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New insights into the functions of anthrax toxin

David J. Banks, Sabrina C. Ward and Kenneth A. Bradley

Anthrax is the disease caused by the Gram-positive bacterium *Bacillus anthracis*. Two toxins secreted by *B. anthracis* – lethal toxin (LT) and oedema toxin (OT) – contribute significantly to virulence. Although these toxins have been studied for half a century, recent evidence indicates that LT and OT have several roles during infection not previously ascribed to them. Research on toxin-induced effects other than cytolysis of target cells has revealed that LT and OT influence cell types previously thought to be insensitive to toxin. Multiple host factors that confer sensitivity to anthrax toxin have been identified recently, and evidence indicates that the toxins probably contribute to colonisation and invasion of the host. Additionally, the toxins are now known to cause a wide spectrum of tissue and organ pathophysiologies associated with anthrax. Taken together, these new findings indicate that anthrax-toxin-associated pathogenesis is much more complex than has been traditionally recognised.

Bacillus anthracis is a soil-borne zoonotic bacterium that causes the disease anthrax in animals. Naturally occurring anthrax in humans is most often a result of contact with infected ruminants such as cattle or sheep: humans do not usually acquire anthrax directly from soil-borne spores. Anthrax was an economically important disease of the 19th century affecting domesticated animals, and people associated with animal products; however, veterinary vaccines developed by Louis

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Institute URL: http://www.mimg.ucla.edu

Accession information: DOI: 10.1017/S1462399406010714; Vol. 8; Issue 7; 11 April 2006

Pasteur in the late 19th century and Maxwell Sterne in the early 20th century successfully controlled *B. anthracis* infections in animals, and consequently reduced the incidence of human anthrax. After World War II, *B. anthracis* was developed as a biological weapon by several countries, thus maintaining efforts in anthrax research. However, by the 1970s, biological weapons programs in the USA and UK were officially abandoned, and so *B. anthracis* received limited attention as the era of modern molecular biology emerged. Recently, though, *B. anthracis* has attracted much interest because of the renewed threat of biological weapons and the efficacy of anthrax as a bioterror-associated disease, and this has led to a resurgence of research efforts aimed at understanding *B. anthracis* pathogenesis.

Disease overview

There are three routes of infection with *B. anthracis*, which result in distinct forms of anthrax in humans: cutaneous, gastrointestinal and inhalation. Cutaneous infection is the most common naturally occurring form of the disease and results from contact of spores with cuts or abrasions on the skin. This form of anthrax responds favourably to antibiotic therapy and has very low rates of mortality when treated. During the US anthrax mail attacks of 2001, 11 of the 22 reported cases were cutaneous, but they accounted for none of the deaths (Ref. 1). Gastrointestinal anthrax has been described in the literature as a much rarer form of the disease, but this may be due to under-reporting rather than a true decreased incidence of this form of the disease (Ref. 2). There is a wide variability in mortality rates associated with separate outbreaks of gastrointestinal anthrax, although more research is needed to understand the reason for this variation. Inhalation anthrax is the most deadly form of the disease, as it almost always progresses to a systemic infection. If left untreated, all three forms of anthrax can result in systemic bacteraemia, which is often fatal, even with aggressive medical treatment. Although antibiotic therapy can effectively clear systemic bacilli, such treatment does not typically alter the outcome of disease, especially if administered after the onset of symptoms (Ref. 1). In the case of experimental animals, the inability of antibiotic treatment to prevent death despite the clearing of infection is attributed to production and release of anthrax toxin by *B. anthracis* (Ref. 3). Therefore, understanding the interactions between anthrax toxin and the host is critical in the development of therapeutics to treat anthrax.

Multiple virulence factors have been identified in *B. anthracis*, each of which contributes to the overall success of this pathogen. Research has mainly focused on two major virulence factors: an antiphagocytic capsule composed of γ-poly-D-glutamate that surrounds the vegetative bacilli; and a tripartite protein exotoxin referred to either as anthrax toxin or, based on individual subunit activities, as lethal toxin (LT) and oedema toxin (OT). Several new *B. anthracis* virulence genes have been identified, and it is likely that renewed research efforts will identify additional virulence factors (Refs 4, 5, 6, 7, 8); however, these novel virulence factors are not examined here. Rather, the focus of this review is on the newly identified targets and functions of anthrax toxin.

