 18 h and harvested onto glass fiber membranes. Radioactivity was quantitated using a direct beta counter (Matrixt 9600, Packard Instrument Company, Meridian, CT), and results are plotted as mean cpm of triplicate wells ± S.D. All experiments were repeated at least twice, and the data for representative experiments are shown.

Results

Kinetics of Presentation of PA Epitopes by Macrophages—We mapped 5 epitopes distributed in domains 1, 3, and 4 of PA and generated T-cell hybridomas specific for each epitope (Fig. 1 and Table II). T-cell hybridomas were assayed for their response to rPA presented by bone marrow macrophages to study the mechanisms of antigen processing of rPA. Macrophages fixed before addition of rPA failed to induce responses in any of the T-cell hybridomas (data not shown), showing that all epitopes required uptake and/or processing of the intact rPA molecule for presentation to occur. Macrophages were treated with rPA for different times before fixation, washing, and addition of T-cell hybridomas to determine the kinetics of presentation of each epitope (Fig. 2). The domain 3 epitope PA64-560 was already presented optimally after only 15 min of exposure to rPA before fixation of macrophages. The responses to epitopes PA64-77, PA154-167, PA659-672, and PA17-730 emerged more slowly and were maximal by 1 to 2 h (Fig. 2b). Presentation of synthetic peptides representing each epitope occurred early and was more uniform in kinetics compared with rPA (Fig. 2b), suggesting that the pattern of responses to rPA was not primarily a result of differences in the affinity of peptide epitopes for MHC class II or the T-cell receptor of the hybridomas. Thus, the data suggest that PA epitopes were processed and presented to T-cells at different rates.

Role of Endosome Acidification in Antigen Presentation of rPA—Progress of endocytosed antigens from early endosomes to the low pH environment of late endosomes or lysosomes is required for presentation of many (17) but not all (24, 42, 43) CD4+ T-cell epitopes. Macrophages were treated with NH₄Cl, a weak base that raises endosomal pH (44) to determine whether a low pH is required for presentation of each of the PA epitopes. Presentation of epitopes PA64-77, PA154-167, PA659-672, and PA17-730 was profoundly inhibited by treatment of APC with ammonium chloride (Fig. 3, c and d), whereas presentation of PA64-77, PA154-167, and PA17-730 was profoundly blocked, independent of antigen dose (Fig. 3, a, b, and e). Ammonium chloride did not affect the ability of macrophages to present synthetic peptides representing each epitope (Fig. 3). The data suggest that presentation of T-cell epitopes of rPA showed a differential dependence on low pH characteristic of late endosomes.

Effect of Cysteine and Aspartic Proteinase Inhibitors on Presentation of rPA—To determine which families of enzymes are involved in processing of T-cell epitopes from rPA, we investigated the effect of treatment of macrophages with broad spectrum proteinase inhibitors. The principal enzymes shown to be required for antigen presentation are lysosomal cathepsins of the cysteine and aspartic proteinase families that mediate proteolytic processing of either the protein antigen or the MHC class II chaperone invariant chain (21, 22). We used broad spectrum inhibitors of these families to determine whether enzymes largely localized to lysosomes were required for presentation of PA epitopes. E-64d (29,35)-trans-epoxysuccinyl-l-leucylamido-3-methylbutane ethyl ester is a membrane-permeable inhibitor that selectively inactivates most cysteine proteinases, including cathepsins L, S, and B (45, 46). E-64d profoundly inhibited presentation of PA64-77 and PA154-167 at

| Inhibitor       | Effect on         | Solvent   | Dose range | Refs. |
|-----------------|-------------------|-----------|------------|-------|
| NH₄Cl           | pH                | H₂O       | 100 mM     | 57    |
| DCl             | Serine            | C₅H₁₀OH   | 40 μM      | 58,59 |
| PMSF            | Serine            | C₅H₁₀OH   | 3 mM       | 60    |
| TPCK            | Chymotrypsin-like serine | Me₅SO-50 | 1–50 μM     | 60    |
| TLCK            | Trypsin-like serine | 1 mM HCl  | 5–165 μM   | 60    |
| E-64d           | Cysteine          | Me₅SO     | 0.1–10 μM  | 46    |
| Pepstatin       | Aspartic          | Me₅SO     | 16–256 μM  | 35    |
| 1,10-Phenanthrol| Metallo           | Me₅SO     | 32–256 μM  | 34    |

