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Biotechnological Approaches to Fight Pathogens at Mucosal Sites

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Introduction: pathogens at mucosal sites

Most interactions between host and pathogens occur at the host mucosal surfaces. Many pathogens, such as the human immunodeficiency virus (HIV), gain entry via the mucosa while others, including *Candida*, *Streptococcus mutans* and *Helicobacter pylori*, must become established at the mucosa to cause damage to the host. Strategies aimed at controlling pathogens at mucosal surfaces and infectious diseases in general are summarized in *Figure 13.1*. These include primarily vaccination and the use of antimicrobial chemotherapy, particularly antibiotics, which have both had an enormous impact on infectious disease (Cohen, 2000). Passive immunization has been used less with the advent of vaccines and antibiotics but is of increasing importance for treatment of immunocompromised patients (Hammarström, 1999) whilst the development and use of topical microbicides is regarded as a potentially important means of preventing infection with HIV (Lange *et al.*, 1993). Pathogens at mucosal sites, however, present particular problems for these measures, e.g. antibiotics can be very effective in clearing systemic infections while being unable to affect mucosal carriage of the pathogen. A limited number of vaccines induce protective mucosal responses and vaccines are not yet available for several microorganisms that infect mucosal surfaces. These observations, together with concern over the spread of antibiotic resistance (Hawkey, 1998; Irvin and Bautista, 1999), have stimulated investigation of additional antimicrobial strategies as well as refinement of existing approaches.

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Abbreviations: CV-N, cyanovirin; ETEC, enteropathogenic *Escherichia coli*; HIV, human immunodeficiency virus; HSV, herpes simplex virus; ICAM-1, intercellular adhesion molecule-1; KT, killer toxin; LNnT, lacto-*N*-noetetraose; Mab, monoclonal antibody; NMR, nuclear magnetic resonance; SA, streptococcal antigen; ScFv, single chain variable region fragment; SIgA, secretory immunoglobulin A; SIV, simian immunodeficiency virus.

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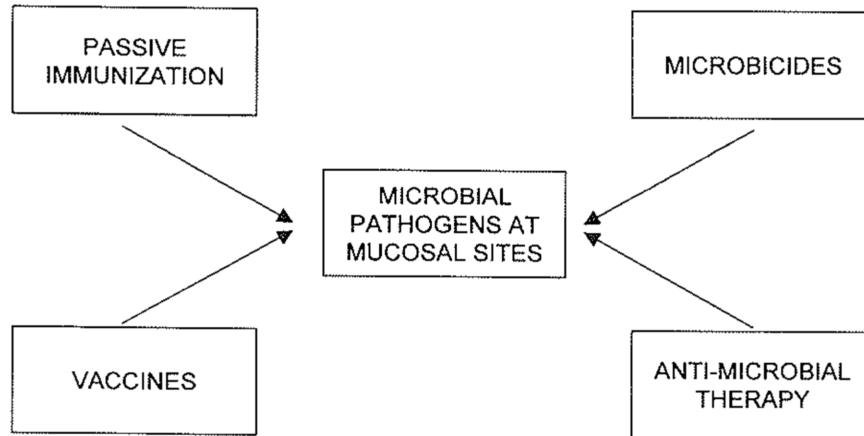

Figure 13.1. Current strategies against pathogens at mucosal sites.

The focus of many studies has been to target early events in infection and, in particular, microbial adhesion. Adhesion or attachment to mucosal surfaces is an essential step in infection or pathogenesis and is mediated by stereospecific interaction between cell surface molecules, termed adhesins, on the surface of the microorganism and receptors on host tissue. Reduced virulence *in vivo* has been demonstrated with specific adhesin-deficient microorganisms (Roberts *et al.*, 1994; Connell *et al.*, 1996) or in animal models where receptors are not expressed (Guruge *et al.*, 1998). Progress in defining the molecular basis of adhesin–receptor interactions has provided the rationale for the design of inhibitors that may selectively block adhesion of pathogens. These inhibitors include both analogues of adhesin molecules and of receptors as well as adhesion-blocking antibodies (*Figure 13.2*). The effectiveness of passive immunization may be improved by use of monoclonal antibodies that target adhesins of specific pathogens directly. Recent developments include use of transgenic plant technology for the expression of secretory IgA (SIgA) forms of

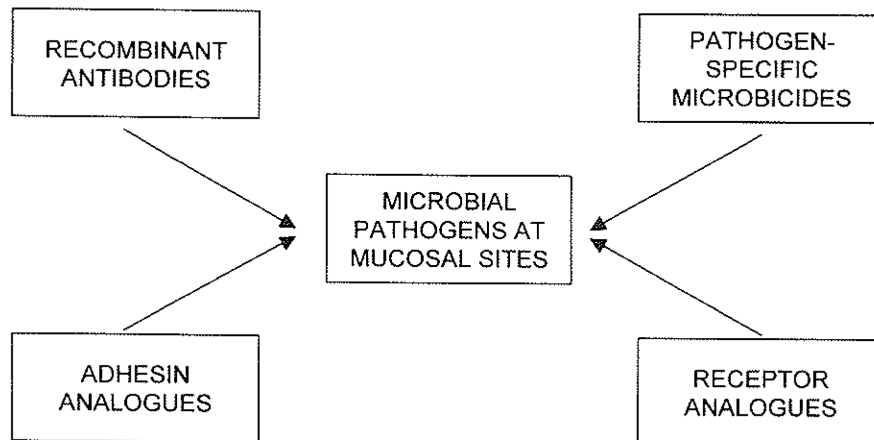

Figure 13.2. Additional targeted strategies aimed at early events in infection.

antibody, as well as combinatorial library technology for the production of novel antibodies. Similarly, targeting of microbicides either by use of improved delivery systems or by development of pathogen-specific antimicrobials has been investigated. In this article we review some of the work that underpins the new approaches to the control of infectious disease.

