Lentivirus Vaccine Development

Antigen presentation by *Salmonella* and iscom

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Lentivirus vaccinontwikkeling
Antigeen presentatie door *Salmonella* en iscom

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. Dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaats vinden op
woensdag 29 oktober 1997 om 13.45 uur

door

Edwin Johannus Tijhaar
geboren te Hilversum

Promotie-commissie

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ISBN 9056770322

The studies described in this thesis were performed at the former Laboratory of Immunobiology (head Prof. Dr. A.D.M.E. Osterhaus) of the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

The work presented in this thesis was supported by the Advisory Council on Health Research (RGO), The Hague, The Netherlands (grant number 88-108/89027).

Printing of this thesis was financially supported by: The National Institute of Public Health and the Environment (RIVM), European Veterinary Laboratory B.V., MSD B.V.

De uitgave van dit proefschrift
is (mede) mogelijk gemaakt
door Intervet International b.v.,
Boxmeer

Cover: Scanning electron micrograph of a lymphocyte. Lymphocytes play a major role in our immune system and are the major targets for infection by lentiviruses.

Drukwerk: Febodruk B.V., Enschede

Aan Himali en mijn ouders

Contents

Abbreviations

AIDS	acquired immunodeficiency syndrome
CA protein	capsid proteins
ConA	concanavalin A
CTL	cytotoxic T lymphocyte
DNA	desoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FIV	feline immunodeficiency virus
FCS	fetal calf serum
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
i.m.	intramuscular
IN protein	integrase protein
i.p.	intraperitoneal
IU	international units
Ig	immunoglobulin
LPS	lipopolysaccharide
LTR	long terminal repeat
MA protein	matrix protein
MHC	major histocompatibility complex
NC protein	nucleocapsid protein
NF-kB	nuclear factor kB
NRE	negative regulatory element
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PR proteins	protease protein
rIL-2	recombinant interleukin-2
RNA	ribonucleic acid
RRE	rev responsive element
RT proteins	reverse transcriptase protein
rVV	recombinant vaccinia virus
SIV	simian immunodeficiency virus
SPF	specified pathogen free
SU	surface unit
TM	transmembrane
TAR	transacting responsive sequences
VN	virus neutralising

CHAPTER 1

General introduction

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), the causative agents of acquired immune deficiency syndrome (AIDS) in humans, are members of the Lentivirinae subfamily of the Retroviridae family. The lentivirus subfamily also includes related members from other species, like monkeys (simian immunodeficiency viruses [SIV]), cats (feline immunodeficiency viruses [FIV]), and the ungulates sheep, goats, horses and cattle.

Structure of lentiviruses

Mature lentiviruses are spherical to ellipsoid particles with a diameter of approximately 100 nm consisting of a lipid envelope surrounding a cone shaped core (Gelderblom et al 1989) (Fig. 1). In HIV-1 the core is formed by a 24 kd capsid protein (p24). It contains two identical strands of positive-sense genomic RNA closely associated with the nucleocapsid proteins (p7 and p9) and several copies of the reverse transcriptase. A membrane associated matrix protein p17 is situated between the core and the envelope. In the envelope a 41 kd transmembrane glycoprotein (gp41) is anchored. The transmembrane protein is non-covalently attached to the 120 kd surface glycoprotein (gp120). The other lentiviruses have a similar structure, with slightly different molecular weights of their proteins.

Genome organisation

The proviral positive strand RNA genomes of lentiviruses are approximately 9500 bp in length and are flanked by two long terminal repeats (LTR) (Fig. 1). The genome contains three large open reading frames (ORF), *gag*, *pol* and *env* that encode structural and enzymatic proteins. Apart from these large ORFs the genome of the primate lentiviruses contains six small genes. Of these auxiliary genes *tat* and *rev* are essential and, *nef*, *vif*, *vpr*, and *vpu* (*vpx* in HIV-2 and SIV) are nonessential for virus replication *in vitro* (for reviews see: Fauci 1993 and Gibbs 1994, Morrow 1994)

LTR. The LTRs direct and control viral DNA and RNA synthesis and the integration of proviral DNA into the genome of the host cell. They contain a polyadenylation signal sequence, a negative regulatory element (NRE), an enhancer region (NF-κB binding region), Sp1 binding sites and transacting responsive sequences (TAR).

gag. The *gag* gene codes for a precursor protein of 55 kd which is cleaved into p17, p24 and p15 by the viral protease.