Table I

Inhibitors of antigen processing
The 14 core residues deduced from T-cell responses to overlapping 20 amino acid synthetic peptides are shown in bold. No allele-specific MHC-binding motifs were identified in the sequence of the deduced epitopes.

| T-cell hybridoma | PA domain | Position | Sequence | MHC restriction |
|------------------|-----------|----------|----------|-----------------|
| Dp7              | 1         | 64–77    | QSAIWGFHKVKSDYAFQ   | A<sup>d</sup>  |
| Dp16a            | 3         | 547–560  | LQPLFLKQKSSNSRKRST | E<sup>4</sup>  |
| Kp55             | 4         | 659–672  | GKDITDFDFNDQDFTSQNIK | A<sup>b</sup> |
| Dp66             | 154–167   |          | INDYDMLNISLRLQDGKTF | A<sup>d</sup>  |
| Kp73             | 717–730   |          | GDTSTINGIKILIFSKKGYE | E<sup>a</sup> |

**FIG. 1. Structure of PA and location of CD4<sup>+</sup> T-cell epitopes.**

Ribbons diagrams of the structure of PA created by DS ViewerPro 5.0 software (Accelrys Inc., Cambridge, UK) using the Protein Data Bank file Acct to show the position of the four domains (a) and the location of the five epitopes shown in Table II (b). Note that PA amino acids 1–13 and 162–174 were not resolved in the crystal and hence are not shown in the structure. Thus only amino acids 154–161 of the epitope PA<sub>154–167</sub> are displayed.

All doses studied. However, presentation of PA<sub>459–672</sub> was more resistant, whereas epitopes PA<sub>547–560</sub> and PA<sub>717–730</sub> were completely unaffected by E-64d treatment (Fig. 4).

Pepstatin is an acylated pentapeptide used to irreversibly block aspartic proteinases including cathepsins D and E (35). Presentation of PA<sub>717–730</sub> was particularly sensitive to treatment of macrophages with pepstatin (Fig. 5), whereas the remaining epitopes were relatively resistant to this inhibitor.

Thus, the most rapidly presented epitope PA<sub>547–560</sub> appeared to be relatively independent of processing by lysosomal cysteine and aspartic proteinases. Furthermore, among the more slowly presented epitopes the two from domain 1 of PA PA<sub>64–77</sub> and PA<sub>154–167</sub> showed a greater dependence on cysteine proteinase activity, whereas the carboxy-terminal epitope of domain 4 (PA<sub>659–730</sub>) was the most profoundly affected by inactivation of aspartic proteinases.

**Effect of Serine and Metalloproteinase Inhibitors on Presentation of rPA—**We first investigated the role of serine proteinases to determine whether they were required for processing and presentation of PA epitopes. Macrophages were treated with the broad spectrum serine proteinase inhibitor 3,4-di-chloro-isocoumarin (DCI), before their use in antigen presentation assays. DCI profoundly inhibited presentation of all epitopes under study from rPA over a range of inhibitor doses (Fig. 7a). TLCK had a differential effect, with PA<sub>64–77</sub> and PA<sub>154–167</sub> showing a greater sensitivity, PA<sub>547–560</sub> intermediate, and PA<sub>659–730</sub> showing at least a 10-fold decreased sensitivity (Fig. 7b). TPCK and TLCK had no effect on peptide presentation. The data suggest that presentation of rPA required serine proteinase activity but that epitopes showed distinct patterns of dependence on chymotrypsin-like and/or trypsin-like serine proteinase activity.

Treatment of macrophages with the broad spectrum metalloproteinase inhibitor 1,10-phenanthroline had a modest inhibitory effect on presentation of PA<sub>64–77</sub>, PA<sub>154–167</sub>, and PA<sub>547–560</sub>, whereas presentation of PA<sub>717–730</sub> was unaffected and a degree of enhancement of presentation of PA<sub>547–560</sub> was seen (Fig. 8), suggesting that metalloproteinases are active during processing of rPA, contributing to presentation of some epitopes, but destruction of others.

