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INTERFERENCE WITH VIRUS AND BACTERIA REPLICATION BY THE TISSUE SPECIFIC EXPRESSION OF ANTIBODIES AND INTERFERING MOLECULES

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SUMMARY

Historically, protection against virus infections has relied on the use of vaccines, but the induction of an immune response requires several days and in certain situations, like in newborn animals that may be infected at birth and die in a few days, there is not sufficient time to elicit a protective immune response. Immediate protection in new born could be provided either by vectors that express virus-interfering molecules in a tissue specific form, or by the production of animals expressing resistance to virus replication. The mucosal surface is the largest body surface susceptible to virus infection that can serve for virus entry. Then, it is of high interest to develop strategies to prevent infections of these areas. Virus growth can be interfered intracellularly, extracellularly or both. The antibodies neutralize virus intra- and extracellularly and their molecular biology is well known. In addition, antibodies efficiently neutralize viruses in the mucosal areas. The autonomy of antibody molecules in virus neutralization makes them functional in cells different from those that produce the antibodies and in the extracellular medium. These properties have identified antibodies as very useful molecules to be expressed by vectors or in transgenic animals to provide resistance to virus infection. A similar role could be played by antimicrobial peptides in the case of bacteria. Intracellular interference with virus growth (intracellular immunity) can be mediated by molecules of very different nature: (i) full length or single chain

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antibodies; (ii) mutant viral proteins that strongly interfere with the replication of the wild type virus (dominant-negative mutants); (iii) antisense RNA and ribozyme sequences; and (iv) the product of antiviral genes such as the Mx proteins. All these molecules inhibiting virus replication may be used to obtain transgenic animals with resistance to viral infection built in their genomes.

We have developed two strategies to target into mucosal areas either antibodies to provide immediate protection, or antigens to elicit immune responses in the enteric or respiratory surfaces in order to prevent virus infection. One strategy is based on the development of expression vectors using coronavirus derived defective RNA minigenomes, and the other relies on the development of transgenic animals providing virus neutralizing antibodies in the milk during lactation. Two types of expression vectors are being engineered based on transmissible gastroenteritis coronavirus (TGEV) defective minigenomes. The first one is a helper virus dependent expression system and the second is based on self-replicating RNAs including the information required to encode the TGEV replicase. The minigenomes expressing the heterologous gene have been improved by using a two-step amplification system based on cytomegalovirus (CMV) and viral promoters. Expression levels around 5 μg per 10^6 cells were obtained. The engineered minigenomes will be useful to understand the mechanism of coronavirus replication and for the tissue specific expression of antigen, antibody or virus interfering molecules.

To protect from viral infections of the enteric tract, transgenic animals secreting virus neutralizing recombinant antibodies in the milk during lactation have been developed. Neutralizing antibodies with isotypes IgG1 or IgA were produced in the milk with titers of 10^6 in RIA that reduced virus infectivity by one million-fold. The recombinant antibodies recognized a conserved epitope apparently essential for virus replication. Antibody expression levels were transgene copy number independent and were related to the transgene integration site. This strategy may be of general use since it could be applied to protect newborn animals against infections of the enteric tract by viruses or bacteria for which a protective MAb has been identified. Alternatively, the same strategy could be used to target the expression of antibiotic peptides to the enteric tract in order to protect against bacterial or virus infections.

1. INTRODUCTION

Historically, protection against virus infections has relied on the use of vaccines, but the induction of an immune response requires several days and in certain situations, like in newborn animals that may be infected at birth and die in a few days, there is not sufficient time to elicit a protective immune response. Immediate protection in new born could be provided either by vectors that express virus-interfering molecules in a tissue specific form, or by the production of animals resistant to virus replication.

The possibility of expressing foreign genes in mammals by gene transfer has opened new venues in animal breeding (Brem et al., 1985; Brem and Muller, 1994; Hammer et al., 1995). An important aspect is the improvement of animal health by the obtention of transgenic animals (Castilla et al., 1998; Muller and Brem, 1996; Sola et al., 1998). Reduction of disease susceptibility of livestock will be a benefit both in terms of animal welfare and from an economical point of view, since the cost of disease has been estimated to account for 10–20% of total production cost (Muller et al., 1997).

