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ANTHRAX IS A DISEASE caused by infection from spores of the bacterium *Bacillus anthracis* (16). *B. anthracis* spores germinate in macrophages located adjacent to the epithelium of primary anthrax infection sites. Depending on the route of infection, the disease presents three different clinical manifestations: cutaneous (in which infection occurs through the skin), gastrointestinal (in which infection occurs through ingestion of spores), and pulmonary anthrax (which occurs through inhalation of spores) (11). Pulmonary anthrax is the most severe form, which often leads to death.

In recent years, many important advances have been achieved in the anthrax field. These findings are expected to stimulate the development of new inhibitors against anthrax toxin (18, 23). The anthrax bacterium releases a toxin that is essential for lethal effects. This virulence factor is composed of three proteins: protective antigen (PA, which binds to cellular receptors), lethal factor (LF, which is a protease), and edema factor (EF, which belongs to the adenylyl cyclase class of proteins). The crystal structures of PA, LF, and EF have been solved (12, 20, 21). LF and EF are individually nontoxic and require association with PA to enter the cytosol and to produce many of the symptoms of anthrax infection. The combination of PA and LF forms the lethal toxin, whereas the association of PA with EF forms the edema toxin. During cellular infection, PA binds anthrax toxin receptor (ATR) and is cleaved at the cell surface by furin and/or a furin-like protease. Cleaved PA oligomerizes and binds to EF or LF or both. The complex is then internalized via endocytosis (1, 2, 14) and trafficked to the acidic environment of endosomes, which promotes channel formation and translocation of LF or EF (10, 17).

The edema factor is an adenylyl cyclase that induces a significant increase in the intracellular concentration of cAMP. Elevated levels of cAMP in the host cells alter water homeostasis, which leads to swelling and edema. The lethal factor is required for cell death and morbidity. LF is a protease that cleaves members of the mitogen-activated protein kinase (MAPK) kinase family, thereby disrupting three MAPK signaling pathways. The direct involvement of these pathways in LT death is unknown (17).

The identification of cellular receptors for anthrax toxin represents valuable progress in understanding the molecular events involved in the intoxication mechanism and in the development of antitoxins. The receptor ATR is a type I...
membrane protein and is expressed at moderately high levels on the cell surface (6). The extracellular region of ATR contains a von Willebrand factor type A (VWA) domain, which may modulate protein-protein interactions. This VWA domain constitutes the direct binding site for PA. In addition, a metal ion-dependent adhesion site motif, localized within the VWA domain, is important for toxin binding (5–7).

ATR is encoded by the tumor endothelial marker 8 (TEM8) gene. Three variants result from alternative splicing of the TEM8 gene. The long isoform (TEM8) is a transmembrane protein of 564 amino acids, with a long proline-rich cytoplasmic tail. The medium isoform (ATR) is a 368-amino acid protein, which possesses a short cytoplasmic tail and diverges from the long isoform at the last four amino acids at the COOH terminus. The long and medium isoforms are identical throughout the extracellular region, the putative transmembrane domain, and a portion of the cytoplasmic tail. Because they both contain the VWA domain that binds to PA, they both function as PA receptors (6). To the contrary, the short isoform lacks any sequence for membrane attachment. As a consequence, this putative secreted protein does not function as a PA receptor (22). Interestingly, ATR/TEM8 is highly conserved in different species, and mouse and human homologs share 98% sequence identity in the extracellular domain (6).