Anthrax toxin

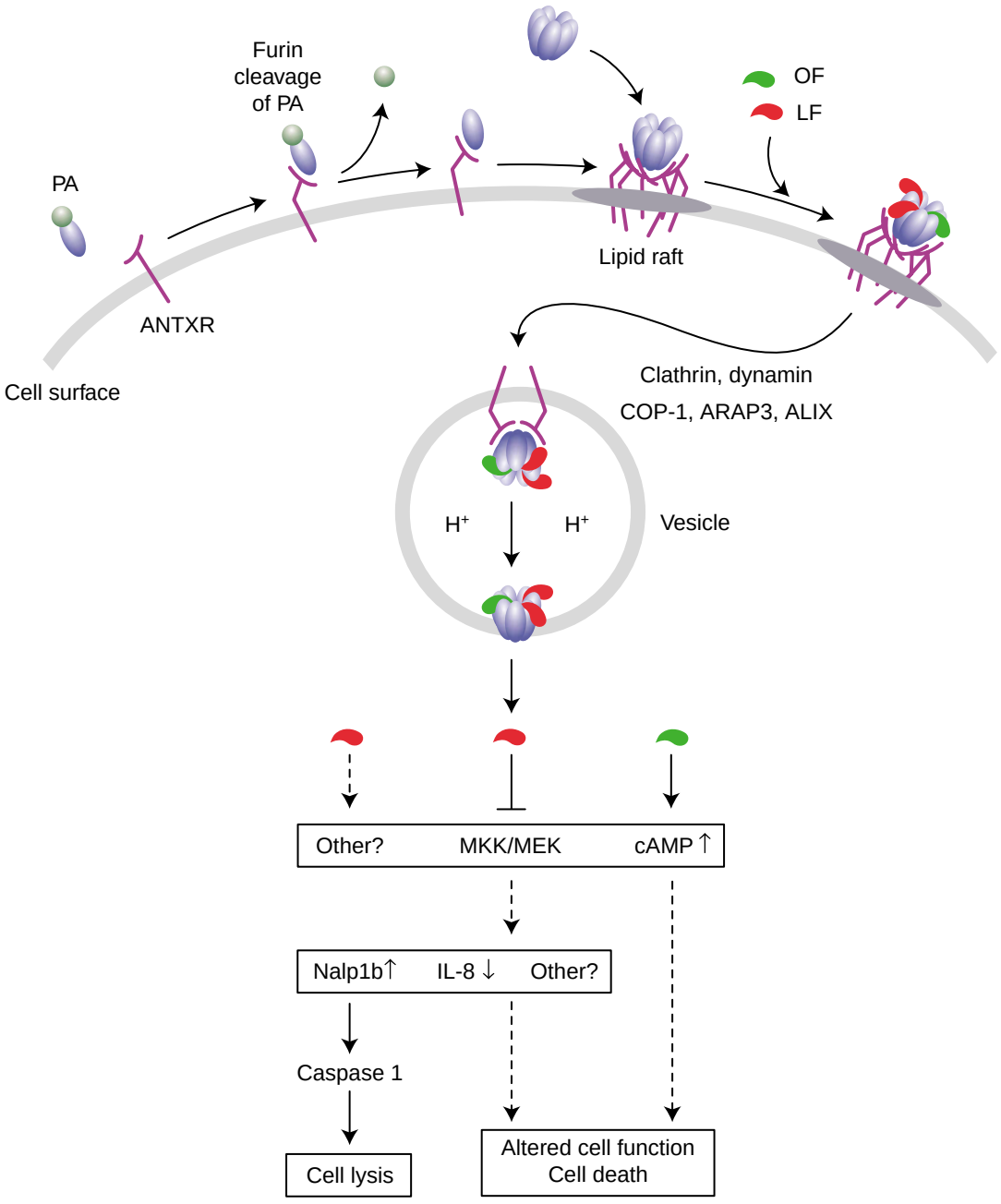
The existence of anthrax toxin was first reported by Smith and Keppie, when they discovered that toxigenicity could be transferred from clarified plasma derived from *B. anthracis*-infected guinea pigs to healthy guinea pigs (Ref. 3). It was later determined that *B. anthracis* secretes three plasmid-encoded toxin proteins that are collectively referred to as anthrax toxin: protective antigen (PA), lethal factor (LF), and oedema factor (OF). Two of these proteins, LF and OF, function individually as alternative catalytic moieties that share a single receptor-binding subunit, PA. The result is that two different binary toxins can be formed from three different toxin subunits: LT, consisting of PA and LF; and OT, consisting of PA and OF. LF and OF do not bind monomeric PA, which is secreted as an 83 kDa protein. Rather, PA must first be processed by a host protease such as furin to remove an N-terminal 20 kDa fragment, and the resulting 63 kDa fragment of PA then assembles into a seven-member ring, or heptamer, that is capable of binding a mixture of up to three molecules of LF or OF (Refs 9, 10, 11, 12) (Fig. 1).

Receptor binding and internalisation

Cellular intoxication begins when PA binds one of two identified cell-surface-exposed anthrax toxin receptors (ANTXRs) on mammalian cells (Refs 13, 14) (Fig. 1). The gene encoding ANTXR-1 was originally identified as tumour endothelial marker 8 (*TEM 8*), whereas that for ANTXR-2 was identified as capillary morphogenesis gene 2 (*CMG2*) (Refs 15, 16). Co-expression of both

Accession information: DOI: 10.1017/S1462399406010714; Vol. 8; Issue 7; 11 April 2006

receptors is not required for intoxication in vitro (Ref. 17), and it is not clear which receptor serves a more significant role during intoxication in vivo. Despite 60% amino acid sequence identity in their toxin-binding domains, PA binds to ANTXR-2 with much greater affinity in vitro and each receptor displays distinct binding properties at low pH (see below) (Refs 18, 19, 20). These



Current model for cellular intoxication by anthrax toxin

Figure 1. Current model for cellular intoxication by anthrax toxin. *(See next page for legend.)*

Accession information: DOI: 10.1017/S1462399406010714; Vol. 8; Issue 7; 11 April 2006

Figure 1. Current model for cellular intoxication by anthrax toxin. *(Legend only; see previous page for figure.)* Cellular intoxication begins when protective antigen (PA) binds membrane-bound surface-exposed anthrax toxin receptor (ANTXR). PA is secreted by *Bacillus anthracis* as an 83 kDa monomer and is proteolytically processed either on the surface of host cells or in the serum (Refs 123, 124) to remove a 20 kDa fragment from the N-terminus. The remaining 63 kDa fragment then heptamerises and binds the catalytic moieties lethal factor (LF) and oedema factor (OF). Oligomerisation of PA induces clustering of ANTXRs, which drives relocalisation into lipid rafts, resulting in dynamin- and clathrin-dependent endocytosis and COP1-, ARAP3-, and ALIX-dependent trafficking of the toxin complex. The endosome becomes acidified, causing the pre-pore structure to insert into the membrane and thus form a pore. LF and OF are delivered to the cytosol through the pore and act on host targets. LF inactivates MKKs/MEKs, promotes IL-8 mRNA decay, and activates Nalp1b. Dashed lines indicate hypothetical or unknown interactions during intoxication. Abbreviations: ALIX, apoptosis-linked gene 2 (ALG-2)-interacting protein X; cAMP, cyclic AMP; ANTXR, anthrax toxin receptor; ARAP3, ankyrin repeat and plekstrin homology domains-containing protein 3; H^+, proton; IL-8, interleukin 8; MKK/MEK, kinase for MAPKs/ERKs (mitogen-activated protein kinases or extracellular-signal-regulated kinases); PA, protective antigen. Adapted from Ref. 44 (Copyright 2004), with permission from Elsevier.

differences may influence target cell type or affect toxin activity. Furthermore, while CMG2 is expressed in normal adult tissues (Refs 13, 17), there are conflicting reports regarding the expression profile of TEM8 (Refs 16, 17, 21, 22, 23, 24, 25, 26, 27). This discrepancy likely results from relatively low-level, if any, expression of TEM8 in normal tissues, making interpretation of negative results difficult. In any event, almost all cells tested express at least one type of receptor, and thus support toxin entry into cells (Refs 28, 29).