Resistance to virus infections can be extracellular or intracellular. Intracellular interference, also named intracellular immunity, can be mediated by molecules of very different nature: (i) full length or single chain antibodies; (ii) mutant viral proteins that strongly interfere with the replication of wild type viruses (dominant-negative mutants); (iii) antisense RNA and ribozyme sequences; and (iv) products of antiviral genes such as the Mx proteins. All these molecules that inhibit virus replication may be used to obtain transgenic animals with resistance to viral infection built in their genomes. Nevertheless, in this chapter we have focused our attention on the expression of molecules such as the antibodies, microbial peptides, and interferon, that are functional either in the extracellular matrix or in cells different to the ones producing them, providing the possibility of protecting the whole tissue.

Resistance to bacteria, in addition to that mediated by well known drugs and by standard immune response, can be mediated by peptide antibiotics such as defensins, magainins, cecropins, and melittins (Boman, 1991; Wachinger et al., 1998). These small peptides were initially described in mammals, insects, and amphibians, but now it has been realized that they are more widely distributed in mammals. In principle, these antibiotics could be used in the generation of transgenic animals to increase their resistance to bacterial infection and, interestingly, to virus infection as well (Wachinger et al., 1998).

The mucosal surface is the largest body area susceptible to virus infection that can serve for virus entry. Then, it is of high interest to develop strategies to prevent infections of these areas. We have developed two strategies to target into mucosal areas molecules interfering with virus growth such as antibodies to provide immediate protection against virus infections. One strategy is based on the development of expression vectors using coronavirus derived defective RNA minigenomes, and the other one relies on the development of transgenic animals providing virus neutralizing antibodies in the milk during lactation.

1.1. Development of Expression Vectors to Target Pathogen Interfering Molecules into the Mucosal Surfaces

Two types of expression vectors are being engineered based on transmissible gastroenteritis coronavirus (TGEV) defective minigenomes (Izeta et al., 1999; Mendez et al., 1996). The first one is a helper virus dependent expression system and the second is based on self-replicating RNAs including the information required to encode the TGEV replicase. TGEV-derived vectors may have several advantages to induce mucosal immunity since: (i) TGEV infects enteric and respiratory mucosal areas (Enjuanes and Van der Zeijst, 1995) which are convenient tissues to induce secretory immunity; (ii) its tropism can be controlled by modifying the spike (S) gene making in principle possible to engineer tissue or species-specificity (Ballesteros et al., 1997); (iii) non-pathogenic strains are available to develop a safe helper virus-dependent expression system (Sánchez et al., 1992); and (iv) coronaviruses are RNA cytoplasmic viruses that replicate without a DNA intermediary making their integration into cellular chromosomes unlikely (Lai and Cavanagh, 1997). Vector systems for the expression of heterologous genes have also been developed from full-length cDNA clones of positive-strand RNA viruses such as alphaviruses including Sindbis virus, Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE) (Frolov and Schlesinger, 1994; Liljeström, 1994; Pushko et al., 1997). These systems have been very useful to elicit humoral and cellular immune responses.

TGEV is a member of the *Coronaviridae* family with a plus-stranded, polyadenylated RNA genome of 28.5 kb (Eleouet et al., 1995). TGEV infects both enteric and respiratory tissues and causes a mortality close to one hundred percent when newborn animals are infected (Saif and Wesley, 1992). To engineer cDNAs encoding TGEV defective RNAs, three deletion mutants of 22, 10.6, and 9.7 kb (DI-A, DI-B, and DI-C, respectively) maintaining the *cis*-signals required for replication and packaging by helper viruses were isolated (Mendez et al., 1996). DI-C RNA was the most abundant and was selected to generate a cDNA encoding synthetic minigenomes that have been used for the tissue specific expression of antigens or molecules interfering with virus replication. These minigenomes expressing heterologous genes have been improved by using a two-step amplification system based on cytomegalovirus (CMV) and viral promoters (Izeta et al., 1998; Penzes et al., 1998). Expression levels around 2 μg per 10^6 cells were obtained and immune responses were elicited by immunizing swine with minigenomes encoding heterologous genes. These expression systems may also serve to express virus neutralizing antibodies in mucosal areas.

