Targeting toxins!

Drug delivery with poisons

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Many organisms produce potently toxic proteins that act on other cells, sometimes with lethal effects. In this way, such proteins help to increase the chance of survival or proliferation of the producing organism. Moreover, a lot of toxins have an exquisitely specific action. For example, proteins studied in the Warwick toxin laboratory — ricin, a toxin from the castor oil seed (Figure 1), and its relatives from the pathogenic *Escherichia coli* 0157 and the dysentery-causing bacterium (*Shigella dysenteriae*), have evolved to selectively target ribosomes within the cells of susceptible organisms, thereby enabling a fatal disruption of protein synthesis. What is very striking is the clever way these particular toxins exploit intracellular transport pathways to travel from the cell surface to their substrates in the cytosol. Once delivered there, each toxin molecule can disable approximately 2000 polysomes per minute, enough to eventually kill the cell. Research is now aimed at elucidating the molecular details of the cellular uptake of ricin and the Shiga family of toxins, and of exploiting their unusual trafficking properties for biotechnological purposes.

Toxin transport into cells

It has become clear that the pathway leading to productive killing of the cell initially involves encasement of the surface-bound toxin in pinched-off membrane vesicles that ferry material between various intracellular organelles. The exact route taken by these vesicles effectively describes the entire secretory pathway, but in reverse[1] (Figure 2A). The molecules that the toxins meet on this inward journey and the nature of the transport carriers at each stage are now being investigated. What is clear is that an hour or so after the initial uptake into the cell, ricin and similar toxins are successfully delivered to the endoplasmic reticulum (ER), a labyrinth of interconnecting chambers that is a major site of protein folding and lipid biosynthesis. To attack ribosomes in the cytosolic matrix, the toxins must escape this membranous enclosure. This they do by taking advantage of a natural pathway to the cytosol, one that is normally undertaken only by unassembled or crippled proteins that have failed to fold properly in the ER. Quite how the toxins 'fool' the stringent ER quality control system into believing that they are genuine candidates for this expulsion process isn't known. It appears that the toxins must become partially unfolded for this to happen, so ER 'unfoldases', chaperones and even the lipids of the membrane could be involved. Once they are shipped across the membrane through conduits that are composed of Sec61 proteins, the toxins must finally avoid the ultimate fate of such expelled proteins, that of proteolysis in the cytosol. Preliminary evidence supports the idea that the lysine-deficient toxins evade being tagged by ubiquitin that would otherwise mark them for destruction by the proteasomal machinery.

With superb irony, it appears that the expelled toxins can be refolded by ribosomes. In this way, they achieve a protease-resistant, active conformation as their substrates effectively commit suicide!

Figure 1. *Ricinus communis* (castor oil) plant. The brightly coloured seed pods protect the developing seeds that contain newly synthesized ricin.

Toxins as intracellular carriers

It is, perhaps, not surprising that over the years proteins with such toxic potency have been eagerly used as components in magic bullets targeted to kill tumour cells. However, in a digression from this more usual application of cytotoxic proteins, a major drive is now being taken to exploit not their toxicity, but their novel intracellular trafficking properties. In this approach, disarmed (non-toxic) versions of toxins are being designed as vectors to deliver viral epitopes or tumour antigens to cellular molecules in order to elicit protective immunity against viruses, other intracellular pathogens or tumours[2,3].

Targeting antigens into the MHC class I-restricted pathway

Vaccination is one of the great success stories of the last century, with most effort focused on the preparation of vaccines comprising live, attenuated or dead pathogens that ultimately cause production of protective antibodies. However, to induce protective immunity against intracellular pathogens such as viruses, which are out of the reach of circulating antibodies, there is a requirement for specific cytotoxic T lymphocytes (CTLs) that can directly kill infected target cells. Unfortunately, vaccine-induced generation of protective CTLs is not particularly easy. This is due to fundamental differences in the way the immune system responds to extracellular and intracellular antigens. These differences centre on the cellular site required for the generation of the immunizing peptides and the nature of the MHC proteins that are required for peptide recognition.