Oligomerisation of PA induces relocalisation of the newly formed receptor–toxin complex into lipid rafts, where it is then internalised via clathrin-dependent, receptor-mediated endocytosis (Refs 30, 31), and trafficked to a low-pH endosome (Fig. 1). Recent data suggest that this trafficking step proceeds through structures alternatively called endosome carrier vesicles or multivesicular bodies (Ref. 31). The acidic pH of the endosome is required to trigger a conformational change in the PA heptamer that allows for conversion of this soluble protein into an integral membrane protein. This structural rearrangement results in formation of a pore in the endosomal membrane through which LF and OF then translocate (Ref. 32). Interestingly, the pH threshold at which this event occurs depends on the receptor to which PA is bound: TEM8 promotes pore formation at pH 6.5, whereas CMG2-bound PA requires a more acidic pH and forms pores only below pH 5.5 (Refs 19, 33).

LF action

Once internalised, LF functions as a zinc metalloproteinase that specifically cleaves the N-termini of MKK/MEK proteins [kinases for MAPKs/ERKs (mitogen-activated protein kinases or extracellular-signal-regulated kinases)], thereby disrupting cell signalling pathways (Refs 34, 35, 36, 37). LF recognises the MKKs/MEKs via a loosely conserved consensus sequence in the N- and C-termini of these substrates that allows for binding of MKKs/MEKs to a 40Å-long groove in LF (Refs 38, 39). In the case of MEK1, LF removes as few as 8 amino acids from the N-terminus, which is a critical region involved in MEK interaction with upstream and downstream signalling partners (Refs 40, 41). Additionally, removal of the N-terminus decreases kinase activity independent of the reduction in substrate binding (Ref. 38). Consequently, LF activity in cells blocks signalling through the three major pathways downstream of MKK/MEK proteins: the ERK1/2, JNK (c-Jun N-terminal kinase) and p38 MAPK pathways. It has been postulated that LF may have additional targets within host cells (Refs 42, 43, 44, 45, 46, 47); however, no such targets have been identified.

OF action

OF is an adenylate cyclase that is produced in *B. anthracis* as an inactive enzyme, requiring calcium and the eukaryotic protein calmodulin for its activation (Refs 48, 49). Upon calmodulin binding, OF undergoes structural rearrangements, unique among adenylate cyclases, that lead to its activation (Refs 50, 51, 52). OF has a specific activity 1000-fold greater than mammalian adenylate cyclases and induces a substantial increase in conversion of intracellular ATP to cyclic AMP (cAMP). Subsequently, water homeostasis and cellular signalling pathways of the host are disrupted, leading to oedema during cutaneous anthrax infection (Refs 48, 49, 53). Additionally, OT inhibits the ability of neutrophils to phagocytose bacilli and produce an oxidative

Accession information: DOI: 10.1017/S1462399406010714; Vol. 8; Issue 7; 11 April 2006

burst (Refs 54, 63). Several pathogenic bacteria encode virulence factors that are either adenylate cyclases or activate host adenylate cyclases (Ref. 55). Despite this convergence on similar virulence mechanisms in distantly related bacterial pathogens, relatively little research has been devoted to OT in the past. However, this is changing rapidly, and several groups have now reported on toxic effects of OT.

New roles for anthrax toxin?

As the research effort on *B. anthracis* increases, many new roles for anthrax toxin are being reported, and it is now appreciated that LT and OT activity on host cells can be much more subtle than previously thought. For example, certain cell types were thought to be LT-insensitive because they do not lyse rapidly upon toxin exposure; nevertheless, their ability to respond appropriately to bacterial stimuli is dramatically altered by anthrax toxin. Thus, it is likely that the source of anthrax virulence does not reside solely within the ability of toxins to kill host tissues outright. Rather, virulence may also derive from toxin-mediated alteration of host immune responses prior to sepsis and toxaemia. This review focuses on recent findings that have led to these newly appreciated roles for anthrax toxin, and we refer the reader to other comprehensive reviews for previously reported aspects of toxin biology (Refs 18, 55, 56, 57, 58, 59, 60, 61). Specifically, here we address four major areas of new understanding of *B. anthracis* toxin: expanded target-cell range; cytokine modulation; novel host cellular factors that influence toxin sensitivity; and in vivo responses of hosts to toxin.

Expanded target-cell range

Historically, LT and OT were believed to target a very limited range of host cells. The first demonstration of in vitro cytotoxicity induced by LT was reported in 1986, when Friedlander showed that murine macrophages lysed rapidly (within ~2 h) in response to LT (Ref. 62). The majority of investigations in the following years concentrated on LT effects on macrophages both in vivo and in vitro, as other cell types were thought to be insensitive (Ref. 63). However, although macrophages play an important role in combating *B. anthracis* infections, and toxin clearly alters macrophage function, it is now apparent that whole-animal sensitivity to LT does not correlate with macrophage death (Refs 64, 65). This has led researchers to consider alternative target cell types affected by LT. Recent studies have uncovered direct LT-induced cytotoxic effects on endothelial cells and dendritic cells (Refs 29, 43, 66, 67, 68), as well as indirect cytotoxicity on red blood cells and endothelial cells (Refs 66, 69) (Fig. 2; Table 1). Additionally, although OT was originally thought to be nontoxic, several groups have now reported cell, tissue, organ and whole-animal pathophysiology or death in response to OT (Refs 70, 71, 72) (Fig. 2; Table 1).