1.2. Interference with Virus Growth by Recombinant Antibodies

The antibodies neutralize virus intra- and extracellularly and their molecular biology is well known. In addition, antibodies efficiently neutralize viruses in the mucosal areas. The autonomy of antibody molecules in virus neutralization makes them functional in cells different from those producing the antibodies and in the extracellular medium. These properties have revealed the antibodies as very useful molecules to be expressed by vectors or in transgenic animals to provide resistance to virus infection.

Advances in antibody engineering have allowed the genes encoding antibodies to be manipulated so that the antigen binding domain can be intracellularly expressed (Jones and Marasco, 1997; Muller and Brem, 1994). The use of intracellular antibodies, or intrabodies, in transgenic animal research and eventual development of pathogen resistant transgenic farm animals is one of the applications. This work has concentrated mostly on the use of single chain antibody (scFv) in which the variable regions of the heavy and light chains are bound together via a linker. By adding known intracellular trafficking signals, the scFvs can be localized to specific cellular compartments such as the nucleus, the endoplasmic reticulum, the inner surface of the plasma membrane, the cytoplasm, and the mitochondria (Jones and Marasco, 1997).

Genes encoding the heavy and light chains of a hapten-specific IgM antibody were modified by site-directed mutagenesis to destroy the hydrophobic leader sequences and allow expression in the cytoplasm of non-lymphoid cells. Mutations were made in which the leader sequence of the light chain was replaced by the nuclear localization signal of the SV-40 larger T antigen. Transfectants in which the heavy chain lacking the hydrophobic leader was expressed together with a light chain carrying the nuclear localization signal were selected and a nuclear distribution of the assembled antibody was found (Biocca et al., 1990). Thus, it should be possible to target a specific antibody to the cell nucleus with the aim of interfering with viruses replicating in this compartment.

Proteins that function in the cytoplasm have also been inhibited by intrabodies. It has been found, however, that the half life of the intrabodies in the cytoplasm is shorter than in the endoplasmic reticulum, and perhaps additional modifications of the intrabody structure would allow a longer half life and therefore greater efficacy (Biocca and Cattaneo, 1995).

In the whole animal, the expression of an antibody can be temporally or spatially restricted by using a tissue-specific promoter only active at a certain time in development. Experiments to test the efficacy of antibody inhibition of HIV-1 replication have been done using scFv antibodies directed against a highly conserved domain of the HIV-1 envelope protein gp120, against the activation domain of the transcription activator protein *tat*, and against the *rev* protein, which is essential for transport of full length viral RNA from the nucleus (Marasco et al., 1993; Richardson and Marasco, 1995). Cultured cells expressing any of these antibodies have shown a marked reduction in viral titer in relationship to untransformed cells that were infected with HIV-1. The antibodies against the nuclear proteins *tat* and *rev* were effective when localized to the cytoplasm, possibly because they inhibited nuclear transport of the proteins (Mhashilkar et al., 1995).

The mucosal immune system and its predominant effector, secretory immunoglobulin A (IgA), provide the initial immunological barriers against most pathogens that invade the body at mucosal surfaces (Mestecky and McGhee, 1987). This is especially true for viruses, since resistance to infection has been strongly correlated with the presence of specific IgA antibody in mucosal secretions (Renegar and Small, 1991). At mucosal surfaces IgA antibodies are particularly stable and, since they are multivalent, might be more protective than IgG (Kilian et al., 1988). Virus neutralization by immunoglobulins is thought to result from the binding of antibody to virion attachment proteins, preventing adherence to epithelial cells. In addition, mucosal antibody interacts intracellularly with viruses preventing their replication possibly by interfering with virus assembly (Mazanec et al., 1992). IgA expressed intracellularly, or as it is transported through the epithelial cell by the polymeric immunoglobulin receptor (pIgR), may be also able to interact with intracellular pathogens such as viruses, preventing replication (Mazanec et al., 1995; Mazanec et al., 1993). It has been shown that IgA monoclonal antibodies against Sendai virus, a parainfluenza virus, colocalize with the viral hemagglutinin-neuraminidase protein within infected epithelial cells and reduce intracellular viral titers. In addition, it has been shown (Mazanec et al., 1995) that IgA interacts with influenza virus hemagglutinin (HA) protein within epithelial cells reducing viral titers in the supernatants and in cell lysates, concluding that this interference with virus replication also takes place intracellularly.