Although the physiological function of ATR/TEM8 is still unknown, multiple lines of evidence suggest a role in the regulation of angiogenesis. In fact, several independent investigators have shown that, in humans, TEM8 is preferentially expressed in endothelial cells within colonic tumors (9, 19, 24). In mice, TEM8 was shown to be highly expressed in tumor vessels, as well as in the vasculature, of developing embryos, but undetectable in normal tissues. Furthermore, a second protein was identified to function as an ATR: the human capillary morphogenesis protein 2 (CMG2) (4, 15, 25). Currently, CMG2 is the protein most similar to ATR/TEM8. CMG2 and ATR possess common characteristics, including a type I transmembrane domain and a VWA domain. These two proteins share 40% amino acid identity throughout their sequence and 60% identity within their VWA domain. Interestingly, similarly to ATR/TEM8, CMG2 was shown to bind PA with its VWA domain. CMG2 is expressed in a variety of tissues, but it is upregulated in human umbilical vein endothelial cells during the process of capillary formation. Taken together, these findings pinpoint that one key function of ATR/TEM8 may be the regulation of the neovascularure.

Before the molecular cloning of ATR, the PA binding receptor was tentatively localized to the basolateral surface of polarized epithelial cells. A series of studies demonstrated that EF enters epithelial cells through the basolateral but not the apical surface, suggesting that the receptor expression is restricted to the basolateral membrane of polarized epithelial cells. As such, the exact cellular distribution of ATR/TEM8 remains unknown (endothelial vs. epithelial). For example, the expression of ATR/TEM8 in epithelial cells has not been reported to date.

In the current study, we have examined the tissue-specific and cellular distribution of ATR/TEM8 in a variety of normal...
mouse and human tissues. For this purpose, we generated and characterized a novel polyclonal antibody that selectively recognizes an extracellular epitope within the VWA domain of the ATR/TEM8 protein. The generation of this novel antibody allowed us to identify the cellular targets of anthrax toxin at the molecular level.

MATERIALS AND METHODS

Materials. Anti-Myc IgG (rabbit polyclonal) was obtained from Santa Cruz Biotechnology. Human lung and skin tissue microarrays sections were from Imgenex.

Animal studies. This study was conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal protocols were preapproved by Institutional Animal Care and Use Committees. For all of these experiments, we used 3-mo-old male wild-type mice in the C57BL/6 background. The mice were housed and maintained in a barrier facility at the Albert Einstein College of Medicine. They were housed under 12:12-h light-dark cycle conditions and had ad libitum access to chow (Picolab, PMI Nutrition International) and water. After the mice were euthanized, the lungs were removed and insulated with 2 ml of 10% neutrally buffered formalin. Small intestine samples and skin biopsies were surgically removed and fixed in 10% neutrally buffered formalin. Small intestine samples and skin biopsies were paraffin embedded, and 4- to 5-μm-thick sections were cut and placed on Super-Frost Plus slides (Fisher) for immunohistochemistry (8).

Construction of ATR/TEM8 cDNA. The cDNAs encoding the long, medium, and short isoforms of ATR/TEM8 were reisolated and subcloned into TOPO, a mammalian expression vector (Invitrogen), according to the manufacturer’s instructions. A Myc-tag epitope was placed at the COOH terminus of each construct. For isolation of the cDNA, we employed human Quick Clone cDNA (Clontech) and the Platinum Taq high-fidelity polymerase (GIBCO-BRL). Primers for amplification were designed based on the following three accession numbers: AF421380, NM032208, and BC012074. Also, a Kozak sequence was placed immediately ahead of the start site (GCCACCATG). The correctness of all clones was verified by DNA sequencing.

Cell culture and transfection. CHO cells were grown in RPMI supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum. CHO cells were transiently transfected using the Lipofectamine transfection reagent (Invitrogen) as per the manufacturer’s instructions, and cellular expression was analyzed 36 h posttransfection.

Antibody production. A polyclonal antibody to ATR/TEM8 was generated by immunization of New Zealand White rabbits with a synthetic peptide (residues 92–107; LMKLTEDREQIRQGLEC) corresponding to a sequence in the extracellular domain of human ATR/TEM8, containing an exogenously added COOH-terminal cysteine residue to facilitate maleimide conjugation to keyhole limpet hemocyanin. The resulting IgGs were purified from serum by ammonium sulfate precipitation, followed by resuspension in PBS, and subjected to peptide affinity chromatography.