Monocytes and macrophages

Despite the focused effort towards understanding LT–macrophage interactions, the role of these cells in anthrax pathogenicity is still not entirely clear. Recently, the effects of LT were studied in immortalised human monocytic cell lines in the undifferentiated state and after differentiation in response to phorbol myristate acetate (PMA), when they acquire a macrophage-like phenotype. LT challenge resulted in MKK/MEK inactivation in both the undifferentiated and differentiated cells, but only the differentiated cells were killed by toxin (Ref. 73). Consistent with a previous report (Ref. 74), programmed cell death was shown to occur in a caspase-independent mechanism, as pan-caspase inhibitors did not protect PMA-differentiated monocytes from LT (Ref. 73). The differential effects of LT on undifferentiated and differentiated monocytes (Ref. 73) and other data suggest that cell death in monocytic cell lines is not only dependent on toxin delivery or substrate cleavage, but also on cell maturation state and activated gene profile. In further support of the idea that sensitivity to LT correlates with activation status or signalling pathways, several reports describe sensitisation of macrophages derived from LT-resistant mice by activation through Toll-like receptor 4 (TLR-4) or by addition of tumour necrosis factor α (TNF-α) (Refs 74, 75, 76, 77).

Although LT treatment did not kill undifferentiated monocytic cells, it did block their proliferation, and their differentiation in response to PMA (Ref. 73). Thus, LT may dampen the innate immune response by simultaneously blocking maturation of monocytes and promoting the death of activated macrophages. Since monocytes can differentiate into both macrophages and dendritic cells when recruited to sites of infection, LT targeting of monocytes could prevent the maturation of two fundamental populations of immune cells.

Accession information: DOI: 10.1017/S1462399406010714; Vol. 8; Issue 7; 11 April 2006

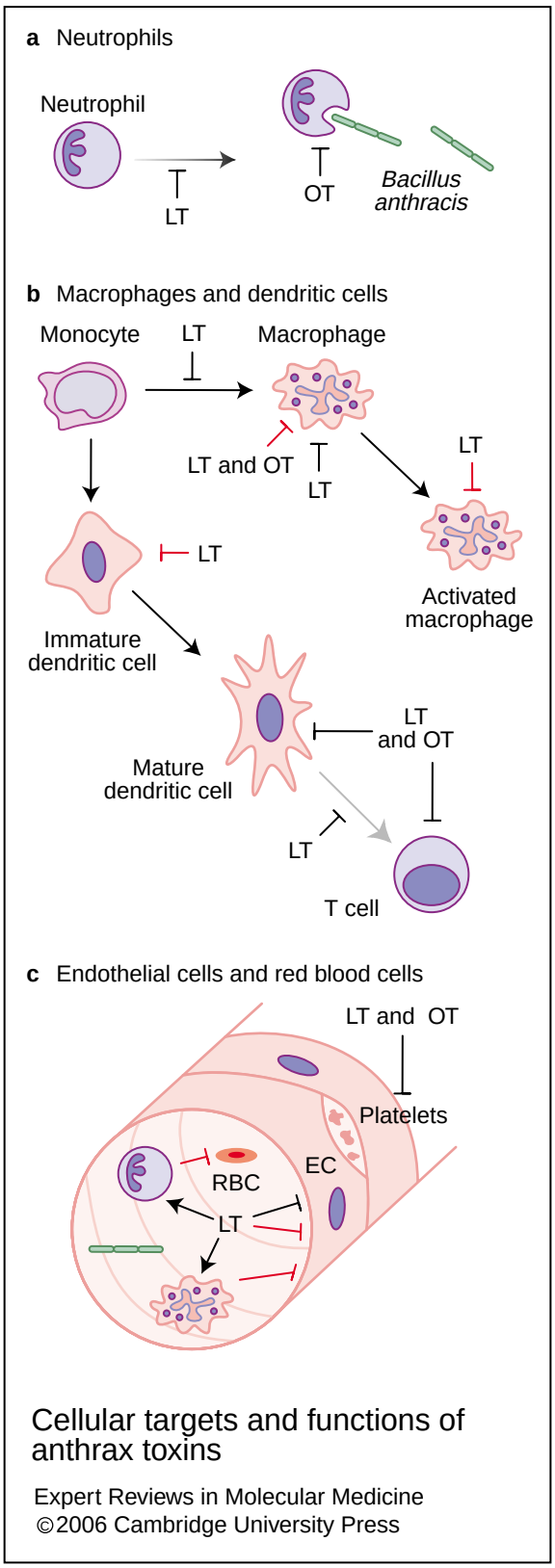


Figure 2. Cellular targets and functions of anthrax toxin. *(See next column for legend.)*

Dendritic cells

In addition to macrophages, many other cell types participate in host innate immunity. In particular, dendritic cells play important roles in activation of the innate immune response and linking the innate and adaptive immune responses. Dendritic cells phagocytose and kill microbial pathogens, produce cytokines, migrate to secondary lymphoid organs where they display microbial antigens to resident naive T cells, and stimulate these T cells to become active. Therefore, *B. anthracis* could substantially increase its fitness during infection if it were able to immunomodulate and/or kill dendritic cells. Indeed, dendritic cells exposed to LT display reduced levels of co-stimulatory molecules, secrete diminished amounts of pro-inflammatory cytokines, and do not effectively stimulate antigen-specific T cells in vivo (Ref. 78). Whereas LT-mediated cytotoxicity towards dendritic cells has not been observed in some investigations (Refs 78, 79), others have recently shown that LT is cytotoxic to both human and murine dendritic cells (Ref. 67). Interestingly, the manner in which cell death occurred was dependent on the source of the dendritic cells. Specifically, dendritic cells from humans and C57BL/6 mice die via apoptosis in a caspase-dependent manner, whereas dendritic cells from BALB/c mice die through a necrotic pathway. This suggests that genetic background influences LT sensitivity in dendritic cells, similar to the case for macrophages (Ref. 80, and see below).

Neutrophils

Neutrophils (polymorphonuclear leukocytes; PMNs) are another population of leukocytes involved in both innate and adaptive immunity. PMNs are the major phagocytic white blood cells involved in the acute inflammatory response,

Figure 2. Cellular targets and functions of anthrax toxin. (a) Neutrophils. Lethal toxin (LT) and oedema toxin (OT) inhibit neutrophil mobility and phagocytosis, respectively. (b) Macrophages and dendritic cells (DCs). LT and OT kill and modulate various immune cells at distinct states of activation and differentiation (see Table 1). (c) Endothelial cells and red blood cells. LT and OT cause endothelial cell (EC) barrier dysregulation, coagulopathy, red blood cell (RBC) death, direct and indirect EC death, and EC cytokine transcript modulation. Black T-bars indicate inhibition or modulation; red T-bars indicate cell death; shaded arrow indicates mobility; grey arrow indicates DC-mediated T-cell activation.