New strategies, new molecules: a biotechnological approach

ANTI-ADHESIN ANTIBODIES

Passive administration of antibody to mucosal surfaces has been used both prophylactically and therapeutically to prevent infection in infants and immunocomprised patients (Heikkinen *et al.*, 1998; Hammarström, 1999). The pooled immunoglobulin preparations that are most frequently used confer some degree of protection against a wide range of microorganisms but the effectiveness of the treatment is limited by the levels of antibody directed towards specific pathogens. Typically, the treatment requires relatively large and repeated doses of immunoglobulin and protection does not extend beyond the period of treatment (Heikkinen *et al.*, 1998). The use of monoclonal antibodies (Mabs) that target microbial adhesins may provide a means of considerably enhancing the effectiveness of this form of treatment with the potential to selectively remove the pathogen. Moreover, the development of technology for engineering antibodies (Winter, 1998) and for large-scale production of such antibodies has increased the availability of these novel antimicrobial agents.

Passive immunization with anti-adhesin Mabs has been shown to be effective against *Streptococcus mutans*, the main cause of dental caries, not only in animal models but also in human trials. Adhesion of *S. mutans* to the tooth surface is mediated by a streptococcal cell surface adhesin, termed streptococcal antigen I/II (SA I/II), that binds to salivary glycoproteins adsorbed to the tooth surface. Mabs raised against SA I/II were applied directly to the tooth surfaces of non-human primates and two Mabs (Guy's 1 and Guy's 13) were effective in preventing both colonization with *S. mutans* and the development of dental caries (Lehner *et al.*, 1985). Subsequently, trials with Mab Guy's 13 were performed in a human model of infection (Ma *et al.*, 1989). Subjects were treated with chlorhexidine gluconate, a broad spectrum antiseptic, to reduce *S. mutans* (and other plaque bacteria) to undetectable levels and Mab or control solution was then applied directly to the teeth on 6 occasions over a period of three weeks. Re-colonization of the oral cavity with *S. mutans* was then followed by sampling dental plaque and saliva at intervals. In control subjects treated with saline or Mab of irrelevant specificity, re-colonization was evident within 1 to 3 days following chlorhexidine treatment and *S. mutans* returned to the original levels or higher within 2–3 months. In contrast, no re-colonization was evident for at least one year in subjects treated with Mab raised against SA I/II. Protection therefore extends for a considerable period after passive immunization has stopped. The long duration of protection could not be attributed to persistence of antibody since this is no longer detectable 24 hours after application (Ma *et al.*, 1998). A model was proposed in which, following clearance with chlorhexidine, any *S. mutans* is bound specifically by anti-SA I/II Mab and prevented from adhering to the tooth surface. The bacteria

may also be opsonized and phagocytosed by neutrophils in the oral cavity. The ecological niche vacated by *S. mutans* is then occupied by other (non-pathogenic) plaque microorganisms that competitively exclude *S. mutans* and so provide long-term protection. In a further study, $F(ab)_2$ fragments were shown to be as effective as the intact antibody, indicating that Fc-mediated effector functions of antibody are not required for protection (Ma *et al.*, 1990).

Adhesion-blocking Mab was also shown to be effective in a human re-colonization model of infection with *Porphyromonas gingivalis* considered to be a possible cause of periodontitis (Booth *et al.*, 1996). Patients were treated with metronidazole to suppress *P. gingivalis* and saline (control) or Mab raised against the adhesin component of an arginine-specific protease, was applied subgingivally on four occasions over 10 days. Subjects treated with Mab were significantly protected against re-colonization with *P. gingivalis* for 9 months.

Protection in animal models of infection

Several studies using animal models of infection indicate that passive immunization with Mabs that block early events in infection may be effective against a variety of infectious diseases including those caused by fungal or viral infection. Vulvovaginitis, caused by infection with *Candida* spp. and most frequently by *Candida albicans*, is one of the most common diseases, with approximately 75% of women experiencing at least one episode and about 5% experiencing recurrent attacks that are not resolved by treatment (Fidel and Sobel, 1996). In the treatment with antifungal drugs, however, the emergence of drug-resistant strains (Denning, 1995) and the deficient immune response in immunocompromised patients present particular problems for therapy and have stimulated research into alternative treatments.