**DISCUSSION**

We mapped 5 epitopes distributed in 3 of the 4 domains of the anthrax protective antigen to study proteolytic processing for MHC class II-restricted antigen presentation by macro-
phages. We used macrophages as APC and treated them with metabolic inhibitors to probe the mechanisms of proteolytic processing for presentation to T-cell hybridomas specific for each of the 5 epitopes (Fig. 1). Two epitopes located in domain 1 (PA64–77 and PA154–167) showed a similar pattern of processing reflected in the greatest sensitivity to inhibitors of both cysteine and trypsin-like serine proteinases, as well as dependence on metalloproteinase activity and sensitivity to raising endosomal pH. PA64–77 is not accessible on the surface of rPA, whereas PA154–167 is located on a loop adjacent to the furin cleavage site (Fig. 1). However, both epitopes seem to require a similar degree of processing for presentation. Of the two epitopes in domain 4, presentation of PA659–672 also showed a dependence on serine, cysteine, and metalloproteinase activity, but was resistant to raising endosomal pH, the latter suggesting processing by members of these enzyme families active at near neutral or intermediate pH (47). In contrast, presentation of PA717–730, located in domain 4 close to the carboxyl terminus of PA, was pH-sensitive but unaffected by inhibitors of both cysteine and metalloproteinases, showing instead the most pronounced dependence on aspartic proteinase activity. Thus, these four epitopes of PA showed differential requirements for proteolytic processing. In particular, PA659–672 and PA717–730 showed significant differences despite the fact that these epitopes are located in close proximity in domain 4, suggesting the local structural context of an epitope is a major factor in the way it is liberated for MHC binding and presentation.

Epitope PA547–560 from domain 3 showed the most distinct pattern of processing based on the rapid kinetics, independence on low pH, and resistance to treatment of macrophages with inhibitors of cysteine, aspartic, and metalloproteinases, strongly suggesting that lysosomal processing was not involved. In fact, the enhanced presentation of PA547–560 by macrophages treated with phenanthroline (Fig. 6) suggests that metalloproteinases destroyed this epitope. PA547–560 was not presented from the intact rPA molecule by pre-fixed macrophages (data not shown), and presentation was dependent on the activity of serine proteinases, suggesting that following uptake, limited proteolytic cleavage of rPA occurred rapidly at an early stage before transport along the endosomal pathway.

Presentation of PA547–560 and PA659–672 share the common feature of resistance to raising endosomal pH. We have demonstrated that both epitopes were presented by mature MHC class II molecules, suggesting presentation of PA547–560 and PA659–672 in early endosomes by recycling MHC class II in the

\[\text{Fig. 3. Role of endosomal acidification in presentation of T-cell epitopes of PA.}\]

Presentation of the indicated doses of rPA (round symbols) or synthetic peptides (square symbols) to the T-cell hybridomas specific for PA64–77 (a), PA154–167 (b), PA547–560 (c), PA659–672 (d), or PA717–730 (e) by macrophages untreated (filled symbols) or treated with 100 mM ammonium chloride (open symbols). Responses are plotted as described in the legend to Fig. 1.

\[\text{Fig. 4. Effect of cysteine proteinase inhibitor on presentation of rPA.}\]

Macrophages were treated with the indicated doses of E-64d (E64d) before pulsing with 0.4 μM rPA and assay of presentation to PA-specific T-cell hybridomas as described in the legend to Fig. 1. E-64d did not inhibit T-cell hybridoma responses to synthetic peptides representing any of the 5 epitopes (data not shown).

\[\text{Fig. 5. Effect of aspartic proteinase inhibitor on presentation of rPA.}\]

Macrophages were treated with the indicated doses of pepstatin before pulsing with 0.6 μM rPA and assay of presentation to PA-specific T-cell hybridomas as described in the legend to Fig. 1. Pepstatin did not inhibit responses to synthetic peptides representing any of the 5 epitopes (data not shown).
so-called recycling pathway. In contrast, presentation of PA64–77, PA154–167, and PA717–730 was dependent on low pH, and we have shown that these epitopes were largely presented by newly synthesized MHC class II molecules, presumably after targeting of rPA to acidic intracellular compartments for presentation by the so-called classical pathway. Lysosomal enzymes such as the cysteine proteinase cathepsin S have a role in degradation of invariant chain which is required for optimal peptide loading of newly synthesized MHC class II molecules (48). Sensitivity of PA epitopes, particularly PA717–730, to cysteine proteinase inhibitors may not indicate a direct role for enzymes such as cathepsin S in cleavage of rPA, but is still compatible with processing for the classical pathway.