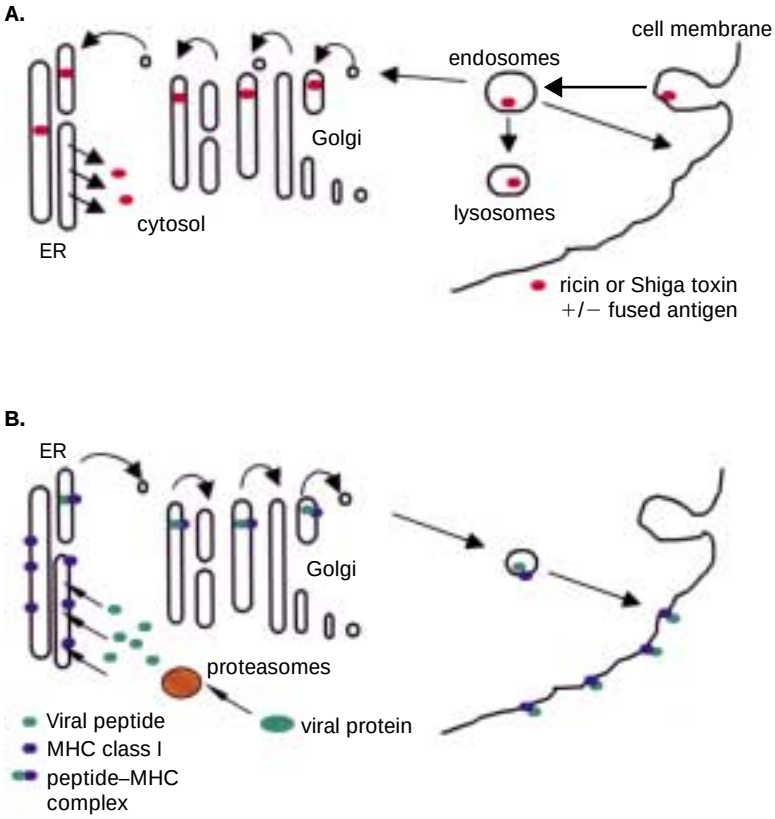
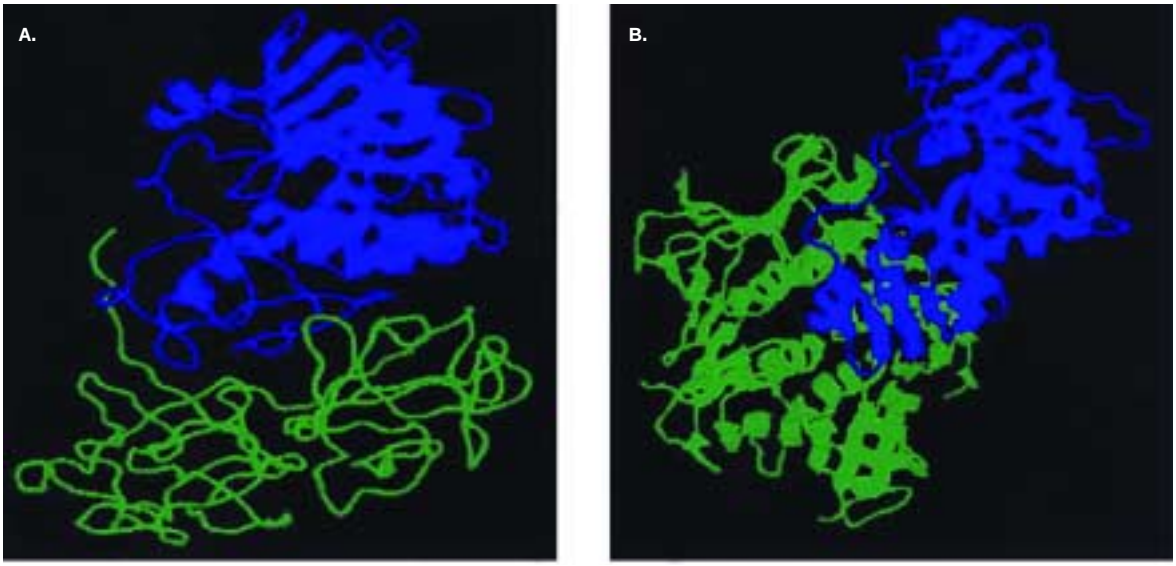


Figure 2. The uptake pathway of certain toxins intersects the MHC class I pathway. (A) The productive cell entry pathway for the holotoxins ricin, Shiga toxin and their derivatives carrying antigens. (B) The classical MHC class I-restricted processing and presentation pathway of nucleated mammalian cells.