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Table 1. Cellular targets and effects of anthrax toxin

Toxin	Target cell	Effect	Refs
Lethal toxin	Neutrophil	Inhibits mobility	42
	Monocyte	Inhibit proliferation and differentiation	73
	Activated macrophage	Causes cell death	73, 74, 75, 76
	Macrophage	Suppresses cytokine production	125
	Immature dendritic cell	Causes cell death	67
	Mature dendritic cell	Suppresses cytokine production, co-stimulatory molecule expression and T-cell stimulation	78, 79, 126
	T cell	Inhibits activation, proliferation, surface-molecule expression and cytokine expression	95, 96, 97
	Red blood cell	Causes cell death	69
	Platelet	Induces coagulopathy	112
	Endothelial cell	Causes cell death; promotes cytokine mRNA degradation; dysregulates barrier function	43, 45, 66, 68 82
Oedema toxin	Neutrophil	Inhibits phagocytosis	63
	Macrophage	Causes cell death	72
	Mature dendritic cell	Suppresses cytokine production	79, 126
	T cell	Inhibits activation, proliferation, surface-molecule expression and cytokine expression	96, 97
	Platelet	Induces coagulopathy	91

and are capable of producing a much greater oxidative burst than macrophages. PMNs are activated primarily by cytokines produced by macrophages and endothelial cells, and they posses receptors for IgGs and complement proteins. Furthermore, PMNs efficiently kill *B. anthracis* and are capable of phagocytosing spores and capsule-producing bacilli (Ref. 81). Whereas the details and consequences of bacilli–PMN interactions in vivo remain unclear, it is apparent that both OT and LT can alter PMN activity. In addition to inhibition of PMN phagocytosis by OT (Ref. 63), it was recently demonstrated that subnanomolar concentrations of LT significantly retard neutrophil mobility, although neutrophils remain viable (Ref. 42). Specifically, PMN chemotaxis towards the chemoattractant FMLP was impaired by LT, which correlates with an overall decrease in new actin assembly (Ref. 42). Additionally, the MEK1 inhibitor PD98059 did not recapitulate these results, suggesting that the inhibitory effect of LT on PMN actin-based motility was independent of LT-mediated MEK1 cleavage. Indeed, there is no known link between MEK1 and actin assembly, further suggesting MEK-independent activity of LT in PMNs.

Red blood cells and endothelial cells

In addition to direct effects of LT on PMNs, LT treatment results in lysis of red blood cells only in the presence of PMNs, implying that LT induces neutrophils to release factors that are haemolytic and/or work in conjunction with LT to kill red blood cells (Ref. 69). The hypothesis that LT induces immune cells to release toxic factors is supported by evidence that conditioned media from LT-treated RAW 264.7 macrophages induced greater cytotoxicity to endothelial cells than LT alone (Ref. 66). These studies highlight the limitations of studying toxin activity on isolated cells in culture.

Vascular damage is emblematic of an anthrax infection. For example, typical symptoms of anthrax-associated vascular injury include vascular leakage and haemorrhages. These clinical presentations of anthrax infection suggest that a focused examination of toxin–host interactions at the vascular level is important. Recent studies have begun to address this topic, and there is now evidence that LT induces caspase-dependent apoptosis in endothelial cells derived from large vessels (human umbilical vein endothelial cells; HUVECs) and small vessels (neonatal dermal vascular endothelial cells; DMVECs) (Ref. 43).

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Direct cytotoxicity to endothelial cells is still somewhat controversial in that some groups have observed less or no cytotoxicity in endothelial cells (Refs 68, 82), whereas others have observed LT-mediated cytotoxicity in HUVECs or specific subtypes of endothelial cells (Refs 29, 66). Not surprisingly, LT cleaves MKKs/MEKs and also prevents phosphorylation of p38 MAPK in HUVECs, suggesting that loss of anti-apoptotic signals in these endothelial cells results in cell death, similar to that described in macrophages and in dendritic cells. However, contrary to what would be predicted, p38 MAPK inhibition with SB202190 actually promoted HUVEC survival, leading to the conclusion that p38 MAPK inhibition in HUVECs does not contribute to LT toxicity (Ref. 43). Therefore, the role of LT in endothelial cell death is not entirely clear, and might involve other MAPK pathways or unknown host targets. Additionally, the effects of LT on HUVECs have been examined in a three-dimensional collagen assay, which is thought to model early stages of blood-vessel development (Ref. 43). LT blocked tubule formation in this assay, which is in agreement with previously described anti-angiogenic and anti-tumour effects of LT (Ref. 83). This is particularly significant because inhibition of endothelial cell survival is known to suppress angiogenesis (Ref. 84). Using LT to target tumours is a very exciting area of cancer therapy research that is reviewed elsewhere in greater detail (Refs 85, 86, 87).

OT toxicity

Historically, the majority of research on anthrax toxin has concentrated on LT, whereas OT has received disproportionately less attention. The source of this discrepancy is probably rooted in the dramatic differences initially observed between LT- and OT-mediated toxicity. For example, whereas macrophages die when challenged with nanomolar concentrations of LT, monocytes challenged with similar concentrations of OT display altered cytokine production yet survive (Ref. 88). Additionally, although CHO cells challenged with similar concentrations of OT respond with dramatically elevated levels of cAMP, they also survive (Ref. 48). Similar phenomena also occur in vivo. For example, subcutaneous injection with purified LT kills Fischer rats within 60 min, whereas initial reports indicated that rabbits and guinea pigs injected with OT develop only localised oedema (Ref. 89). Variable degrees of LT and OT toxicity are also observed in mice following subcutaneous injection of spores from *B. anthracis* strains lacking capsule and specific toxin components: spores lacking OF were only tenfold less virulent as measured by LD_{50} increase, whereas spores lacking LF displayed more than 1000-fold attenuation (Ref. 90). This suggests that OT contributes less to overall anthrax virulence than LT, at least in subcutaneous spore challenge of mice.