Western blot analysis. Cells were lysed in hot sample buffer (13). Samples were then collected and homogenized with the use of a 26-gauge needle and 1-ml syringe. Murine tissue lysates were mixed with an appropriate volume of sample buffer containing a reducing agent (5–20 mM DTT final concentration). Protein lysates were resolved by SDS-PAGE (10% acrylamide) under reducing conditions and transferred to nitrocellulose membranes (Schleicher and Schuell). The protein bands were visualized with Ponceau S (Sigma). Membranes were blocked with 4% nonfat dried milk in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) supplemented with 1% bovine serum albumin. Blots were then incubated at room temperature for 1 h with primary antibody diluted in TBST/1% bovine serum albumin. Horseradish peroxidase-conjugated secondary antibodies were used to visualize bound primary antibodies with the SuperSignal enhanced chemiluminescence substrate (Pierce).

Peptide competition. CHO cells were transiently transfected with cDNA encoding the long isoform and subjected to preparative SDS-PAGE (19). Peptide competition was performed with synthetic α-LMKLTEDREQIRQGLEC peptide at 20 μg/ml in the absence of reducing agent. The resulting protein bands were visualized with Ponceau S (Sigma). Membranes were blocked with 4% nonfat dried milk in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) supplemented with 1% bovine serum albumin. Blots were then incubated at room temperature for 1 h with primary antibody diluted in TBST/1% bovine serum albumin. Horseradish peroxidase-conjugated secondary antibodies were used to visualize bound primary antibodies with the SuperSignal enhanced chemiluminescence substrate (Pierce).
PAGE gel. After transfer, the nitrocellulose membrane was cut into strips and incubated with the anti-ATR/TEM8 polyclonal antibody alone or in combination with nonspecific or ATR/TEM8-specific peptides. As expected, the anti-ATR/TEM8 antibody alone recognizes the long isoform as an ~80- to 85-kDa doublet. Note that after preincubation with the peptide antigen used for the antibody production (the immunogen), the anti-ATR/TEM8 antibody no longer recognizes the ~80- to 85-kDa doublet. In contrast, preincubation of anti-ATR/TEM8 IgG with an irrelevant peptide did not affect the immunoreactivity of the antibody.

**Fig. 3.** The anti-ATR/TEM8 antibody is sensitive to peptide competition with the immunogen. CHO cells were transiently transfected with the cDNA encoding the long ATR/TEM8 isoform and subjected to separation with the use of a preparative SDS-PAGE gel. After being transferred, the nitrocellulose membrane was cut into strips and incubated with the anti-ATR/TEM8 polyclonal antibody, either alone or in combination with nonspecific or ATR/TEM8-specific peptides. As expected, the anti-ATR/TEM8 antibody alone recognizes the long isoform as an ~80- to 85-kDa doublet. Note that after preincubation with the peptide antigen used for the antibody production (the immunogen), the anti-ATR/TEM8 antibody no longer recognizes the ~80- to 85-kDa doublet. In contrast, preincubation of anti-ATR/TEM8 IgG with an irrelevant peptide did not affect the immunoreactivity of the antibody.

**Immunohistochemistry.** Paraffin sections were deparaffinized in xylene (twice, 10 min each), hydrated through a graded series of ethanol washes and placed in PBS. Antigen retrieval was performed by heating at 95°C for 15 min in citrate buffer. Endogenous peroxide activity was quenched by incubation with the peroxidase blocking reagent (DAKO). Sections were incubated overnight with the anti-ATR/TEM8 polyclonal antibody. After being washed, the slides were incubated with a biotin-conjugated secondary antibody. The slides were then incubated with horseradish peroxidase-conjugated streptavidin (DAKO). Bound antibodies were visualized using diaminobenzidine as the substrate. The sections were then counterstained with hematoxylin and dehydrated. The slides were mounted with a xylene-based mounting medium (Micromount, Surgipath) and observed with an Olympus IX 70 inverted microscope.