The introduction of antibody genes into cells to protect them against virus infection has been recently explored in model tissue culture systems to determine whether this strategy may potentially be applied to *in vivo* protection of mucosal surfaces by gene therapy with antibody-encoding genes, since monoclonal antibodies (MAbs) are now available for a vast range of viruses. Virus neutralizing MAbs may protect mucosal tissues against viral infections, however it is not known whether the transformation of a small percentage of the cells from a given tissue using a vector, leading to the formation of antibody secreting cells, will provide protection to the neighboring tissues.

To clarify this point, we have studied the protection of epithelial cell monolayers against TGEV infection using expression plasmids encoding virus neutralizing MAbs (Castilla et al., 1997). Immunoglobulin gene fragments encoding the variable modules of the heavy (VH) and light (VL) chains of TGEV neutralizing MAb 6A.C3 have been cloned and sequenced. The selected MAb recognizes an epitope that is located in the globular portion of the spike (S) protein (Gebauer et al., 1991). Studies by our laboratory on the mechanisms of TGEV neutralization (Suñé et al., 1990) and on its antigenic and genetic variability (Sánchez et al., 1992; Sánchez et al., 1990) have led to the

identification of a mouse MAb which neutralized all the tested TGEV isolates collected in three continents throughout 40 years, and also TGEV-related coronaviruses which infect at least three animal species: porcine, canine, and feline. This MAb, 6A.C3, probably binds to an epitope essential for virus replication since no neutralization escape mutants appeared in tissue culture when this MAb was employed (Gebauer et al., 1991). A Mab with similar characteristics has been independently isolated by another group (Delmas et al., 1990).

The sequences of MAb 6A.C3 kappa and gamma 1 modules were identified as subgroup V and subgroup IIIC, respectively (Castilla et al., 1997). These chimeric immunoglobulin genes with the variable modules from the murine MAb and constant modules of human gamma 1 and kappa chains were constructed using RT-PCR. Chimeric immunoglobulins were stably or transiently expressed in murine myelomas and COS cells, respectively. The secreted recombinant antibodies had radioimmunoassay (RIA) titers higher than 10^3 and reduced the infectious virus more than 10^4-fold. Recombinant dimeric IgA showed a 50-fold enhanced neutralization of TGEV relative to a recombinant monomeric IgG_1 which contained the identical antigen binding site. Stably-transformed epithelial cloned cell lines which expressed either recombinant IgG or IgA TGEV neutralizing antibodies reduced virus production by $>10^5$-fold after infection with homologous virus, although a residual level of virus production ($<10^2$ PFU/ml) remained in less than 0.1 % of the cells. This low level persistent infection was shown not to be due to the selection of neutralization escape mutants. Although some residual virus synthesis persisted in less than 0.1% of the cells in culture, it is possible that *in vivo* defense mechanisms apart from virus neutralization, such as cytolytic T cells, could completely control virus infection. This new strategy may be particularly useful in infections where quick immune intervention is required in a defined tissue, since viral vectors could express antibody genes within two to three hours after inoculation and this expression may be targeted to specific tissues.

Three mechanisms may be responsible for the reduction in virus production: i) extracellular neutralization; ii) intracellular neutralization, and iii) modulation of virus production by antibody (Fujinami and Oldstone, 1984). Extracellular neutralization is likely to be responsible for the results of this study, because the antibody is continuously released into the medium, even during virus infection, and in the immunoglobulin gene-transformed uncloned cells about 85 to 90% of the cells did not produce the antibody yet they were still protected from TGEV infection.