gag-pol. The 3'-end of the *gag* gene overlaps the 5'-end of the *pol* gene

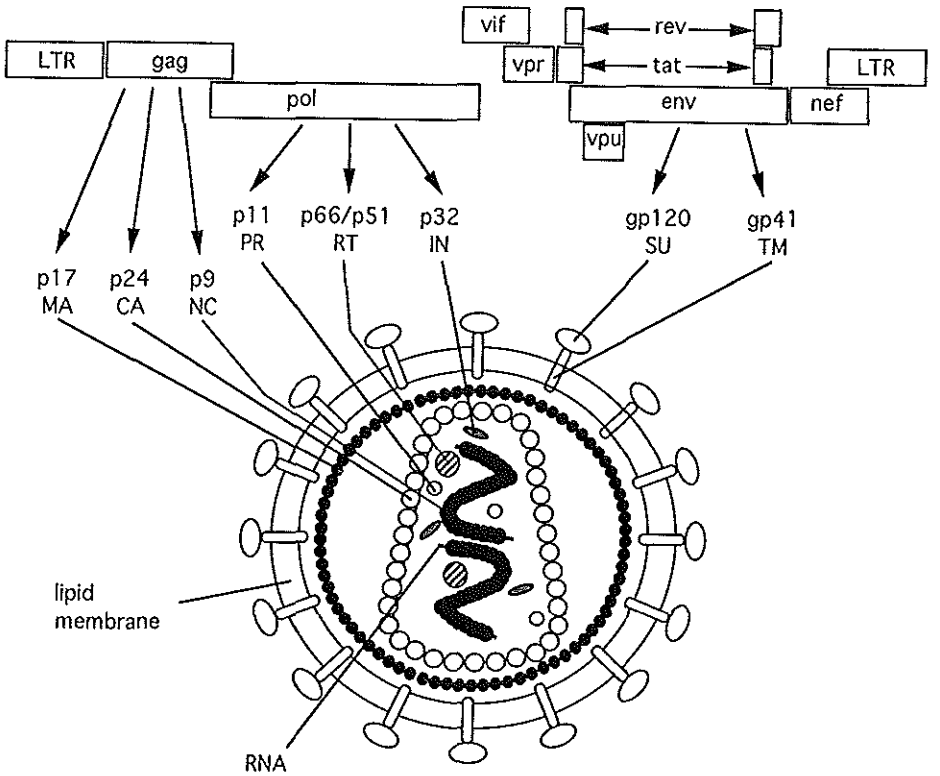

Fig. 1 Genome organisation and virion structure of HIV-1. Abbreviations: MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface glycoprotein gp120; TM, transmembrane protein gp41.

by a few hundred nucleotides. The *pol* gene, which has a reading frame different from the *gag* gene, is translated as a result of a frame shift of 5% of the ribosomes translating the *gag-pol* mRNA (Jacks 1988). By autocleavage a 10 kD viral protease is released from the resulting gag-pol precursor polyprotein (Ratner 1985, Sanchez-Pescador 1985, Kramer 1986, Debouck 1987). This protease cleaves the polyprotein into a functional reverse transcriptase, RNase H and integrase (Farmerie 1987, Peng 1989). The same protease is also responsible for the cleavage of the Gag precursor protein mentioned above.

env. The *env* gene codes for a glycoprotein (160 kD for HIV-1), which after endo-proteolytic cleavage forms the surface unit (SU) protein (gp120 for

HIV-1) and the transmembrane (TM) protein gp41 for HIV-1). In primate lentiviruses the *env* gene products have been shown to control many functions which are important for viral pathogenesis (for review: Levy 1993, Sattentau 1996, Chirmule and Pahwa, 1996).

tat. The *tat* gene (transactivator) consists of two exons in the *env* region of the genome. Its transcription into mRNA involves two splicing events (Arya S.K. 1985; Sodroski1985). Tat is a nuclear protein of 15 kd that acts by enhancing the rate of transcription from the 5' LTR (Hauber1987, Kao1987, Ruben1989), boosting the expression of viral genes 50 to 1000 times the level seen in HIV mutants lacking the *tat* gene. Its effect depends on a short sequence within the LTR, the trans acting responsive (TAR) sequence, that is included in the mRNA transcripts of every gene of the primate lentiviruses.

rev. The Rev protein is encoded by a spliced mRNA of which the first coding exon overlaps the *tat* gene and the second exon the *env* gene (Sadaie 1988). It is a nuclear protein that binds to the Rev responsive element (RRE), which is present in viral mRNA before splicing. It facilitates translocation of full length or single spliced viral mRNAs from the nucleus to the cytoplasm (Malim 1989). These mRNAs encode structural proteins. At low concentrations of Rev the viral mRNAs are predominantly small and multiple spliced (Emerman 1989, Felber 1989). These small mRNAs encode regulatory proteins. The concentration of the Rev protein therefore plays an important role in determining whether a lentivirus infection is latent or productive.

nef. The nef gene encodes a protein on the cell surface of infected cells. The protein is believed to increase viral load and hence to allow the virus to overcome the host immune response. It may act as a superantigen and a mitogen for T-cells (Torres 1996a, 1996b).

vif. Vif for virion infectivity factor is a small hydrophobic protein that is essential for cell free infection.