Recent research on the role of OT in *B. anthracis* pathogenesis suggests that OF likely has a more significant role in anthrax pathogenesis than previously thought (Refs 70, 71,72, 91). For example, the effect of OT in the developing zebrafish embryo model, as well as on multiple mammalian macrophage and non-macrophage cell lines, at toxin concentrations between 20–400 nM has been examined (Ref. 72). Developing zebrafish embryos incubated with OT exhibited necrosis of the liver, cranium and gastrointestinal tract, and reduced swim-bladder inflation, and died within 7 days of toxin challenge. Additionally, cell-type specific, apoptosis-independent cytotoxicity in response to OT was observed (Ref. 72). Specifically, fibroblast (NIH/3T3) cells were OT-insensitive whereas macrophages (RAW264.7 and IC-21) were OT-sensitive, with 80–90% cell death occurring 72 h after intoxication. Interestingly, there seemed to be no direct correlation between OT-generated cAMP levels and cell death when comparing responses between multiple cell lines. The ability of CHO cells to survive dramatic increases in cAMP during OT intoxication could be related to their ability to adapt to unusually high cAMP levels (Ref. 72). Alternatively, OT-mediated toxicity may be independent of its adenylate cyclase activity (Ref. 72).

Cytokine modulation

Cytokines link the innate and adaptive branches of immunity, and are important in combating infection. Although anthrax toxin is believed to target cytokine production, evidence from one recent study questions the role of toxin in modulating cytokines following spore challenge (Ref. 92). In this case, mice were able to mount similar cytokine responses when challenged with toxin-producing or atoxigenic strains of *B. anthracis*. It is not clear how these results relate to previous studies showing clear effects of toxins on cytokine production by isolated immune cells.

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In any case, cytokines are likely to be important for fighting *B. anthracis* infections (Refs 93, 94), and the adaptive branch of the immune system may be important when sublethal doses of spores are inhaled, or during either cutaneous or gastrointestinal anthrax.

How might *B. anthracis* modulate an adaptive immune response? A major component of adaptive immunity is T cells. Specifically, naive $CD4^+$ T cells are activated by antigen-presenting cells such as dendritic cells, and differentiate into antigen-specific helper T (Th) cells. Differentiated T cells produce cytokines, activate cytotoxic and Th cells, and initiate antigen-specific antibody production in B cells. Therefore, if host adaptive immunity is subverted during anthrax infection, one likely target would be T cells. Indeed, LT inhibits the ability of dendritic cells to activate T cells, thus shutting down an important mechanism of inducing adaptive immunity (Ref. 78). Additionally, anthrax toxin may contribute to modulation of adaptive immunity during anthrax infection by directly inhibiting human T-cell activation, proliferation, and cytokine gene expression (Refs 95, 96, 97). Specifically, LT and OT each inhibited expression of the early-activation surface-marker CD69 and the interleukin (IL)-2 receptor CD25 in T cells activated by CD3 crosslinking. Also, both toxins inhibited key aspects of T-cell signalling: expression of the cytokines TNF-α, interferon (IFN)-γ, IL-2 and IL-5; activation of the transcription factors NFAT and AP-1, which are essential for cytokine gene expression; and MKK/MEK pathways. Clearly, anthrax toxin can have a profound inhibitory effect on T-cell activation and signalling.

Anthrax toxin suppresses cytokine secretion in multiple cell types, including macrophages, dendritic cells, T cells, and endothelial cells. Only recently, though, was insight provided regarding the mechanism by which cytokine downregulation occurs (Ref. 82). Specifically, LT was shown to reduce the amount of the pro-inflammatory cytokine IL-8 produced and secreted by human endothelial cells by increasing the rate of IL-8 mRNA decay. By contrast, stability of other cytokine transcripts, such as IL-6, was not affected by LT, suggesting specificity in LT-mediated mRNA decay. The authors speculate that this phenomenon could be related to transcript stability signals that are transmitted through MAPK pathways. These signals lead to the modification of proteins that bind directly to AU-rich elements (AREs) in the transcript 3' untranslated region (3' UTR) (Ref. 82). Decreased cytokine mRNA stability might enhance bacterial fitness during the initial phase of a systemic anthrax infection because, at this early stage, bacilli are in the bloodstream and the immune system is intact. Moreover, endothelial cells that line blood vessels would likely be exposed to anthrax toxin during infection and release IL-8 into the bloodstream to attract immune cells. Therefore, anthrax toxin suppression of innate immune cell recruitment to a focal point infection might enable a nascent colonisation event to expand before becoming a systemic infection. Although the exact mechanism of increased IL-8 mRNA decay was not identified, the authors propose that previously observed decreases in cytokine transcript production in macrophages and dendritic cells during LT challenge might be the result of increased mRNA instability (Ref. 82).

Host cell factors influencing toxin sensitivity

Several host factors that confer sensitivity to anthrax toxin have been identified, most of which are involved in processing and cellular uptake of toxin. These factors include furin, which can proteolytically cleave PA leading to heptamerisation (Ref. 98), and anthrax toxin receptors (Refs 13, 14). In addition, the vesicle-associated proteins clathrin, dynamin, epsilon-COP, ALIX [apoptosis-linked gene 2 (ALG-2)-interacting protein X] and ARAP3 function in endocytosis and trafficking of toxin to late endosomes (Refs 30, 31, 99, 100).

Host factors that confer sensitivity to anthrax toxin following toxin internalisation are not as well defined. However, three quantitative trait loci, termed Ltxs1–3, have been described in mice that control macrophage sensitivity to LT. The first, and most influential, quantitative trait locus is Ltxs1: animals with a sensitivity allele at this locus have macrophages that lyse rapidly when challenged with LT. Ltxs1 was mapped to chromosome 11 by backcrossing LT-susceptible and LT-resistant inbred mice (Ref. 101). This locus was later shown to contain the gene *Kif1C*, which encodes a kinesin-like motor protein implicated in retrograde vesicular transport from the Golgi complex to the endoplasmic reticulum (Ref. 102). *Kif1C* allelic polymorphisms correlated with macrophage sensitivity to toxin at a step downstream of toxin binding, internalisation and MKK/MEK cleavage. However, the mechanism

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of resistance conferred by a functional *Kif1C* gene was not identified and overexpression of a *Kif1C* resistance allele in sensitive cells provided only modest protection (Ref. 102). Now, new studies by the same group have revealed that a previously unidentified gene in the same region more likely controls LT sensitivity (Ref. 103). This gene, *Nalp1b*, is a member of the NLR family of proteins involved in caspase activation, inflammation, and innate immunity to infectious diseases (Refs 104, 105). The presence of the *Nalp1b* sensitivity allele correlates perfectly with caspase-1 activation and macrophage sensitivity to LT, strongly suggesting that this gene controls host susceptibility to LT. Although the mechanism by which LT activates Nalp1b remains unknown, this discovery characterises a fundamental source of host response to LT and will undoubtedly stimulate further research in host innate immunity and *B. anthracis*.