**RESULTS**

A cellular receptor for PA was recently identified as ATR, a transmembrane protein that results from alternative splicing of the TEM8 gene. The physiological function of ATR/TEM8 has not yet been fully elucidated, but several lines of evidence suggest that ATR/TEM8 may play a role in angiogenesis. For example, TEM8 transcript levels were discovered to be elevated selectively in colorectal cancer endothelium (19, 24). In addition, ATR/TEM8 is abundantly expressed in tumor-associated blood vessels as well as in the vasculature of developing murine embryos (19, 24). In contrast, ATR “activity” has been localized to the basolateral surface of polarized epithelial cells (3). As such, the cellular localization and tissue distribution of the ATR/TEM8 protein remain controversial.

In this report, we attempt to elucidate the distribution of ATR/TEM8 in tissues that constitute the primary targets for anthrax infection. For this purpose, we generated and characterized a novel anti-ATR/TEM8 polyclonal antibody that selectively recognizes all three ATR/TEM8 isoforms (long, medium, and short).

**Novel anti-ATR/TEM8 polyclonal antibody detects all three ATR/TEM8 isoforms.** Figure 1A shows a schematic diagram of the three ATR/TEM8 isoforms resulting from alternative splicing of the TEM8 gene. The long isoform (TEM8) contains 564 amino acids with a transmembrane region and a long proline-...
rich cytoplasmic tail. The medium isoform (ATR) encodes a 368-amino acid protein with a transmembrane region and a short cytoplasmic tail. The long and medium isoforms are identical throughout the extracellular and transmembrane domains but diverge at the last four amino acids of the cytoplasmic tail at the COOH terminus. As such, the medium and long isoforms both function as receptors for the anthrax toxin (6). To the contrary, the short isoform encodes a 333-amino acid protein, which is identical to the other two isoforms in the extracellular domain but profoundly diverges just before the

Fig. 5. ATR/TEM8 is highly expressed in the epithelial cells of murine lung. Paraffin-embedded sections of mouse lung were subjected to immunohistochemistry with anti-ATR/TEM8 polyclonal antibody and counterstained with hematoxylin. ATR/TEM8 is strongly expressed in the respiratory epithelium of the bronchi (b) of the lung. Intense staining is evident in the ciliated cells surrounding the lumen of the bronchial tube (C and D). The endothelial cells lining the vessels (v) show weak or undetectable staining (A, B, and D), whereas the smooth muscle cells surrounding the vessels highly express the ATR/TEM8 proteins (A). A higher magnification view is shown to better illustrate this point (E).

Fig. 6. Epithelial cells of the human lung express high levels of ATR/TEM8. Immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody was performed on human lung paraffin-embedded sections. ATR/TEM8 is highly expressed in the respiratory epithelium of the bronchi (A). Intense ATR/TEM8 staining occurs in the ciliated epithelial cells lining the lumen of the bronchial tube (B).
putative transmembrane domain and is lacking any sequence for membrane attachment. As a consequence, the short putative secreted isoform does not function as a receptor for the anthrax toxin.

All three ATR/TEM8 isoforms share a common extracellular VWA domain (residues 44–216). To generate a novel rabbit polyclonal antibody directed against ATR/TEM8, we used a sequence within the VWA domain of ATR/TEM8 (residues 92–107; LMKLTEDREQIRQGLE) (Fig. 1A). Importantly, we chose this ATR-epitope because it is completely divergent from an ATR-related protein, namely, CMG2 (ILPLTGDRGKISKGLE), at this position; note that 8 of the 16 amino acid residues are not conserved.