Adhesion of *C. albicans* to epithelial cells may involve a variety of adhesins, including the mannan component of yeast mannoprotein (Kanbe and Cutler, 1994) and secreted aspartyl proteinases or 'Saps' (Watts *et al.*, 1998). The latter may modify host surface molecules but are also thought to act directly as adhesins. In a rat model of vaginal candidiasis (De Bernardis *et al.*, 1999), intra-vaginal administration of Mab (IgM isotype) raised against a carbohydrate epitope of the mannoprotein or anti-Sap Mab (IgG1 isotype), thirty minutes before intravaginal challenge with 10^7 yeast cells of *Candida albicans*, reduced fungal burden. In contrast, two Mabs (both IgG1) recognizing polypeptide epitopes of the mannoprotein conferred no protection (De Bernardis *et al.*, 1997). Most significantly, in the group treated with anti-Sap Mab only 1/5 rats was still infected by day 10 compared with 5/5 in the control group. These findings suggest that the epitope-specificity of Mabs is important for protection and are consistent with a mechanism that involves blocking of adhesion. The importance of antibody specificity was also suggested by separate studies using a murine model of vaginal candidiasis where two IgM Mabs and an IgG3 Mab were protective (Han *et al.*, 1998, 2000). The Mabs were again directed against epitopes within the adhesion-mediating carbohydrate moiety of the mannoprotein. As in the treatment of oral infections, protection in these models of vaginal candidiasis extended well beyond the period of antibody administration. The vagina, like the oral cavity, is a mucosal compartment that is normally colonized by a diverse flora, thus the relatively long duration of protection in this model may also be the result of competitive exclusion by other members of the vaginal flora.

Attachment of herpes simplex virus (HSV) involves initial binding of surface glycoprotein C to heparan sulphate proteoglycans followed by binding of glycoproteins B and D (Fuller and Lee, 1992). Recombinant human Fabs against glycoprotein D were selected from a phage display combinatorial antibody library generated using bone marrow lymphocyte RNA from a long-term asymptomatic HIV-1-positive individual with serum antibodies against HSV-1 and HSV-2 (Burioni *et al.*, 1994). One Fab that possessed *in vitro* virus-neutralizing activity and inhibited plaque formation was engineered in an IgG1 form of the antibody. The IgG1 Mab was compared with Fab and $F(ab)_2$ fragments in a murine model of vaginal transmission in which antibody or fragments were administered intravaginally immediately before inoculation with HSV-2 by the same route (Sanna *et al.*, 1996). Complete protection was achieved with both intact IgG1 and fragments, indicating that Fc-mediated effector functions are not required for protection in this model.

That anti-adhesin antibodies may also be effective in preventing bacterial infection at a mucosal site that is not normally colonized has been demonstrated in a murine cystitis model. Passive immunization with antiserum raised against the pilus-associated adhesin FimH produced a 100- to 150-fold reduction in the number of uropathogenic *Eschericia coli* recovered from the bladder (two days after challenge) compared with serum from mice immunized with adjuvant alone (Langermann *et al.*, 1997).

Transgenic plant technology in the production of recombinant antibody

Expression in transgenic plants provides a potential means of producing adhesion-blocking antibodies in the quantity that would be required for large-scale use of this form of antimicrobial. Transgenic plants are able to assemble immunoglobulin molecules efficiently (Hiatt *et al.*, 1989; Ma *et al.*, 1994) and, of particular relevance to the production of antibodies for administration at mucosal sites, can assemble SIgA (Ma *et al.*, 1995). SIgA is the main isotype associated with mucosal surfaces and because of increased avidity (due to tetravalency) and increased resistance to proteolysis (due to the presence of secretory component), may be more potent than IgG as a mucosal antimicrobial agent.

The effectiveness of both classes of antibody was compared in the human model of infection with *S. mutans* described above. The SIgA form of the protective Mab, Guy's 13, was expressed in transgenic tobacco plants and purified (Ma *et al.*, 1998). In a human trial, the plant-derived antibody proved to be as effective as the parent murine IgG antibody in preventing re-colonization with *S. mutans*. Persistence of the secretory antibody in the oral cavity was increased compared with IgG (three days compared with one day). The increased persistence of SIgA at mucosal surfaces should allow longer intervals between applications and this form of antibody may be particularly appropriate for application at sites that are less accessible than the oral cavity. In this study as in previous trials, no serum antibody responses were detected to the topically applied Mab.

The efficacy of transgenic plant-derived antibodies has also been demonstrated in the murine model of vaginal HSV infection. In this example, a murine Mab raised against HSV glycoprotein B was humanized (Co *et al.*, 1991) and subsequently expressed as an IgG1 antibody in soybean or a myeloma cell line (Zeitlin *et al.*, 1998).

Both forms of antibody were stable in human semen and cervical mucus and both were protective in the murine vaginal model.

ADHESIN-ANALOGUES

Soluble forms of a microbial adhesin or of an adhesin fragment may be used as competitive inhibitors to block adhesion. In several studies, adhesion epitopes have been mapped by determining the inhibitory activity of synthetic peptides or recombinant polypeptide fragments derived from the sequence of microbial adhesins, using *in vitro* adhesion assays. Filamentous haemagglutinin (FHA) and pertactin, both adhesins of *Bordetella pertussis*, bind to integrins by means of RGD sequences and binding *in vitro* is inhibited by peptides comprising the respective RGD-containing sequences (Relman *et al.*, 1990; Leininger *et al.*, 1991). Similarly, pilus-mediated adhesion of *Pseudomonas aeruginosa* is inhibited by a peptide from the semi-conserved C-terminal region of the pilin monomer (Lee *et al.*, 1994) and a recombinant 38-residue peptide from the fibronectin-binding protein of *Streptococcus pyogenes* inhibited bacterial adhesion *in vitro* (Talay *et al.*, 1992).