In vivo response of hosts to toxin

Antibiotic treatment is effective at clearing culturable *B. anthracis* from the bloodstream of a bacteraemic host. However, the host remains vulnerable to toxaemia, as evidenced by the high rates of mortality even with supportive care. This is the main rationale for development of antitoxins for treating anthrax. Although our understanding of toxin–host interactions at the cellular level is rapidly advancing, there is a clear need to relate these responses to the events that occur at the organismal level.

Vascular barrier dysfunction

Tissues from human inhalation anthrax victims (Ref. 106), and from spore-challenge inhalation anthrax models in nonhuman primates (Ref. 107) and mice (Ref. 108), confirm findings from earlier studies that pleural effusions and lung haemorrhages are hallmarks of systemic anthrax. Since the vascular endothelium regulates vessel permeability, and is in direct contact with the bloodstream, it was predicted that endothelial cells are a likely target of anthrax toxin during infection (Ref. 68). Primary human lung microvascular endothelial cells challenged with LT showed reduced trans-endothelial electrical resistance and increased permeability to labelled albumin (Ref. 68), demonstrating that the integrity of the vascular membrane had been compromised by toxin. Additionally, LT induced rearrangement of the actin cytoskeleton and altered distribution of the cellular adhesion protein VE-cadherin, which are both indicative of compromised barrier function (Ref. 68). These results suggest that pleural effusions and lung haemorrhages associated with systemic anthrax infection could be related to increased vascular endothelium permeability and barrier dysregulation. Although the exact molecular targets of LT and/or affected pathways that lead to barrier dysfunction are unknown, recent work by the same group (Ref. 45) showed that LT induces up-regulation of vascular cell adhesion molecule 1 (VCAM-1) and enhances monocyte adhesion to endothelial monolayers in the presence of TNF-α. The authors speculate that these events might disrupt recruitment and activation of leukocytes to the cell wall, presumably suppressing the host immune and inflammatory response to infection. Further, increased serum titres of secreted VCAM-1 (sVCAM-1) were observed upon treatment with LT and TNF-α (Ref. 45). Presence of sVCAM-1 in serum is commonly associated with vasculitis, and suggests that LT might be contributing towards this clinical presentation.

Coagulopathy

Host coagulopathy as a result of toxaemia is relatively uncommon, yet occurs with the highly virulent, shiga-toxin-producing *Escherichia coli* (STEC) O157:H7 strain, which causes haemolytic uraemic syndrome (HUS) (Ref. 109). Additionally, *Helicobacter pylori* infections often correlate with idiopathic thrombocytopenic pupura (ITP), wherein host platelet counts increase after antibiotics are administered and infection is cleared (Ref. 110). The functions of pathogen-induced host coagulopathy during infection are unclear, but have been hypothesised to promote host colonisation and invasion, particularly into sterile-site tissues. Since haemorrhage and increased vessel permeability are symptoms frequently observed during invasive anthrax infections, *B. anthracis* virulence factors likely target host clotting ability and endothelial cells (see above). Indeed, a recent report indicates that LT-challenged mice display decreased platelet and fibrinogen levels, which are consistent with disseminated intravascular coagulopathy (DIC) (Ref. 111). However, other researchers concluded that DIC does not occur when mice are challenged intravenously with LT (Ref. 65). Despite these contradictory reports, results from in vitro studies indicate that both OT and LT can promote

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coagulopathy. First, OT inhibits rabbit platelet aggregation, which is probably mediated through protein kinase A activation (Ref. 91). Second, mice injected intravenously with OT have a substantially protracted bleeding time after tail incision, relative to control mice, indicating that clotting ability is suppressed (Ref. 91). Third, clotting of whole human blood, surface expression of P-selectin on platelets, and platelet adhesion are inhibited by LT in vitro (Ref. 112). Taken together, these results strongly suggest a link between LT and OT activities and host coagulopathy during anthrax infection. Although the mechanisms of how LT and OT induce host coagulopathy are not characterised, these toxins have apparently converged on disrupting host coagulation, despite their completely different catalytic activities. These results indicate that *B. anthracis* employs both LT and OT to induce host coagulopathy during infection, and further suggest that both toxins participate in host sterile-site invasion.

Toxin interactions with the endocrine system

Variability in host response to infectious disease can influence the outcome of infection (Refs 113, 114). Recent identification of host factors that confer susceptibility to LT, in addition to those discussed above, suggests that such a phenomenon might be involved in host susceptibility to *B. anthracis*. For example, LT was found to specifically repress glucocorticoid nuclear hormone receptor (GR) activity (Refs 46, 47). In certain animal models, the host is relatively resistant to inflammatory disease due in part to hypothalamic–pituitary–adrenal (HPA) axis hyper-responsiveness and resultant hyper-secretion of glucocorticoids from the adrenal glands in response to pro-inflammatory stimuli (Ref. 47). Typically, this responsiveness protects the host through anti-inflammatory and immunosuppressive effects of glucocorticoids. However, some inflammation-resistant rodent strains are highly susceptible to pro-inflammatory stimuli after repression of GR activity. That is, loss of GR activity during infection might render the host more susceptible to virulence factors produced by a microbial pathogen. Consistent with this hypothesis, rodents that have been adrenalectomised (ADX) or treated with a GR antagonist (RU486) and simultaneously exposed to infectious agents or pro-inflammatory bacterial products display increased mortality rates (Refs 115, 116). Recently, ADX mice were shown to have increased susceptibility to LT that is independent of macrophage sensitivity and cytokine response (Ref. 46). Furthermore, LT resistance in DBA/2J mice can be reversed upon ADX or RU486 treatment, suggesting that the neuroendocrine system plays a significant role in mouse sensitivity to LT.