Macrophages (49) and dendritic cells (50) are the principal targets of anthrax lethal toxin. During toxin assembly, PA binds to a cell surface receptor (8) followed by cleavage into 20 and 63 kDa fragments by cell surface-associated serine proteinases, particularly furin (51). Furin cleavage of PA leads to heptamerization of the larger PA63 fragment to form a pore, although the smaller PA20 fragment remains associated by non-covalent interactions (52). The epitopes in domain 1 are both located within the PA20 fragment, and their presentation was dependent on serine proteinase activity consistent with a requirement for furin cleavage. Indeed, in a separate study of the mechanisms of uptake of rPA for presentation to CD4+ T-cells, a specific furin inhibitor blocked presentation of PA64–77 and PA154–167, but not those in other domains of PA.3 Thus, furin cleavage may be required to unlock domain 1 for presentation of epitopes PA64–77 and PA154–167, but not for efficient uptake of PA for presentation of the remaining PA epitopes.

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Fig. 6. Effect of a serine proteinase inhibitor on presentation of rPA. Presentation of the indicated doses of rPA (round symbols), or synthetic peptides (square symbols), to T-cell hybridomas specific for epitopes PA64–77 (a), PA154–167 (b), PA659–672 (c), PA717–730 (d), or 717–730 (e) by macrophages untreated (filled symbols) or treated with 40 μM DCI. Responses are plotted as described in the legend to Fig. 1.

Fig. 7. Effect of trypsin-like and chymotrypsin-like serine proteinase inhibitors on presentation of rPA. Macrophages were treated with the indicated doses of TPCK (a) or TLCK (b) before pulsing with 0.6 μM rPA and assay of presentation to PA-specific T-cell hybridomas as described in the legend to Fig. 1. As controls, TPCK and TLCK did not inhibit T-cell hybridoma responses to synthetic peptides representing each epitope; representative examples of the effect of TPCK on presentation of the synthetic peptide 717–730 to T-cell hybridoma Kp73 (broken line in a) and the effect of TLCK on presentation of the synthetic peptide 154–167 to T-cell hybridoma Dp16a (broken line in b) are shown.

Fig. 8. Effect of a metalloproteinase inhibitor on presentation of rPA. Macrophages were treated with the indicated doses of 1,10-phenanthroline before pulsing with 0.6 μM rPA and assay of presentation to PA-specific T-cell hybridomas as described in the legend to Fig. 1. Phenanthroline did not inhibit T-cell hybridoma responses to synthetic peptides representing any of the 5 epitopes (data not shown).

The rapid kinetics of presentation of PA547–560 from domain 3 compared with the other 4 epitopes of PA studied here, provides strong evidence that epitopes are liberated from rPA at different rates. The requirement for serine proteinase activity independent of low pH for presentation of PA547–560 suggests processing of rPA by neutral proteinases present in early endosomes or at the cell surface during uptake of rPA. The majority of serine proteinases have near-neutral pH optima,
and those active at low pH, such as members of the carboxypeptidase C family (53), largely lack the endopeptidase activity for initial cleavage within protein antigens required for antigen presentation. Presentation of the remaining 4 epitopes of PA also required serine proteinase activity, but in these cases this was in addition to the activity of cysteine, aspartic or metalloproteinases. The cysteine and aspartic proteinase activity is very likely to be of lysosomal origin, but this is not necessarily the case for the metalloproteinase activity, as some members of this enzyme family have near-neutral pH optima, such as aminopeptidase N located at the plasma membrane (39). Thus, metalloproteinases could participate in events prior to lysosomal processing during antigen presentation.

Collectively, the data suggest that processing of rPA occurs sequentially in a series of steps beginning with cleavage by neutral proteinases before rPA-containing endosomes make contact with lysosomal enzymes. For epitopes such as PA427-560 this initial step is sufficient for presentation to occur which is consistent with the exposed location of PA427-560 on the surface of domain 3 of PA. However, for the majority of epitopes processing may be initiated by neutral proteinases followed by further cleavage during subsequent lysosomal processing and loading of MHC class II. Serine proteinases have been implicated in cell-free or extracellular antigen processing for loading MHC class II molecules at the cell surface (32, 33, 54), but their involvement in endosomal processing within APC has not been studied in detail. Treatment of APC with some inhibitors of serine proteinase activity was shown to have a limited effect on presentation of epitopes of sperm whale myoglobin (34) or hen egg lysozyme (35). However, we have demonstrated sensitivity of presentation of T-cell epitopes of the surface M protein of Streptococcus pyogenes of vaccine antigens to induce optimal immune responses upon vaccination against infectious diseases that still pose a threat to humans.

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