A synthetic peptide (p1025) corresponding to residues 1025–1044 of SA I/II, which inhibited adhesion *in vitro* of *S. mutans* to salivary receptor (Kelly *et al.*, 1995), was also shown to prevent infection with *S. mutans* in a human clinical trial (Kelly *et al.*, 1999) using the re-colonization model described above. Following treatment with chlorhexidine to eliminate *S. mutans*, p1025 (synthesized as an acetylated peptide amide) was applied directly to the teeth surfaces of subjects on six occasions over three weeks. In addition, a mouthwash containing p1025 was used daily for two weeks. Control subjects received buffer only or a non-inhibitory peptide, p1125 (spanning residues 1125–1144 of SA I/II). Re-colonization with *S. mutans* was then followed by sampling plaque and saliva as before. In control groups, re-colonization was evident by the end of the three weeks of administration of buffer or non-inhibitory peptide. By the end of the experiment (120 days), all members of the group receiving p1125 had re-colonized, as had three of the four receiving buffer. In contrast, none of the subjects treated with p1025 had re-colonized by day 88 and only one of four had re-colonized by the end of the experiment. The effect of administration of p1025 on *Actinomyces naeslundii*, a non-pathogenic member of the oral flora, was also investigated. Both the control group receiving p1125 and the group receiving p1025 had re-colonized by the end of peptide administration (day 21) suggesting that p1025 specifically targeted *S. mutans*. The peptide was not toxic to *S. mutans* and was detectable in the oral cavity for only 6 hours after application, thus the extended protection cannot be explained by continuing action of the peptide. As proposed for the protective Mab, initial inhibition of *S. mutans* adhesion to the tooth surface (by p1025) may be followed by competitive exclusion by other members of the oral flora to provide long-term protection.

That synthetic peptides may be used to prevent infection at other mucosal sites was demonstrated in a murine model of rotavirus infection (Ijaz *et al.*, 1998). Rotavirus is the main cause of viral enteritis. Viral attachment requires proteolytic cleavage of virus protein 4 (VP4) into subunits VP5 and VP8, the latter component being the adhesion domain. A synthetic peptide (residues 232–255) corresponding to the conserved sequence spanning the cleavage site inhibited virus attachment to cells *in*

vitro and was investigated as an antimicrobial *in vivo*. Peptide was administered orally to mice one hour before challenge with virus and mice were then monitored for clinical symptoms. At 72 hours, mice were killed and intestines were removed to determine viral load. Mice receiving the highest dose of peptide (1 mg) were completely protected from rotavirus infection and showed no symptoms compared with buffer-treated controls. The protease cleavage site was not previously identified as an adhesion epitope and it was suggested that inhibition of rotavirus binding may either be the result of competitive inhibition of binding to epithelial cells or of competition with rotavirus VP4 for proteolytic enzymes required for generation of the VP8 subunit. Irrespective of the mechanism, this study demonstrates that effective levels of inhibitory synthetic peptide can be delivered to the intestine.

Synthetic adhesion-blocking peptides: a new type of antimicrobial?

Synthetic peptides corresponding to adhesion epitopes can therefore be used to prevent infection at mucosal sites. These observations provide a basis for the rational design of adhesion-blocking peptides that may be applied to a variety of mucosal infections. The finding that administration of a single peptide confers protection is of interest. For *S. mutans*, at least one other adhesion epitope has been identified within SA I/II (Crowley *et al.*, 1993; Kelly *et al.*, 1999) and genome sequencing suggests there may be at least six further adhesin molecules (http://www.genome.ou.edu/smutans.html). Thus, in an environment such as the oral cavity where there may be competition for a limited niche, even partial inhibition of adhesion may be sufficient to prevent establishment of *S. mutans*.

In these 'proof of principle', *in vivo* studies, the effect of varying dose of inhibitor was examined either not at all or only to a limited extent. However, the affinity of a synthetic peptide for the specific host receptor is likely to be considerably lower than that of the intact adhesin and even lower than that of the microorganism which may be effectively polyvalent. There may be exceptions to this. Values for the dissociation constant (K_d) of the pilin adhesion peptide of *P. aeruginosa* were in the μM range and were similar to those of the pilin monomer (Lee *et al.*, 1994). The presence of a disulphide bond may stabilize conformation of this peptide and NMR spectroscopic analyses indicate the presence of a type I and type II β-turn in the solution structure (Campbell *et al.*, 1997). In contrast, NMR spectroscopy indicated that p1025 has no defined solution structure, an observation that may be consistent with much lower affinity of the p1025 interaction with salivary receptor than that of intact SA I/II (Kelly *et al.*, 1999). Delivery and maintenance of a sufficiently high dose of peptide to compete with the pathogen is therefore an important issue if this form of treatment is to be successful. In addition, binding of peptides to host receptors may induce some degree of pathology by the same signalling processes that occur on binding of the pathogen. It may, however, be possible to modify adhesion-blocking peptides so that they bind to receptor but do not induce signalling, as for altered peptide ligands that are used to downregulate T-cell responses (Sloan-Lancaster and Allen, 1996).

RECEPTOR ANALOGUES

The corollary to use of adhesin-analogues is the use of soluble forms of host receptor

molecules as inhibitors of microbial adhesion. Many studies have investigated soluble carbohydrate receptor analogues reflecting the frequency with which these structures are recognized by microbial adhesins. The development of technology for large-scale synthesis of oligosaccharides has contributed significantly to this approach (Zopf and Roth, 1996). Protein receptors have also been identified and treatment with soluble forms of intercellular adhesion molecule 1 (ICAM-1) has provided promising results against rhinovirus infection.