vpr and *vpx.* These genes are related (Tristen 1990,1992). All the primate lentiviruses contain at least one homologue of *vpr/vpx.* The SIV_{mac}/HIV-2/SIV_{sm}, group of primate lentiviruses is the only one to contain both *vpr* and *vpx* genes. The *vpr* and *vpx* products are both virion associated (Cohen 1990), but their functional roles have not been elucidated. However, they seem to play a role in replication of the virus *in vivo*, as a single *vpx* deletion mutant and a *vpr/vpx* double mutant of SIV_{mac} were slightly, respectively severely attenuated *in vivo*. No attenuation was observed for a single *vpr* deletion mutant (Gibbs 1995).

vpu. Viral protein U (Vpu) is a protein unique to HIV-1 and the SIV strain

found in chimpanzees. It is a cytoplasmic protein that facilitates assembly and export of virions and the degradation of CD4 in the endoplasmatic reticulum (Lenburg and Landau 1993, Raja 1994). Deletion of *vpu* had a significant effect of infectivity and moderate effects on pathogenicity in a SCID-hu model system (Aldrovandi and Zack, 1996).

Lentivirus replication cycle.

Attachment of the primate lentiviruses to the target cell is achieved by the high affinity interaction between the SU part of the virus glycoproteins and the cellular surface antigen CD4 (Stein 1987). This binding alone is insufficient to allow entry of the virus into the cell and for nearly 10 years researches have tried to identify the HIV co-receptor. Recently a number of co-receptors have been identified that are responsible for bringing the surface membranes of the host cell and virus together to allow fusion. The first identified co-receptor for macrophage tropic (M-tropic) strains is the chemokine receptor CCR5 (Alkhatib 1996a, 1996b , Deng 1996, Dragic 1996) and for T cell tropic (T-tropic) strains of HIV, a chemokine receptor named fusin (also termed CXCR4 or LESTR) (Feng 1996 Science 272: 872-877). Other chemokine receptors that have been implicated as co-receptors for HIV-1 are CCR3 and CCR2b (Doranz 1996 Cell 85:1149-1158, Choe 1996). The newly described co-receptors are all seven transmembrane receptors from the large family of G protein coupled receptors in man, which respond to chemokines. Chemokine receptor CCR5 binds RANTES, MIP-1α and MIP-1β what explains the inhibition of these chemokines on PBMC infection by HIV-1, HIV-2 and SIV observed by Cocchi et al (Cocchi 1995 Science 270:1811-1815). The importance of the CCR5 receptor for HIV-1 infection is further emphasised by the finding that individuals homozygous for a 32-base-pair deletion in the CCR5 gene are highly resistant to infection with M-tropic, but not T tropic viruses. This deletion produces a truncated version of CCR5 that is not expressed at the cell surface (for a review on host factors and pathogenesis of HIV see Fauci 1996).

The structural changes induced in the SU protein by its primary interaction with CD4 and than with its co-receptor, trigger a conformational change in the TM protein. As a result the fusion peptide of the TM protein penetrates the target cell (Lifson 1986, Weissenhorn 1997, Binley 1997). The transmembrane domain and the fusion domain of the TM protein are than brought into close proximity, facilitating the coalescence of the viral and cell membranes and, ultimately, membrane fusion. Upon fusion the viral core containing the two identical genomic RNA strands, reverse transcriptase and RNase H, protease, and integrase

(Varmus and Swanstrom 1985, Farnet and Haseltine, 1990), is released into the cell's cytoplasm. The single stranded RNA is here converted by the action of reverse transcriptase (DNA polymerase and RNase H together) into a double stranded DNA intermediate, flanked at both ends by long terminal repeats (LTR). Formation of the provirus is completed once the double stranded DNA intermediate is integrated into the host genome by the action of the viral integrase. Expression of the genes of this provirus is a complex process controlled by both host and viral factors. In the latent stage of the infection short double spliced mRNAs coding for the regulatory proteins reach the cytoplasm (Feinberg 1986, Malim 1989). Later in infection the long unspliced mRNAs coding for the structural proteins and replicative enzymes are dominant. During this lytic stage of the infection the capsid precursor protein and the gag-pol precursor protein become inserted into the cell membrane via the myristic acid attached to their N-termini (Veronese 1988) where they co-assemble. The capsid precursor protein contains two copies of a viral RNA-binding motif, the so called cysteine-histidine box (Marie and Spahr, 1986). This cysteine-histidine box specifically recognises the ψ packaging signal located at the 5'-end of the genomic RNA and ensures that the RNA is incorporated in the resulting closed spherical particles (Lever 1989). Assembled nucleocapsids bud through the cell membrane, which contains the already digested virus envelope glycoprotein. Late maturation events include the proteolytic cleavage of capsid protein and replicative enzyme precursors (Oroszlan and Luftig 1990).