Modulation of virus production by antibody binding to viral antigens on the surface of virus-infected cells is a phenomenon that operates in several virus systems (Fujinami and Oldstone, 1984). This mechanism may modulate TGEV synthesis in antibody producing ST cells. This modulation of virus synthesis probably leads to the establishment of a persistent infection, since residual virus production was often observed for at least three weeks post infection (results not shown). Antibody-induced modulation is a mechanism by which other viruses persist and escape immunologic surveillance (Fujinami and Oldstone, 1984). Experimental evidence has suggested such mechanism for measles virus, herpes simplex virus, and retrovirus.

1.3. Transgenic Animals Expressing Infectious Pathogen Specific rMAbs on B Cells

Transgenic animals to improve health and disease resistance based on specific immunity have been described (Kooyman and Pinkert, 1994; Lo et al., 1991; Storb, 1987;

Weidle et al., 1991). Pioneering studies showed the construction of transgenic mice producing functional anti-phosphorylcholine antibodies (Storb et al., 1986). Later, transgenic mice expressing chimeric anti-*Escherichia coli* immunoglobulin α heavy chain gene were developed in order to induce constitutive immunity against a pathogenic strain of *E. coli* (K99) (Kooyman and Pinkert, 1994). Because the route of *E. coli* infection was enteric, an IgA transgene was desirable. A chimeric gene construct was cloned that coded for a HC that recognized a specific *E. coli* pilus antigen. The construct comprised a κ gene promoter, murine VDJ, and bovine α-HC constant region. Expression of the immunoglobulin gene mRNA was detected before and, in some cases, only after challenge. As no differences were found when sera were analyzed for bovine IgA in control and transgenic mice, protein expression was assessed by challenge of HC founders with K99 *E. coli*. Protection was observed in the transgenic mice, however, the transgene was not transferred to the progeny.

Transgenic mice, sheep and pigs harboring genes encoding the murine α-HC and κ-LC from two different monoclonal antibodies specific for a polysaccharide such as phosphorylcholine (PC), which is potentially protective against pathogenic bacteria were generated (Lo et al., 1991). Two transgenic founders with the integration of one or both intact transgenes were produced in mice, sheep and pig. High serum levels (ranging from 0.3 to 1.3 mg/ml) of transgene rIgA were detected in transgenic mice and pigs. In the transgenic sheep no serum expression of anti-PC rMAb was found. Despite the absence of any functional mouse LC in the pigs, both animals secreted mouse transgene antibodies into their serum. The secreted rMAbs presumably included endogenous pig L chains. Allelic exclusion, i.e. the suppression of endogenous immunoglobulin rearrangement and expression, was observed in only one transgenic mouse, the endogenous immunoglobulin production of the other transgenic animals was unaffected. Unfortunately, little if any of the transgene immunoglobulin in the sera examined showed binding specificity for the antigen. Thus, a functionality in terms of the transgene rMAb could not be demonstrated.

In a second generation of transgenic animals expressing rMAbs (Weidle et al., 1991), the genes for the H (γ) and L (κ) chains of a MAb specific for 4-hydroxy-3-nitro-phenylacetate (NP) were introduced into the germ line of mice, rabbits, and pigs. During the second lactation rMAb titers higher than 0.1 mg/ml of milk were found early in lactation, and the levels declined thereafter. The transgenic rMAb bound the antigen in an ELISA test. However, in isoelectric focusing, only a small fraction of the antibody matched the mouse MAb, probably because the association of the transgenic and endogenous antibody chains (Muller et al., 1997).

1.4. Transgenic Animals Secreting Virus Neutralizing Antibodies in the Mammary Gland

To protect the enteric tract from viral infections, transgenic animals secreting virus neutralizing recombinant antibodies in the milk during lactation have been developed. Neutralizing antibodies with IgG1 or IgA isotypes were produced in the milk of transgenic mice. To this end, we used TGEV as the experimental system.