Clinical trial

Infection with rhinoviruses is the most frequent cause of the common cold. The cellular receptor for rhinovirus was identified as ICAM-1 (Greve *et al.*, 1989; Staunton *et al.*, 1989) and soluble ICAM-1 prevented infection of cultured cells with rhinovirus *in vitro* (Marlin *et al.*, 1990). In a chimpanzee model, soluble ICAM-1 was administered intranasally and prevented infection with rhinovirus (Huguenel *et al.*, 1997). Soluble ICAM-1 was then used in a four-centred clinical trial (Turner *et al.*, 1999) where it was applied intranasally to subjects who were given six doses/day (total 4.4 mg/day) at three hour intervals for a total of seven days. Experimental infection with rhinovirus was either 7 hours before the initial application of soluble ICAM-1 or 12 hours after initial application. In either case, there were significant reductions in disease severity and viral shedding compared with placebo controls.

Carbohydrate analogues in animal models of respiratory and gastrointestinal infection

Streptococcus pneumoniae is a major cause of pneumonia, otitis media, meningitis and sepsis. The bacterium can colonize the nasopharynx without causing disease but may subsequently spread and cause disease. Adhesion of *S. pneumoniae* to human cell lines and to primary respiratory epithelial cells *in vitro* was inhibited by sialylated oligosaccharides terminating in NeuAcα2-3(or -6)Galβ1 (Barthelson *et al.*, 1998). Concentrations of oligosaccharide required for 50% inhibition were approximately 2 mM. Much lower concentrations (approximately 500-fold less) were required with inhibitors in polyvalent form, prepared by covalent linkage of the oligosaccharides to human serum albumin (approximately 20 molecules per molecule of protein).

Sialylated forms of lacto-*N*-neotetraose (LNnT, Galβ1-4 GlcNacβ1-3 Galβ1-4 Glc) as well as non-sialylated LNnT were subsequently investigated as inhibitors of pneumococcal infection in a rabbit model of pneumonia (Idänpään-Heikkilä *et al.*, 1997). To demonstrate whether the oligosaccharides could prevent adhesion *in vivo*, *S. pneumoniae* was incubated with the sugars (100 μM) for 15 minutes before intratracheal administration. Co-administration of bacteria with either LNnT or the α2-6-sialylated derivative (LSTc) significantly reduced the bacterial load in the lungs (determined in bronchoalveolar lavage fluid) compared with saline-treated controls and by 96 hours after challenge, infection was cleared in rabbits receiving LNnT. Significant reductions in lung pathology and in the number of animals with bacteraemia were also evident. To determine therapeutic potential of the inhibitors, a single dose of oligosaccharide was administered intratracheally at 6 hours or 24 hours after pneumococcal challenge. Although less effective than co-administration with

bacteria, treatment with LNnT produced a significant reduction in bacterial load or bacteraemia.

A protective effect was also observed if the oligosaccharides were administered 24 hours before pneumococcal challenge. These results cannot be simply explained by inhibition of adhesion since persistence of the oligosaccharides for 96 or even 24 hours is unlikely. The possibility that the oligosaccharides may directly affect cells was investigated. Short-term (5–30 minutes) exposure of a human lung cell line to LNnT transiently reduced the binding capacity for *S. pneumoniae*. It was suggested that the protective effect may therefore be due to both adhesion-inhibition and a direct effect on epithelial cells, such that they are made transiently refractory to pneumococcal adhesion. Neoglycoproteins, i.e. oligosaccharides coupled to human serum albumin, were also investigated in this model but, despite their increased molar potency *in vitro*, were no more effective on a molar basis than the monomeric oligosaccharides.

Helicobacter pylori, a human specific gastric pathogen, is the causative agent in chronic active and peptic ulcer disease and a risk factor in the development of gastric adenocarcinoma, one of the most common forms of cancer in humans. In a transgenic mouse model of infection, development of pathology was associated with adhesion to the gastric epithelium (Guruge *et al.*, 1998). Several different adhesins may be involved in adhesion of *H. pylori* to gastric epithelial cells, including HpaA which is specific for sialoglyconjugates (Evans *et al.*, 1993). To investigate the potential of these oligosaccharides to prevent or treat infection, the oligosaccharide 3'-sialyllactose (NeuNAcα2-3Galβ1-4Glc), which inhibits *in vitro* adhesion of *H. pylori*, was given to rhesus monkeys that had been persistently colonized with the bacterium for at least 12 months (Mysore *et al.*, 1999). Oligosaccharide mixed with food was given 3 times daily for a period of 94 days. Infection was cleared permanently in 2 out of 6 monkeys and transiently in one further animal. This study therefore showed some effect but the limited degree of protection may reflect a need for other receptor analogues to be used in combination. In particular, the blood-group antigen-binding adhesin (BabA) that binds to Lewis^b antigen may play an important role in adhesion of *H. pylori* to gastric epithelium and expression of the gene in clinical isolates is significantly correlated with disease (Gerhard *et al.*, 1999).

Carbohydrate receptor analogues were also investigated in a model of diarrhoea resulting from infection with enterotoxigenic *E. coli* (ETEC) in calves (Mouricout *et al.*, 1990). A mixture of oligosaccharides prepared from the non-immunoglobulin fraction of bovine plasma inhibited adhesion *in vitro* and was administered orally in water as soon as symptoms were evident following challenge with a lethal dose of ETEC. Treatment was repeated 3–5 times over 2–3 days. Treated calves were protected (11 out of 13) whereas untreated calves were not (0 out of 7) and numbers of adherent ETEC were reduced by 2 orders of magnitude in treated calves.