Pathogenesis

HIV is a sexually transmitted and blood-borne pathogen. Infection with this virus can have a number of diverse manifestations ranging from subclinical abnormalities to opportunistic infections and malignancies that define the acquired immunodeficiency syndrome (AIDS), the end-stage of HIV infection (reviewed by Levy, 1993). From the very first description, AIDS has been associated with the depletion of CD4+ T-lymphocytes (Gottlieb 1981). The virus infects CD4+ T lymphocytes and cells of the monocytes/macrophage lineage, dendritic cells and certain glial cells. The mechanisms by which HIV causes immunodeficiency are not entirely clear, but they are driven by a persistent HIV infection (Pantaleo 1993, Embretson 1993). Increasing evidence suggests that the decline in CD4+ lymphocytes that finally results in the collapse of the immune system is not solely due to lytic infection of these cells. Defective antigen presentation and inappropriate signalling by antigen presenting cells resulting in T cell anergy and priming cells for activation induced apoptosis contribute to the

lymphocytes (Stanley and Fauci 1993, Meyaard 1993, Oyaizu 1995).

Animal models for HIV infection in man

The ideal animal model for AIDS would be an inexpensive readily available laboratory animal that can be infected with HIV-1 and subsequently develops an AIDS-like disease. Apes like the chimpanzee (*Pan troglodytes*) and the gibbon (*Hylobates spp.*) have been shown to be susceptible to experimental HIV-1 infection (Alter1984, Fultz J. Virol. 1986, Goudsmit 1988, Arthur 1989, Lusso, 1988). However, these animals have not been shown to develop AIDS upon infection. This aspect, together with the endangered status, the high costs and the ethical objections against the use of animals so closely related to humans, makes these models unsuitable for extensive AIDS research. It has been shown that HIV-1 can replicate in severe combined immunodeficiency (SCID) mice, reconstituted with human lymphocytes or human foetal thymus and liver or lymph nodes (McCune 1988, Aldrovandi and Zack 1996). However, functional CD8+ human lymphocytes do not persist in these mice and HIV-1 spreads rapidly and ablate T cells rather than replicate in a chronic, low grade fashion (Mosier 1989). Nowadays the most widely used primate model for HIV infection in man is simian immunodeficiency virus (SIV) infection of macaques (*reviewed in* Schultz and Hu, 1993). SIVmac, isolated from a rhesus macaque (*Macaca Mulatta*) (Letvin 1985) and SIVsm from sooty mangabeys (*Cercocebus atys*) (Fultz, PNAS 1986) show remarkable similarities with the human immunodeficiency viruses in virus morphology, genome organisation, replication cycle, tropism and pathology of disease that they cause in their natural hosts. The SIV and HIV-1 envelope glycoproteins are relatively distinct. Therefore chimeric SIVs carrying HIV-1 *tat*, *rev*, *vpu*, and *env* genes were constructed. These so-called SHIVs are able to replicate, albeit poorly, in cynomolgus macaques (*Macaca fascicularis*) (Shibata 1991, Li 1992, Sakuragi 1992) and may offer a system for testing HIV-1 based vaccines.

Of the non-primate animal models FIV infection in cats is by far the most promising and widely studied. Like HIV-1 and SIV, FIV is T lymphotropic, causes a progressive loss of $CD4^+$ T cells, can infect macrophages and astrocytes, and eventually causes severe immunodeficiency or AIDS in its natural host (Barlough 1991, Dow 1990, Hoffmann-Frezer 1992, Hopper 1989, Hurtrel 1992, Pedersen Science 1987, Sparger E.E., (review) AIDS 1989, Yamamoto Am J Vet Res 1988, Torten 1991). Like in the clinical course of HIV infection in man, five different stages can be recognised in the clinical course of FIV infection in cats (Ishida 1990). In the acute phase after infection, experimentally

infected cats develop a transient low grade fever, neutropenia which may persist for some days up to several weeks and generalised lymphoadenopathy which can persist for several months (Pedersen [review] 1989, Yamamoto 1988). This phase is followed by the asymptomatic (AC) phase, which can last for years. The similarities of FIV infection of cats with HIV-1 infection of man render it an attractive small animal model, that is relatively inexpensive and poses less ethical problems than the use of primates. However, it also has a number of drawbacks. Of these probably the most important one is that, unlike the primate lentiviruses, FIV does not use CD4 as the primary receptor.

Anti-viral immunity

The immune system can combat viral infections by a number of specific and non-specific strategies. The specific immune response can be divided in antibody and cell mediated immunity, which both may exert their functions systemically and at mucosal surfaces.