Another line of evidence suggests that a similar phenomenon may occur during infection as a result of OT activity. In particular, OT was recently shown to kill mice at concentrations lower than those required for lethality by LT, and OT-induced death results from multi-organ failure in the absence of gross oedema (Ref. 71). Importantly, at sublethal toxin doses, the only organ affected by OT was found to be the adrenal gland. Thus, OT disruption of the HPA axis might sensitise the host to LT, and these two toxins might thus work in concert to cripple the host at multiple levels.

Clinical implications

Since the anthrax bioterror attacks of 2001, there has been a concerted effort to develop enhanced detection, vaccine and antitoxin-treatment strategies for *B. anthracis*. However, many of these new therapeutic approaches are still in the development stage. By contrast to the rapid advances made in understanding anthrax toxin functions at the molecular and cellular levels, translation of these findings to the clinical setting has not yet occurred. Presently, pre-exposure vaccination or antibiotics administered immediately after a suspected exposure remain the best defences against anthrax. Ciprofloxacin and doxycycline are the antibiotics of choice for treating anthrax. The current anthrax vaccines used in the USA and UK are based on filtered culture supernatants from toxin-producing strains of *B. anthracis*, and are associated with adverse side effects. Replacement vaccines based on purified, recombinant PA are currently being tested with the hope that they will have fewer side effects. Several antitoxin approaches have been described, including those based on a dominant negative mutant PA, polyvalent peptides that block toxin assembly, soluble ANTXRs, or small molecules (reviewed in Ref. 117). However, the antitoxins nearest to clinical use are those based on humanised monoclonal antibodies against PA. Several of these antitoxins are currently in preclinical or clinical trials, and have shown efficacy in primate models of inhalation anthrax.

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One problem associated with treatment of inhalation anthrax is the difficulty in diagnosing this disease. Initial symptoms are flu-like and often overlooked. A characteristic widening of the mediastinum as determined by chest X-ray is informative if anthrax is suspected. Future tests will likely be based on PCR or immuno-detection of *B. anthracis* antigens such as PA. Another possible strategy to monitor a suspected anthrax victim for signs of infection is to measure the prothrombin time (PT) for repressed clotting ability. We predict this may be useful to identify the onset of anthrax toxaemia. However, it is not clear if measurable clotting defects would occur early enough to allow for treatment.

Conclusion

The reasons why anthrax is such an extremely virulent disease are not entirely clear. Current evidence points towards toxin-mediated immunomodulation of the host, toxin-induced necrosis or apoptosis of various host cell types, and capsule-mediated inhibition of phagocytosis as being important for virulence. However, other, as yet unidentified, virulence factors are likely to exist. Interestingly, *B. anthracis* is the only known bacterial pathogen to encode a virulence factor that proteolytically cleaves MKKs/MEKs, but is not the only pathogen that inactivates MAPK signalling pathways. For example, *Yersinia pestis*, the plague bacterium, blocks MKK/MEK and IκB kinase subunit β activation with YopJ, a type III secreted effector protein that belongs to a superfamily of functionally related molecules found in both plant and animal bacterial pathogens (Refs 118, 119, 120). YopJ binds MKKs/MEKs (Ref. 121) and also has ubiquitin-like cysteine protease activity (Ref. 122), but these two functions are apparently independent. Interestingly, *Y. pestis* also encodes three understudied adenylate cyclases that may interfere with immune-cell function, similar to *B. anthracis* OT (Ref. 55). It is conceivable that adenylate cyclase activity and MKK/MEK inactivation act synergistically to enhance the fitness and pathogenicity of both *Y. pestis* and *B. anthracis*, and might explain why these unrelated bacteria produce virulence factors whose biological activities are strikingly similar. Indeed, some evidence seems to support synergistic toxicity of LT and OT (Refs 79, 89, 90, 96).

Here we have reviewed recent findings that anthrax toxin can induce a diverse set of cellular responses, depending on target cell type. LT can induce cytotoxicity, block immune cell maturation and/or cytokine production, alter cytoskeletal function, interfere with endothelial cell barrier function, and disrupt neuroendocrine signalling by blocking GR activity. These divergent effects of LT may relate to distinct arrays of MKK/MEK pathways activated in different host tissues. Since ANTXRs are expressed in virtually all tissues where examined, we suspect that the outcome of intoxication may not be limited solely by toxin access to the host cytosol, but rather by the array of activated MKKs/MEKs in intoxicated cells. In support of this hypothesis, it was recently demonstrated that LT cytotoxicity correlates with elevated phosphorylation levels of MEK1/2 in both melanoma cell lines and normal cells, and does not correlate with ANTXR expression levels (Ref. 29). Similarly, we further suspect that OT probably has tissue-specific effects, since cAMP regulates a litany of cellular functions through multiple signalling pathways. Indeed, OT-induced cytotoxicity is apparently cell-type-specific and does not correlate with cAMP levels, indicating other host cell factors influence the outcome of intoxication (Ref. 72). Therefore, we anticipate that novel, unconventional host targets and pathogenic effects of LT and OT will continue to be discovered as toxigenicity on distinct host cell populations, tissues and organs are evaluated further.

Acknowledgements and funding

This work was supported by National Institutes of Health Grant AI-057870 awarded to K.A.B. D.J.B. was supported by National Institutes of Health Microbial Pathogenesis Training Grant 2-T32-AI-07323. We thank the anonymous peer reviewers of this article for their comments.

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Further reading, resources and contacts

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David J. Banks, Sabrina C. Ward and Kenneth A. Bradley (2006) New insights into the functions of anthrax toxin. Expert Rev. Mol. Med. Vol. 8, Issue 7, 11 April, DOI: 10.1017/S1462399406010714

Accession information: DOI: 10.1017/S1462399406010714; Vol. 8; Issue 7; 11 April 2006