MICROBICIDES

Conventional microbicides are broad-spectrum agents, such as the spermicide nonoxynol-9 that has a detergent-type mode of action by which it disrupts the membranes of microorganisms, and other surface-active agents, including benzalkonium chloride and bile salts (Herold *et al.*, 1999). Other broad-spectrum antibiotics include sulphated compounds such as dextran sulphate and sodium lauryl sulphate

which block microbial adhesion (Herold *et al.*, 1997; Piret *et al.*, 2000). A major drawback of these broad-spectrum agents is interference with the normal microbial flora which, in the long run, may favour the onset of infections at mucosal sites (Rosenstein *et al.*, 1998; Stafford *et al.*, 1998).

Development of more targeted or pathogen-specific microbicides could overcome some of these difficulties. Two approaches that have been adopted to produce microbicides that may be more specifically targeted are the use of anti-idiotypic antibodies that mimic microbial toxins and screening of natural compounds to identify novel microbicides.

A Mab that neutralizes the activity of a killer toxin (KT) from the yeast *Pichia anomala* has been used in vaccination studies to induce anti-idiotypic antibody that mimics the activity of the original KT (Polonelli *et al.*, 1993). Such KT-like antibodies were considered to have potential as agents that could be applied topically. Using a phage display library from splenic lymphocytes of mice immunized with the KT-neutralizing Mab, an anti-idiotype scFv, H6, was isolated that possessed candidacidal activity (Magliani *et al.*, 1997). In the rat model of candidal vaginitis, described above, intravaginal administration of the H6 scFv significantly decreased the time required for resolution of infection. Fourteen days after challenge with *C. albicans*, none of the mice receiving H6 (Mab or scFv) were still infected, whereas all mice in the control groups remained infected. H6 scFv was also effective *in vitro* against a multidrug-resistant isolate of *Mycobacterium tuberculosis* (Conti *et al.*, 1998).

Cyanovirin-N, CV-N, a low M_r protein, was identified from screening extracts of the blue-green alga *Nostoc ellipsosporum* for compounds that inactivated diverse strains of HIV-1, HIV-2 and SIV (Boyd *et al.*, 1997). At nanomolar concentrations, CV-N irreversibly inactivates primary isolates as well as laboratory-adapted strains and also inhibits cell–cell fusion and transmission between infected and non-infected cells. CV-N binds with high affinity to HIV gp120, although this does not prevent binding of CD4 nor attachment of intact HIV virus to target T-cell lines (Mariner *et al.*, 1998). The virucidal activity may be due to inhibition of post-attachment fusion events. Treatment of CV-N with denaturants or high temperature (boiling for 15 minutes) did not result in significant loss of anti-HIV activity. Structural analyses provide a rationale for this remarkable stability which augurs well for use of CV-N *in vivo*. The primary sequence of CV-N, 101 amino acid residues, comprises two internal repeats of 50 and 51 residues that are well conserved (Gustafson *et al.*, 1997) and the solution structure determined by NMR is that of a largely β-sheet protein comprising two symmetrically related domains formed by strand exchange between the sequence repeats (Bewley *et al.*, 1998). Each domain is further stabilized by a disulphide bond.

That these novel microbicides are proteins has also allowed development of a novel means of delivery to the mucosa as discussed below.

Local delivery systems

Delivery of the adhesion-blocking antimicrobials to mucosal surfaces and maintenance of sufficiently high concentrations of antimicrobial at mucosal surfaces has generally been achieved by repeated doses with relatively large quantities of inhibitor. Development of delivery systems that allow prolonged local release of the

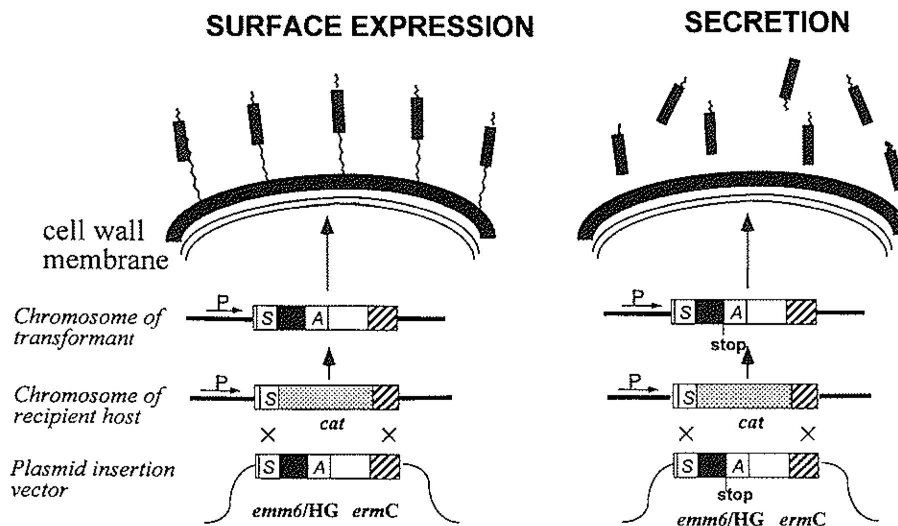

Figure 13.3. Schematic representation of the strategy used for surface expression or secretion of heterologous antigens in *Streptococcus gordonii*. The recombinant insertion vector contains a resistance marker (*ermC*), and an *emm6*-based gene fusion (*emm6*-HG). The regions of *emm6* encoding for the signal sequence (S) and for the C-terminal anchor (A) of the M6 protein are indicated. In the chromosome of the recipient, two regions of homology with the insertion vector are flanking a different resistance marker (*cat*). The DNA of the insertion vector is linearized during the transformation process, and recombination occurs at both the homologous segments. In the chromosome of the transformants, the DNA fragment containing both *emm6*-HG and *erm*C is integrated into the chromosome, *cat* is deleted, and *emm6*-HG is downstream a strong resident promoter (P). When the region encoding the anchor is part of the fusion, the recombinant protein is expressed on the bacterial surface (left panel) while, when a stop codon (stop) is inserted after the heterologous gene, the anchor is not part of the fusion and the recombinant protein is secreted in the supernatant (right panel).