Antibody mediated immunity

The effector molecules of humoral immunity are the immunoglobulins (Igs or antibodies) secreted by activated B cells. They can prevent virus infectivity by binding to specific antigens at the surface of viruses (virus neutralisation), or by activating the complement system which can lead to viral cytolysis and uptake by macrophages. Once a virus has released its genetic information into the cytoplasm of a host cell, leading to the production of viral protein, antibodies can limit virus replication by causing lysis of the infected cell, either by utilising the complement pathway or through antibody-dependent-cellular cytotoxicity (ADCC).

Naive B cells bear membrane bound IgM and IgD molecules on their surfaces with specificity for certain antigenic epitopes. The B cells proliferate upon encounter of the corresponding epitope (clonal expansion) creating many progeny B cells with antibodies having identical antigenic specificity. These B cells than differentiate into either effector (plasma) cells or memory B cells. During B cell proliferation B cells undergo a process called class switching whereby the class of antibody produced changes (for review on class switching see Snapper and Mond 1993). Different classes of immunoglobulins have different effector functions. For example, the pentameric structure of IgM makes it very suitable to activate the complement system by the classical pathway, IgG can target viruses for lysis by FcR bearing macrophages and secretory IgA antibodies play a major role in the mucosal immune response.

Antigenic stimulation of B cells alone is usually not enough to induce B cells to proliferate. This requires also cytokines secreted by activated T helper lymphocytes.

T-cell mediated immunity and antigen presentation

Contrary to B cells that can recognise free antigen, T lymphocytes (T cells) have receptors that recognise peptide fragments of degraded proteins associated with major histocompatibility complex (MHC) proteins (Townsend 1989). Different classes of T cells exist and can be distinguished by the type of cluster differentiation marker (CD) that they carry on their surface (Knapp 1989). CD4-CD8+ T cells interact with peptides presented in association with MHC class I molecules (Norment 1988), whereas CD4+CD8- T cells recognise peptides bound to MHC class II molecules (Gay 1987). Exogenously delivered proteins are endocytosed by specialised antigen presenting cells like macrophages, dendritic cells or B lymphocytes. In the acidic endosomes the proteins are reduced and proteolytically cleaved into peptides (Van Noort 1991, Vidard 1991). The endosomal processing and class II MHC molecule biosynthetic pathways intersect (Peters 1991, Harding 1991) and the peptides bind to the a and b subunits of the class II molecular complex, displacing the invariant chain. The peptide loaded class II MHC complex is then expressed on the cell surface.

Proteins delivered to the cytosol of cells, e.g. following a viral infection, are processed by the endogenous pathway. In this pathway proteins are proteolytically degraded into peptides by proteasomes (Brown 1991, Martinez and Monaco 1991, Goldberg and Rock 1992), and transported by transporter molecules (TAP1 and TAP2) to the lumen of the endoplasmatic reticulum, where they are further degraded. In the ER, newly synthesised MHC I heavy chains and β2-microglobulin are assembled into heterodimeric MHC class I molecules through the activity of accessory proteins such as p88. Each of these units can bind a peptide and the peptide-loaded MHC class I heterodimers are transported to the cell surface.

The different pathways of antigen processing may not be totally separate. For example, Jarquemada et al. have demonstrated that the endogenously produced influenza A virus matrix protein can probably gain access to endosomal/lysosomal compartments via an intracellular route, leading to the processing and presentation in a class II-restricted manner. Increasing evidence also suggests that their is an alternative route for particulate antigens that results in a class I restricted presentation of these antigens (for review see Rock 1996).

Peptides presented in the context of class I molecules will induce CD8+

cytotoxic T-lymphocytes (CTL) which can specifically kill virus infected cells, whereas peptides presented in association with class II MHC molecules will induce CD4+ T-helper (Th) cells. Different types of CTL and Th cells exist and they can mediate different types of immune responses through the action of their cytokines. A response that is often referred to as a type 1 response, preferentially mediates cellular immune responses, like delayed type hypersensitivity (DTH) responses and CD8+ CTL responses, whereas a type 2 response provides help for antibody production (Cher and Mosman, 1987, Boon 1988., Del Prete 1991, 1994, Parronchi 1991).

Mucosal immunity

The mucosal immune system is anatomically and functionally divided into separate regions. These include areas where antigens are encountered, processed and initial B- and T-cell triggering occurs (called inductive sites) and areas where immune cells actually function (called effector sites) (McGhee et al. 1992). In humans the major inductive sites are probably the Peyer's patches of the gastrointestinal tract and the tonsils in the upper respiratory tract, with the lamina propria and salivary/mammary glands constituting the effector sites in the gastrointestinal and respiratory tracts, respectively. Following oral entry, antigens may be taken up from the small intestine by the specialised microfold or "M" cells. These cells can even take up whole bacteria, and transport them to the follicle beneath, known as Peyer's patch. The induction of T- and B-cell responses to the antigens occurs here (inductive sites) and is followed by migration of the induced lymphocytes to the mesenteric lymph nodes. These lymphocytes then travel via the efferent lymphatics to the thoracic duct, where they enter the blood circulation. Later, cells migrate to various effector sites in the gastrointestinal, respiratory and genitourinary tracts. This is known as "the common mucosal immune system (reviewed by Brandtzaeg 1989, McGhee 1992, Kiyono 1995, Abreumartin 1996). IgA plasma cells initially stimulated by cognate B and T cell interactions in gut-associated lymphoid tissue (GALT) secrete local antibody that is specifically taken up by epithelial cells, transported to the mucosal surface, and released with a part of the receptor as secretory IgA (Bergmann and Waldman, 1988, Dahlgren et al 1989). Secretory IgA can protect against pathogens that replicate at or enter via mucosal surfaces (Michetti et al. 1992, Walker et al 1992).