The immune response to TGEV has been characterized (Antón et al., 1995; Brim et al., 1994; VanCott et al., 1994) and full protection against this virus was provided by lactogenic immunity from immune sows (Saif and Wesley, 1992). It has also been shown that the passive oral administration of serum elicited by recombinant

adenoviruses expressing the spike protein, completely protected piglets against virulent virus challenge (Torres et al., 1995).

Conventional approaches such as lactogenic immunity and artificial feeding may target the antibody to epithelial surfaces providing protection against enteric virus infections (Saif and Wesley, 1992). Alternatively, transgenic animals secreting virus neutralizing antibodies into their milk during lactation should provide immediate protection to piglets against enteric coronavirus infection (Castilla et al., 1998; Sola et al., 1998).

The mammary gland expression system is by nature very suitable for the production of proteins that function in the gastrointestinal tract and which can be orally administered (Lee et al., 1994; Lee and De Boer, 1994). Milk is now known to contain an array of bioactivities which extends the range of influence of mother over young beyond nutrition alone. Bioactivities in milk include microbial growth control, immunoregulation, and non-immune disease defense, such as lactoperoxidase, lysozyme, and lactoferrin (Schanbacher et al., 1997).

We have reported the production of the recombinant TGEV neutralizing MAb 6A.C3 of human IgG1 or porcine IgA isotypes and their secretion in the mammary gland of transgenic mice (Castilla et al., 1998; Sola et al., 1998).

To express the rIgG1 in transgenic mice, 18 founders secreting a recombinant monoclonal antibody (rMAb) neutralizing TGEV into the milk were generated (Castilla et al., 1998). The genes encoding a chimeric MAb with the variable modules of the murine TGEV specific MAb 6A.C3 and the constant modules of a human IgG_1 isotype MAb were expressed under the control of regulatory sequences derived from the whey acidic protein (WAP) which is an abundant milk protein. Antibody expression titers of 10^6 by RIA were obtained in the milk of transgenic mice which reduced TGEV infectivity with a neutralization index of 6 (10^6-fold). The antibody was synthesized at high levels throughout lactation. Integration of matrix attachment regions (MAR) sequences with the antibody genes led to a 20- to 10,000-fold increase in the antibody titer in 50% of the transgenic animals. Antibody expression levels were transgene copy number independent and were related to the site of integration. These data suggested that the MAR sequences were acting as transcription enhancer as previously suggested (Poljak et al., 1994), more than by isolating the expression cassette from negative regulatory signals from flanking genes (Bonifer et al., 1990; Jenuwein et al., 1997). The generation of transgenic animals producing virus neutralizing antibodies in milk could be a general approach to provide protection against neonatal infections of the enteric tract.

To express the rIgA, ten lines of transgenic mice secreting TGEV neutralizing rMAbs into the milk were generated (Castilla et al., 1998; Sola et al., 1998). The rMAb light and heavy chain genes were assembled by fusing the genes encoding the variable modules of the murine MAb 6A.C3, and a constant module from a porcine myeloma of IgA isotype. Seventeen out of 23 transgenic mice integrated both light and heavy chains, and at least 10 of them transmitted both genes to the progeny leading to one hundred per cent of animals secreting functional TGEV neutralizing antibody during lactation. Selected mice produced milk with TGEV specific antibody titers higher than one million as determined by RIA, neutralized virus infectivity by one million-fold, and produced up to 6 mg of antibody per ml. Antibody expression levels were transgene copy number independent and integration site dependent. Co-microinjection of genomic β-lactoglobulin gene with rMAb light and heavy chain genes led to the generation of transgenic mice carrying the three transgenes. Highest antibody titers were

produced by transgenic mice that had integrated the antibody and β-lactoglobulin genes, although the number of transgenic animals generated does not allow a definitive conclusion on the enhancing effect of β-lactoglobulin co-integration. This approach may also lead to the generation of transgenic animals providing lactogenic immunity to their progeny against enteric pathogens.

The production of an active anti-CD6 rMAb in the mammary gland of transgenic mice has also been reported (Limonta et al., 1995). However, the antibody expressed by these transgenic animals did not have protective activity against infectious agents, and the antibody levels achieved were considerably lower than the TGEV neutralizing rMAbs reported above.