An alignment of the COOH-terminal domain sequences of the three isoforms illustrates that the three isoforms profoundly diverge at the COOH terminus (Fig. 1B). Note that the short isoform lacks a transmembrane domain, whereas the medium isoform has a 25-amino acid cytoplasmic tail, compared with the 221-amino acid cytoplasmic tail of the long isoform.

To test the immunoreactivity of our novel anti-ATR/TEM8 antibody, we transiently transfected CHO cells with Myc-tagged cDNA encoding the short, medium, and long ATR/TEM8 isoforms. Western blot analysis revealed that the ATR/TEM8 antibody successfully recognizes all three isoforms (Fig. 2A). Importantly, the different isoforms can be distinguished based on their respective molecular weights. The short isoform is a 45-kDa protein, the medium isoform is a ~60-kDa protein, and the long isoform is an ~80- to 85-kDa doublet. This doublet is probably due to multiple glycosylation events. Interestingly, this doublet was also detected in untransfected

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**Fig. 7.** ATR/TEM8 is highly expressed in the epithelial cells (keratinocytes and follicular cells) of murine skin. Immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody was performed on murine skin paraffin-embedded sections. A reveals strong ATR/TEM8 expression in the thin epithelium of the epidermis, as well as in the hair follicles. B shows a section of A at higher magnification.

**Fig. 8.** ATR/TEM8 is highly expressed in the epithelial cells of human skin. Immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody was performed on paraffin-embedded sections of human skin. Robust ATR/TEM8 expression is evident in the thick epithelium of the epidermis (A). B shows a section of A at higher magnification.
CHO cells, suggesting that the ATR/TEM8 antibody recognizes the long endogenous isoform. To independently verify these results, we used three isoforms containing a Myc tag at the COOH terminal and performed immunoblotting with an anti-Myc polyclonal antibody. Figure 2B shows that all three isoforms are strongly expressed in CHO cells. These results demonstrate the specificity and the strong affinity of the novel anti-ATR/TEM8 polyclonal antibody, as it detects exogenous as well as endogenous ATR/TEM8 isoforms.

Anti-ATR/TEM8 antibody is sensitive to peptide competition with immunogen. We further analyzed the specificity of the anti-ATR/TEM8 antibody by competition with the peptide used for antibody production (the immunogen) and with an irrelevant peptide. CHO cells were transiently transfected with the cDNA encoding the long isoform. As expected, Western blot analysis with the anti-ATR/TEM8 antibody detected an ~80- to 85-kDa doublet corresponding to the long endogenous isoform (Fig. 3). However, immunoreactivity was abolished by preincubation of the anti-ATR/TEM8 antibody with a 100 M excess of the antigenic peptide (Fig. 3). Importantly, the immunoreactivity of the antibody was not affected by an irrelevant peptide (Fig. 3). These results demonstrate the high selectivity of the novel ATR/TEM8 antibody in detecting ATR/TEM8 isoforms.

ATR/TEM8 isoforms are widely expressed in a variety of mouse tissues. To gain insight into the pathogenesis of anthrax infection, we next examined the tissue distribution of the ATR/TEM8. We performed Western blot analysis on a variety of mouse tissue lysates. Figure 4 shows that the ATR/TEM8 antibody successfully detects the endogenous expression of all three ATR/TEM8 isoforms, distinguishable by their different molecular weights. We found that the ATR/TEM8 isoforms are widely and differentially expressed. The long isoform is predominantly expressed in the spleen, lung, and kidney, whereas the medium isoform is mainly expressed in the liver and kidney. Finally, the short isoform is particularly enriched in the heart, brain, and kidney (summarized in Table 1).