The major route of entry of HIV is the mucosa of the urino-genital tract and the rectum. The Langerhans cells in the genital mucosa express CD4 and may be the first host cells were HIV replication will take place. Langerhans cells are

not present in the rectal mucosa (Hussain 1995) and it is thought that the target for HIV in rectal transmission may be colorectal or ileal epithelial cells. The importance of an adequate mucosal immune response to lentiviruses has been highlighted by studies in which vaccinated animals that were protected against a parental challenge failed to be protected when they we challenged via their mucosa (Sutjipto 1990).

Approaches for the development of lentivirus vaccines.

An effective profilactic HIV vaccine is urgently needed, especially for use in the third world. The ideal vaccine should be safe, confer long-lasting protection with a minimum of vaccinations, be inexpensive to produce and stable without cooling. Virtually all classic and new vaccine approaches are being studied for their effectiveness in the control of lentivirus infections, which by itself is indicative for the huge problems encountered. The most intensively explored approaches are listed here:

Live attenuated vaccines

Vaccines based upon live attenuated viruses are generally still the most effective vaccines. They have enabled the eradication of small pox and are expected to enable or facilitate the eradication of poliomyelitis and measles in due time. Since attenuated viruses replicate *in vivo* they induce MHC class I restricted CTL responses. Furthermore, all the viral proteins can be presented to the immune system, thus inducing a wide spectrum of humoral and cell mediated immune responses, thereby reducing the risk of emerging escape mutants. The most impressive protection of macaques against SIV infection so far, has been obtained by vaccination with a live attenuated SIVmac239, which had a deletion in the *nef* gene (Daniel et al Science 1992). Despite the potential advantages of live, attenuated viruses as vaccines there are a number of concerns about their safety. For example, an attenuated virus may revert to its virulent form *in vivo*, as has been shown for the *nef* deletion mutant SIVmac32H (pC8) (Dittmer 1995). Modern recombinant DNA techniques can now reduce this risk by the introduction of defined deletion mutations, but these deletions do not rule out the possibility that recombination *in vivo* with other (persistent) viruses will create more virulent viruses. Because infection with *nef*-deleted viruses persists for live, (temporary) loss of cellular immune competence due to intercurrent illness or due to ageing could result in an increased level of viral replication, which might result in the development of disease. Indeed, a SIVmac-*nef* deletion mutant that protected adult macaques against wild type SIVmac infection was pathogenic in

protected adult macaques against wild type SIVmac infection was pathogenic in neonates (Baba 1995). The integration of provirus in the host genome, poses another problem in the development of live attenuated lentiviruses.

Inactivated whole virus vaccines

These vaccines are based on complete viruses and can therefore contain several antigens which may favour induction of a protective immune response thereby reducing the risk of the emergence of escape mutants. However, they may contain undesirable antigens that induce an autoimmune response or enhance infection. They also generally fail to induce MHC class I restricted CTL responses, which may be essential in the clearance of lentivirus infections (for review: Gotch 1996). In the FIV-cat system, vaccination of cats with paraformaldehyde fixed T cells (FL-4 cells) persistently infected with FIV and with paraformaldehyde inactivated FIV derived from the same cells, proved to be protective against homologous and to a lesser extent heterologous challenge (Yamamoto 1991, 1993). In the SIV-macaque system, vaccination with whole inactivated virus induced partial protection against challenge with PBMC from a SIVmac infected macaque (De Vries, 1994). Earlier reported protection against challenge with cell-free virus after immunising with inactivated whole SIVmac was almost certainly mediated by the immune response to cellular proteins incorporated into both the immunogen and the challenge viruses (De Vries 1994, Stott 1991).