Generally, exogenous antigens (such as foreign recombinant protein or bacterial proteins) are taken into antigen-presenting cells and are broken down in endosomal compartments. The resulting peptides become bound to empty MHC class II proteins that are then transported to the cell surface where they can be recognized by $CD4^+$ T cells (T helper cells). This can lead to the activation of $CD4^+$ cells, which in turn triggers a process that results in the activation of B cells and antibody production. By contrast, in nucleated cells, peptides derived from the breakdown of even a small number of intracellular viruses or other intracellular pathogens are transported into the ER lumen by peptide transporters associated with antigen processing before becoming associated with MHC class I proteins in the ER membrane (Figure 2B). The peptide–MHC class I complexes are then transported to the cell surface where they may be recognized by $CD8^+$ T cells. Such cells can then proliferate and differentiate into memory cells or CTLs that can destroy the cell before the pathogen that gave rise to the peptide is able to propagate and escape. Since the MHC class I and class II pathways are largely segregated, it can be difficult to raise a CTL response against an external protein. This is clearly problematic for a vaccine candidate where it is desirable to induce a CTL response to aid the clearance of intracellular pathogens or, indeed, of unwanted self antigens.

This problem has led to the emergence of a number of technologies that could have profound impact on vaccine administration[4]. Most rely on efficient delivery of antigen into the MHC class I pathway rather than the MHC class II pathway. These approaches include the use of synthetic peptide vaccines, virus-like particles, DNA vaccines, dendritic cell adjuvants and protein toxin vectors. It



Toxin structures. The backbone structures of (A) ricin and (B) Shiga toxin, showing the domain structure of this type of toxin, with cell binding subunits in green and the related catalytic subunits in blue.

is the last of these that is being studied at the Warwick laboratory in a collaboration with Professor Vincenzo Cerundolo of the Institute of Molecular Medicine in Oxford.

Toxins as non-live delivery vectors

Since ricin and Shiga toxin can be routed from the cell surface to the ER (Figure 2A) and on to the cytosol, one idea in developing a new generation of vaccines is to exploit these trafficking properties for the delivery of peptides directly into the MHC class I-restricted pathway (Figure 2B). Obviously, to use toxins purely as vectors rather than as killing agents in their own right, it is necessary to use catalytically-inactive versions. This is made possible because the ribosome-inactivating catalytic site is well characterized, and minimal structural disruption is required to produce so-called disarmed versions. Genetic fusion of defined peptide epitopes or even whole viral/tumour proteins with the desired toxin variant can then be made. Continued investigation of this approach has been encouraged by the fact that ricin and the Shiga proteins are tolerant of extra sequences (even whole proteins), they are inherently stable and resistant to extracellular proteases, and they have already been shown to work well in delivering model antigenic peptides to MHC class I molecules *in vitro*.

It would be simplistic to assume that all antigens carried by these proteins would be correctly delivered, processed and presented in exactly the same way in all cell types. Already differences are emerging. Whereas ricin can potentially deliver antigens to class I MHC proteins in the ER of many types of cell, Shiga-peptide fusions can induce CTLs from a limited number of cell types owing to the more restricted distribution of the toxin receptor[2,3]. CTLs can be induced via Shiga B chain–peptide fusions that are preferentially taken up by professional antigen-presenting cells[3] which, interestingly, are insensitive to the action of active Shiga holotoxin. This implies differences in the trafficking routes in the different cell lines[5]. Moreover, internalized Shiga B chain can reach the nucleus[6]. These observations may broaden the applicability of toxins as vehicles such that they may be engineered to ferry different types of macromolecules to different parts of different cell types. As ever, future exploration of their potential utility promises to be an interesting venture.

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