These results are in conflict with previous findings, in which TEM8 expression is restricted to tumor endothelium and developing vasculature and is undetectable in normal tissues. ATR/TEM8 is highly expressed in epithelial cells lining B. anthracis’ three sites of entry: lung, skin, and intestine. Next, we attempted to evaluate the detailed tissue distribution of ATR/TEM8 by immunohistochemistry. We tested the ATR/TEM8 expression in the three tissues that represent the main routes of entry of the anthrax bacterium, i.e., lung, skin, and intestine. Because the pulmonary form is the most lethal form of the anthrax disease, we first assessed ATR/TEM8 expression on paraffin-embedded sections from mouse lung. Surprisingly,
ATR/TEM8 expression was strongly detectable in the respiratory epithelium of the bronchi of the lung (Fig. 5). In particular, an intense staining was evident in the ciliated epithelial cells surrounding the luminal surface. In addition, ATR/TEM8 was also expressed in the smooth muscle cells surrounding the vessels, as well as in epithelial cells lining the alveoli. We observed a weak or undetectable staining in the endothelial cells lining the pulmonary vessels (Fig. 5). Taken together, the tissue distribution of ATR/TEM8, as determined by immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody, correlated with the primary uptake sites of the anthrax bacterium.

To evaluate whether the ATR/TEM8 epithelial localization is mouse specific or a more general phenomenon, we performed immunohistochemistry with the ATR/TEM8 polyclonal antibody on human lung paraffin-embedded sections. Figure 6 shows that ATR/TEM8 staining is concentrated in the respiratory epithelium of the bronchi in the lung. In particular, the ciliated epithelial cells lining the bronchi display strong ATR/TEM8 expression. Taken together, these results suggest that normal respiratory epithelial cells can effectively bind and internalize more molecules of anthrax toxin than other cell types, causing the lethal pulmonary form of the disease.

Microscopic or gross breaks of the skin represent another important route for anthrax infection. For this reason, we evaluated ATR/TEM8 expression in skin biopsies. Immunohistochemistry with the ATR/TEM8 polyclonal antibody on mouse skin paraffin-embedded sections revealed that ATR/TEM8 is strongly expressed in the epithelial keratinocytes of the epidermis (Fig. 7). To verify the epithelial localization of the ATR/TEM8 in other species, we also analyzed the ATR/TEM8 expression in humans. Figure 8 shows that ATR/TEM8 displays a similar expression pattern in both mouse and human skin. ATR/TEM8 staining is particularly intense in the thick layer of epithelial cells of the epidermis.

The intestine represents the third main route of infection of the anthrax bacillus. As such, we attempted to evaluate the expression pattern of ATR/TEM8 in the small intestine. Interestingly, immunohistochemistry with the ATR/TEM8 polyclonal antibody on mouse small intestine paraffin-embedded sections revealed that ATR/TEM8 is highly expressed in the epithelial cells lining the intestinal crypts (Fig. 9).

**ATR/TEM8 isofrom expression in lung, skin, and small intestine.** We next performed Western blot analysis on lysates prepared from normal mouse lung, skin, and small intestine (Fig. 10). Interestingly, the long isofrom is predominantly expressed in the lung and in the small intestine but is still present at lower levels in skin. In contrast, the medium isofrom is absent from the lung but is highly expressed in the small intestine and to a lesser extent in the skin. Finally, the short isofrom is present only in skin (summarized in Table 1). Thus these three sites of entry also differentially express all three ATR/TEM8 isofroms in a tissue-specific fashion.

**DISCUSSION**

Taken together, our results clearly show that the receptor for anthrax toxin is highly expressed in the epithelial cells lining organs that represent the major uptake routes of *B. anthracis* spores. Strikingly, increased sensitivity of certain organs, i.e., lung, skin, and intestine, to anthrax infection correlates with high expression levels of the anthrax toxin receptor. This implies that the initial entry sites of the bacterium also represent sites for increased anthrax toxin uptake. High levels of anthrax toxin receptors on lung epithelium may be responsible for edema formation and accumulation of pleural fluids, which are hallmarks of anthrax pathogenesis. As such, these findings provide new important clues for understanding the function of ATR/TEM8 and the mechanisms that underlie the pathogenesis of anthrax infection.

**REFERENCES**