Subunit vaccines

Subunit vaccines are based on the principle of using selected well-defined antigens. They do not pose the safety risks of live attenuated or whole inactivated virus vaccines, because they are non-infectious and the composition of the vaccine may be well defined and controlled. This makes it possible to include only those antigens that are important for the induction of protection. Antigens that may induce adverse effects like autoimmune reactions or enhancement of infection can be left out. Unfortunately subunit vaccines per se are generally poorly immunogenic when administered alone. They usually also fail to induce class I restricted CTL responses. Therefore a large number of adjuvant formulations (e.g. alum, muramyl-dipeptide, Quil A) or carrier vehicles (e.g. iscom, liposomes, protein cochleates) have been developed which potentiate their immunogenicity (Vaccine Design, The subunit and adjuvant approach, 1995). However, most adjuvants have not been released for human use. A discussion of all adjuvants and adjuvant and adjuvant systems is outside the scope of this thesis. However, one of these, the immune stimulating complex (iscom), shows

The iscom is a cage-like structure with a diameter of 30-40 nm into which antigens can be incorporated (Morein 1984). It consists of glycosides of the adjuvant Quil A, cholesterol, the antigen, and in most cases phospholipids. Most viral membrane proteins with their hydrophobic anchor sequences are readily incorporated during iscom preparation. Proteins lacking hydrophobic transmembrane sequences can be incorporated by linking them to a hydrophobic carrier molecule such as palmitic acid (Reid 1992). Alternatively, proteins can be treated with low pH to expose hydrophobic stretches in the molecule that are normally hidden (Morein 1990, Heeg 1991). The iscom is a highly effective antigen presentation form for viral antigens. It is a potent inducer of B and T cell responses, including MHC class I restricted CTL responses, leading to solid and long-lasting immunity against many viral infections (for review see Rimmelzwaan and Osterhaus 1995). When administered by the intranasal route, iscoms can induce mucosal immune responses in the lung, including specific IgA and CTLs. The induction of CD8+ CTLs by iscoms is most likely a result of entry into the endogenous pathway of antigen processing and presentation. This was shown *in vitro* by the stimulation of a CD8+ CTL clone specific for the fusion (F) protein of measles virus (MV) by F protein incorporated in iscom, but not by purified protein or UV inactivated MV. This stimulation was insensitive to the action of chloroquine, which inhibits the exogenous pathway of processing by raising the pH of the endosomal compartment. In addition, the presentation of F protein by iscom to CD8+ T cell clones was abrogated when a TAP negative antigen presenting cell was used (Van Binnendijk 1992).

Macaques immunized with iscom containing envelope and core protein and Nef peptides of SIVmac developed virus neutralising antibodies and MHC class I restricted CTL (Hulskotte, Virology 1996). Unfortunately, this was insufficient to protect the macaques against a cell-free SIVmac challenge. A vaccine consisting of a mixture of iscom prepared from inactivated whole SIVmac (32H) grown in a human T-cell line, a gp120-enriched iscom preparation and p27 protein failed to protect macaques against cell-free SIVmac produced in PBMC from a macaque, but did protect two out of four macaques against intravenous challenge with PBMC from a SIV infected macaque (Osterhaus 1992, De Vries 1994). Interestingly, the protected monkeys shared a MHC class I allele with the PBMC donor monkey, suggesting that SIV specific CTL played a role in the observed protection (Heeney 1994).

Virus like particles and self-aggregating proteins.

Certain proteins that spontaneously associate into virus-like-particles (VLP) are

Certain proteins that spontaneously associate into virus-like-particles (VLP) are also evaluated as carriers of viral proteins. One approach is the Ty:VLP system (Mellor 1985) wherein a foreign DNA sequence is fused in-frame to the TY A gene. Following expression in yeast the VLP are formed which can then be purified and used for immunisation experiments. Using this system a MHC class I restricted CD8+ CTL response was induced against the principle antibody neutralising domain, V3, of HIV-1 (Layton 1993).

The hepatitis B virus core antigen (HBcAg) and surface antigen (HBsAg) are examples of self-aggregating viral proteins that are evaluated as potential carriers for foreign antigens (Francis et al. 1990). HBcAg spontaneously self-aggregates into characteristic 27 nm particles. Chimeric core particles can be created by the insertion of peptide sequences onto the N- or C-termini of HBcAg (Clarke, 1987 and Stahl, 1989). Fusions of the V3 domain from HIV-1 to either the N- or C-terminal of HBcAg, resulted in the induction of neutralising antibodies to HIV-1 (Von Brunn 1993) and specific MHC class I restricted CD8+ CTL responses were induced upon immunisation with the V3 domain fused to HBsAg (Schlienger 1992).

Live, recombinant viral and bacterial carriers.