In principle, the expression of antibodies in transgenic animals could be regulated by an inducible promoter or it could be activated by the infectious agent itself. In that case, the cloning of the antibody genes after a viral promoter will be required. The expression of this transgene will only be induced when the infection takes place and the viral transcriptase in produced. Experiments in which the expression of an scFv antibody HIV-1 specific was controlled by the HIV-1 LTR promoter have shown that this is an effective method of inhibiting viral replication (Chen et al., 1994). This approach requires a precise characterization of the viral promoter.

1.5. Protection against Bacterial and Viral Infection by Antimicrobial Peptides

The strategy described for the protection against virus infections in mucosal areas could be extended to their protection against bacterial infection by using molecules that, such as the antibodies, inactivate the infectious agent. This is the case of antimicrobial peptides, which are key components in immunity. There are as least four types of antimicrobial peptides: defensins, magainins, cecropins, and melittins, the two first found in mammals and amphibians, respectively, and the other two found in insects. These molecules are the base of an ancient antimicrobial defense found in both animal and plant kingdoms (Boman, 1991; Lehrer et al., 1991). These peptides have been classified based on their sequence and on structural features.

Animal peptide antibiotics can be rapidly activated after injury or invasion of the host by microbial agents, combating parasitic growth immediately after infection. Antimicrobial peptides thus provide an important defense mechanism in lower animals and the first line of host defense during the time required for mobilization of specific immunity in vertebrates.

Defensins and β-defensins are found in neutrophils and in epithelial cells of mucosal tissues of mammals. They are predicted to function as the first line of host defense against microbial pathogens. Impairment of defensin activity has been implicated in chronic bacterial infections in cystic fibrosis patients (Smith et al., 1996). Interestingly, there is evidence that defensins may also be active in protection against virus infections (Wachinger et al., 1998).

Defensins act on a wide variety of bacteria but usually more efficiently on gram-positive than on gram-negative bacteria. They also work on fungi and some of them display a small degree of cytotoxicity for normal eukaryotic cells. Defensins are made as preproteins of 93–95 residues containing one defensin copy. A smaller peptide with only 12 residues and one intramolecular disulfide bond was found in bovine neutrophils (Boman, 1991).

Extracts of bovine tracheal mucosa have an abundant peptide with potent antimicrobial activity (Diamond et al., 1991). The 38-amino acid peptide, which has been named tracheal antimicrobial peptide (TAP), is produced in a proportion of 2 μg/g of wet mucosa. The size, basic charge, and presence of three intramolecular disulfide bonds is similar to, but clearly distinct from, the defensins and it has been classified as a member of the β-defensin family of antibiotic peptides found in the tracheal mucosa of the cow. The putative TAP precursor is predicted to be relative small (64 amino acids), and the mature peptide resides in the extreme carboxyterminus. The mRNA encoding this peptide is more abundant in the respiratory mucosa than in whole lung tissue. The purified peptide has antibacterial activity *in vitro* against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, and Pseudomonas aeruginosa.* In addition, the peptide was active against *Candida albicans*, indicating a broad spectrum of activity. This peptide appears to be, based on structure and activity, a member of a group of cysteine-rich, cationic, antimicrobial peptides found in animals, insects, and plants.

TAP gene expression in the bovine airway is inducible by lypopolysaccharide and inflammatory mediators, suggesting that it functions to protect the upper airway from infection. Limited availability of bovine TAP has precluded investigation of its potential utility in agriculture and medicine. To overcome this problem, transgenic mice expressing bTAP using an expression vector driven by control sequences from the WAP gene have been generated (Yarus et al., 1996). bTAP was purified to homogeneity from milk and showed antimicrobial activity against *E. coli.* The bTAP available from a mammary gland bioreactor will allow evaluation of bTAP for use as an antibiotic in agriculture and medicine.