antimicrobial molecule in a bioactive form at mucosal surfaces could reduce the number of repeat doses and might allow more effective targeting to less accessible mucosal sites within the gastrointestinal tract. One strategy that has been exploited successfully is the use of non-pathogenic commensal bacteria capable of colonizing mucosal surfaces as live vectors for the delivery of microbicidal molecules (Beninati *et al.*, 2000; Oggioni *et al.*, 2000; Pozzi *et al.*, 2000).

THE *STREPTOCOCCUS GORDONII* HOST-VECTOR SYSTEM

S. gordonii is a human commensal which was developed as a model system for expression of heterologous proteins and mucosal delivery of heterologous antigens for vaccine purposes (Medaglini *et al.*, 1997a; Pozzi *et al.*, 1997). *S. gordonii* is naturally competent for transformation and therefore easily amenable to genetic manipulation (Pozzi *et al.*, 1990). In this system, the heterologous DNA encoding the recombinant protein is integrated into the bacterial chromosome downstream from strong resident promoters, yielding stable genetic constructs and avoiding the problems connected with the cloning in *E. coli* of promoters from Gram-positive bacteria. To deliver heterologous antigens to the bacterial surface, the M6 protein, a fibrillar surface protein of *Streptococcus pyogenes,* was chosen as the vector

molecule due to its conserved signal and anchoring sequences (Fischetti, 1989). The signal sequence allows export of the M6-based fusion proteins, whereas the presence of the anchor determines the cell-surface display of the recombinant proteins. When the anchor is not part of the fusion, the recombinant proteins are not attached to the cell surface but instead are secreted in the supernatant (Medaglini *et al.,* 1993). The system devised for heterologous gene expression in *S. gordonii* uses a two step procedure (Oggioni and Pozzi, 1996; Oggioni *et al.*, 1999a) (*Figure 13.3*): (i) construction of translational fusions of the heterologous gene to the M6 protein in *E. coli* plasmid vectors, and (ii) transformation of the recombinant vectors into specially engineered recipient strains of *S. gordonii* where regions of homology allow for integration into the chromosome downstream of a resident promoter. Using this system, a wide variety of antigens, ranging in size from 15 to over 400 amino acids, were expressed in *S. gordonii* and were efficiently recognized by monoclonal and polyclonal antibodies as well as human T-cell clones (Pozzi *et al.*, 1997). Furthermore, the recombinant antigens expressed on the bacterial surfaces were also immunogenic in different animal models following both local or systemic immunizations (Oggioni *et al.*, 1995, 1999b; Medaglini *et al.*, 1997b, 2000; Di Fabio *et al.*, 1998; Ricci *et al.*, 2000).

Recently, the system was extended to allow the expression of two different fusion molecules (fusion-proteins) on the surface of *S. gordonii*. The integration of the two different gene-fusions downstream of two different chromosomal promoters ensures stable expression of the two fusion proteins. Using this strategy, it could be possible to express on the same bacterium two different microbicidal molecules with potentially a stronger chance of success in fighting the infectious agent.

MUCOSAL COLONIZATION AND *IN VIVO* EXPRESSION OF HETEROLOGOUS PROTEINS BY RECOMBINANT BACTERIA

Although *S. gordonii* is a commensal bacterium commonly present in the human oral cavity, it was also shown to stably colonize the oral mucosa of mice (Medaglini *et al.*, 1995; Oggioni *et al.*, 1995), the vaginal mucosa of both mice (Medaglini *et al.*, 1997b, 1998) and rats (Beninati *et al.*, 2000) and to persist in the vagina of cynomolgous monkeys (Di Fabio *et al.*, 1998). *S. gordonii,* after a single inoculum, consistently colonize the vaginal or oral mucosa for 8 and 10 weeks respectively, with recombinant and wild-type strains being equally effective in colonization (Medaglini *et al.*, 1995, 1997b, 1998; Oggioni *et al.*, 1995). Importantly, the recombinant strains stably express the heterologous antigen *in vivo* during all the period of colonization (Medaglini *et al.*, 1997b, 1998), a crucial issue when constructing genetically engineered bacteria to be used as delivery system. Colonization, therefore, allows prolonged exposure to the bacterial-produced molecule after a single administration and is essential for stimulation of the host immune system since killed recombinant bacteria were not effective in inducing an immune response (Medaglini *et al.*, 1995, 1997b). Use of colonizing commensal bacteria for the delivery of microbicidal molecules may, therefore, offer the strong advantage of prolonged exposure to the microbicide with a single administration.

EXPRESSION AND DELIVERY OF MICROBICIDES BY RECOMBINANT *S. GORDONII*

In order to exploit the possibility of a prolonged local delivery of microbicidal antibodies by colonizing commensal bacteria, *S. gordonii* was engineered to secrete or display on the surface the H6 anti-idiotypic single-chain antibody mimicking a yeast killer toxin, described above (Beninati *et al.*, 2000). Intact bacterial cells expressing the cell surface H6 or the culture supernatant of the H6-secreting bacteria were shown to exert a strong dose-dependent candidacidal activity *in vitro*. *In vivo* candidacidal activity of the two H6-producing strains was assayed in the model of oestrogen-dependent rat vaginal candidiasis (De Bernardis *et al.*, 1999). Colonization of the rat vagina with recombinant *S. gordonii* expressing H6 cleared *Candida* infection in 75% of animals treated with the H6-secreting strain and 37% of animals treated with the strain expressing H6 on the surface while 100% of animals treated with control strain were still infected at day 21 (Beninati *et al.*, 2000). The observed candidacidal effect was comparable with that of the antimycotic drug fluconazole. *In vivo* production by human commensal bacteria of bioactive antimicrobial molecules may therefore circumvent the problem of short half-life of polypeptide-based microbicides.

To further investigate the feasibility of using recombinant commensal bacteria for vaginal delivery of microbicides, the potent HIV-inactivating protein CV-N was expressed in *S. gordonii* (Pozzi *et al.*, 2000). Recombinant *S. gordonii* strains expressing CV-N on the surface or secreting it in the supernatant were constructed. The *in vitro* HIV-inactivating capability and the *in vivo* microbicidal activity in animal models of CV-N-producing commensals are currently under investigation.

Perspective

Human clinical trials have demonstrated that administration at mucosal surfaces of adhesin analogues, receptor analogues or antibodies, directed against microbial adhesins, can prevent or reduce severity of infectious disease. Furthermore, several studies using animal models of infection indicate that these approaches may be applied to a variety of infections at mucosal surfaces. As such, these approaches may provide a useful alternative to more established therapies or preventive measures. Targeting of treatment such that pathogens are selectively removed or prevented from colonizing a mucosal surface has the advantage over more broad-spectrum antimicrobial approaches that the remaining normal non-pathogenic microorganisms may contribute to long-term protection by competitive exclusion. However, the success of passive immunization against uropathogenic *E. coli* suggests that treatment may also be applied to mucosal surfaces that are not normally colonized, although it does not seem likely that the protective effect would be extended significantly beyond the time of administration of the antimicrobial in such cases. We have also argued above that strategies aimed at this initial stage in the process of infection may be less likely to select for resistant strains of microorganism.

A common feature of many of the studies discussed in this review is that targeting of a single microbial adhesin is effective in preventing or reducing infection. This is somewhat unexpected since many bacteria possess several adhesins and suggests that becoming established in a mucosal niche can be a precarious process. Turnover of

mucous and epithelial cells, competition with indigenous flora, peristalsis and/or fluid flow may present major obstacles to pathogens at mucosal sites. Addition of a further obstacle by blocking an important adhesin–receptor interaction may be sufficient to prevent the microorganism from becoming established. Experimentally, these treatments are effective when given prophylactically or shortly after challenge infection. It remains to be seen whether they can be effective therapeutically, although the use of recombinant *S. gordonii* expressing candidacidal antibody suggests that therapeutic treatment may be possible.

A number of potential problems with treatments that target adhesins or host receptors have been identified. Concerns that mucosal administration of receptor analogues will induce auto-reactive antibody have not been substantiated to date. In several of the studies described above, serum and, in some cases, saliva have been analysed for antibodies against the applied antimicrobial. No such antibodies were detected. Of particular interest, intranasal application of soluble ICAM-1 (often regarded as an effective route of mucosal immunization) induced no detectable serum antibodies in any of 90 subjects (Turner *et al.*, 1999). The possibility that adhesin analogues may bind to host receptors and by signalling induce pathology similar to that induced by the pathogen has been discussed above. Conversely, receptor analogues may bind to specific microbial adhesins and induce expression of alternative adhesins since most microorganisms possess multiple adhesins. Although this does not appear to be a major problem in the studies reviewed here, the effectiveness of the treatment may be increased by use of more than one receptor analogue to guard against the possibility. Use of monovalent inhibitors may reduce the likelihood of signalling and, in this context, the finding that monomeric carbohydrate receptor analogues were as effective as polymeric forms in preventing experimental infection with *S. pneumoniae* (Idänpään-Heikkilä *et al.*, 1997) is significant.

For administration of antimicrobials at mucosal surfaces, repeated doses of relatively large amounts are typically used so as to maintain effective levels of the agent. This in turn creates a requirement for large-scale production. Use of transgenic plant technology offers one potential solution to this problem. Moreover, this is currently the most effective means of producing SIgA forms of antibody, so providing a further means of increasing persistence of anti-adhesin antibodies at mucosal surfaces. The use of recombinant *S. gordonii* to deliver ScFv *in vivo* (Beninati *et al.*, 2000) is an important development. Engineering of commensal bacteria to secrete antimicrobials offers a potential solution to the problems of producing large amounts of material as well as delivering and maintaining effective concentrations of antimicrobial at mucosal surfaces.

Mucosally administered adhesion-blocking molecules appear to be generally well-tolerated and effective. Given the rapid progress in this field and the expected enormous increase of potential targets that have been or will be identified as the result of genomic sequencing, several such compounds are likely to emerge as a new class of antimicrobial.

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