The cecropins were originally identified as highly potent antibacterial peptides in immune hemolymph from the cecropia moth. All cecropins are 31–39 residues, are devoid of cysteine, and have a strongly basic N-terminal half. Although cecropins were thought to be unique to insects, a cecropin has been found in pig intestine (Lee et al., 1989), which implies that cecropins are widespread in the animal kingdom.

Cecropins A and B are highly active against several gram-positive and gram-negative bacteria, while the porcine form shows high activity only against gram-negatives. Cecropins also lyse artificial liposomes composed of phospholipids having zwitterionic or negatively charged head groups but did not lyse eukaryotic cells. The primary targets of defensins, magainins, cecropins, and melittins are the inner and outer bacterial membranes. All four groups of peptides have also been shown to form channels in artificial membranes, but it is not yet clear whether channel formation is the mechanism by which these peptides kill microorganisms.

Previously, it was reported that most cationic peptides do not induce resistance mutants *in vitro* and enhance antimicrobial activity of classical antibiotics in resistant bacteria, thus serving as anti-resistance compounds (Hancock, 1997). However, it has been recently reported (Guo et al., 1998) that increased acylation of lipid A, the major component of the outer leaflet of the outer membrane, is a cationic antimicrobial peptide resistance mechanism. In addition to antibacterial activities, amphipathic antimicrobial peptides have also been reported to act against fungi (Ahmad et al., 1995), protozoa (Bevins and Zasloff, 1990), and viruses (Wachinger et al., 1998).

Cecropins are relatively non-toxic to normal cells from multicellular organisms but, in addition to bacteria, they are toxic to protozoa and fungi, as well as infected and abnormal cells. Transgenic mice have been produced with interleukin 2 promoter/enhancer controlled expression of a synthetic cecropin-class lytic peptide that were subsequently analyzed for their resistance to *Brucella abortus* (Reed et al., 1997).

The lymphocytes of the transgenic mice could be induced to transcribe and mature cecropin mRNA after exposure to Con A. The transgenic mice showed an increased resistance to *B. abortus* as compared with non-transgenic mice.

Melittin and cecropin inhibit replication of animal viruses. Melittin is a 26 amino acid amphipathic α-helical peptide, which is a major component of bee venom (Bazzo et al., 1988; Habermann and Jentsch, 1967; Terwilliger and Eisenberg, 1982). Melittin and cecropins consist of two α-helices linked by a flexible segment and contain amphipathic structures. Whereas melittin is lytic for red blood cells at high concentrations, cecropins do not lyse erythrocytes or other eukaryotic cells (Steiner et al., 1981; Wade et al., 1992) and appear to be non-toxic for mammalian cells. Melittin has been reported to inhibit replication of murine retroviruses, tobacco mosaic virus (Marcos et al., 1995) and herpes simplex virus (Baghian et al., 1997), suggesting that melittin also displays antiviral activity. Analogous to antibacterial activity, the antiviral activity of melittin has been attributed to direct lysis of viral membranes, as demonstrated for murine retroviruses (Esser et al., 1979). However, melittin also displays antiviral activity at much lower, non-virolytic concentrations, as shown for T cells chronically infected with human immunodeficiency virus 1 (HIV-1) (Wachinger et al., 1992). Melittin and cecropins are shown to suppress production of HIV-1 by acutely infected cells. Melittin treatment of T cells reduces levels of intracellular Gag and viral mRNAs, and decrease HIV long terminal repeat (LTR) activity (Wachinger et al., 1998). HIV LTR activity is also reduced in human cells stably transfected with melittin and cecropin genes. These results indicate that antimicrobial peptides such as melittin and cecropin suppress HIV-1 replication by interfering with host cell-directed viral gene expression.

1.6. Transgenic Animals Producing γ-Interferon

(Dobrovolsky et al., 1993). The human γ-interferon (hIFN-γ) is an immunomodulator displaying antiviral and antiproliferative properties. Large amounts of glycosylated hIFN-γ have been produced in the mammary gland of transgenic mice (Dobrovolsky et al., 1993). The concentration of hIFN-γ in the milk was 1800 IU/ml. These transgenic animals could protect their progeny from virus infections of the enteric tract during lactation.

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