Several viruses and bacteria have been evaluated as potential live carrier systems to present heterologous antigens to the immune system. Some of the viral vectors, like attenuated vaccinia virus (Andrew 1991) and adenovirus (Johnson 1988) replicate *in vivo*, whereas others, like poliovirus minireplicons (Burke 1988; Evans 1989; Dedieu 1992) and avipox virus (Radaelli 1994), are engineered to have a restricted or abortive replication. Since they all infect host cells, both humoral and cell-mediated immunity, including class I restricted CTL responses, can be induced. A chimeric influenza virus has been used to induce neutralising sIgA against HIV-1 after intranasal immunisation, which was detectable for more than a year (Munster 1995). A combined vaccination scheme using a live vaccinia virus expressing the SIV envelope protein followed by a boost with purified protein protected macaques against a challenge with SIVmne (Hu 1992), but failed to protect against challenge with SIVmac (Giavedoni 1993). Some viral carriers like influenza and poliovirus can only accommodate relatively small inserts, limiting the number of heterologous B and T cell epitopes that can be presented. Live viral carriers may pose similar safety risks as live attenuated viruses, like the generation of more virulent viruses from the carrier by mutations or recombination *in vivo*.

The most widely studied bacterial carriers are BCG (*Bacillus Calmette-*

Guerin) and attenuated *Salmonella* strains. BCG has been used as an attenuated vaccine against *Mycobacterium tuberculosis* infections for many years. It is an avirulent derivative of *M.bovis* and is the most widely used live human vaccine. BCG has been administered to about a billion people within the first months of life with remarkable few side effects. Its adjuvant qualities have been well documented. Killed BCG included in Freund's adjuvant stimulates cellular immunity against other included antigens. The safety of BCG and its strong adjuvant properties prompted researchers to develop it as a live carrier for heterologous antigens (Stover 1991). BCG expressing the principal neutralising domain of the envelope of HIV induced VN antibodies in mice and was able to protect against HIV infection in a hu-SCID mouse model (Honda 1995).

Of the attenuated *Salmonella* strains used as live carriers, the *aro* mutants have received most attention. The *aro* genes encode enzymes of the prechorismate pathway, which is the sole route of biosynthesis of several key aromatic metabolites. Mammals do not possess an equivalent pathway and must extract the aromatic components from their diet. The concentration of these aromatic compounds in mammals is insufficient for *Salmonella aroA*, *C* or *D* mutants, resulting in a self-limiting infection. *S. typhimurium aroA* mutants are highly attenuated in mice, yet proved to be excellent single dose vaccines against salmonellosis (Hoiseth and Stocker, 1981). *Salmonella typhimurium aroA* strains have been used successfully to present heterologous bacterial (Clements 1986, Poirier 1988), viral (Charbit 1993, Schödel 1994, Tite 1990) and protozoal (Aggarwal 1990, Sadoff 1988, Yang 1990) antigens to the immune system of mammals. Systemic humoral and cellular responses, including class I restricted cytotoxic T lymphocytes (CTL), and mucosal humoral responses have been shown to result from vaccination with recombinant *Salmonella* strains (Aggarwal 1990, Flynn 1990, Gao 1992, Turner 1993, Pfeifer 1993). The induction of local as well as systemic immune responses following oral administration most likely depends on their ability to proliferate in the GALT as well as in the liver and spleen. Induction of a mucosal immune response may act as a first line of defence against pathogens like HIV-1, of which the major port of entry are the mucosae of the urogenital and gastro-intestinal tracts. *S. typhimurium aroA* expressing the Gag protein of SIV induced specific secretory IgA responses and systemic CTL responses, but failed to induce specific serum antibodies, following oral administration (Valentine 1996).

A potential disadvantage of bacterial carriers is their inability to glycosilate proteins. Also, the conformation of viral proteins expressed by bacteria is not necessarily identical to their natural conformation in the virus. These drawbacks

may reduce the range of virus neutralising epitopes presented to the immune system.

The induction of local and systemic immune responses, the low production costs and the ease of (oral) administration make attenuated *Salmonella* strains attractive vaccine candidates. However their development as live carriers for heterologous antigens, has been hampered considerably by problems with instability of expression or poor production levels caused by the toxicity of the antigens for the producing *Salmonella* strains. A number of strategies have been developed to ensure stable expression of the heterologous antigens, like chromosomal integration of the gene encoding the heterologous antigen (Hone 1988, Strugnell 1990) or linkage of an essential gene to the expression plasmid (Nakayama 1988, Curtiss 1989). Although these approaches have proven to be useful for the stable expression of a number of antigens, they may result in over-attenuation of bacteria expressing antigens at toxic levels. Another approach is the use of promoters that are induced *in vivo*. This kind of promoters may delay antigen expression till the bacteria reaches tissues relevant for the induction of protective immunity, resulting in much better humoral responses compared to a constitutive promoter (Chatfield 1992). However, also this strategy does not allow the production toxic antigens during the complete route of infection nor will it allow further growth of the bacterial population once antigen expression reaches toxic levels. For the induction of antibodies against the B subunit of the heat labile toxin (LTB) of *E.coli* the initial amount of antigen produced appeared to be of more importance than the stability of expression (Cardénas 1993, 1994). For the induction of class I restricted CTL responses abundant as well as stable antigen expression have both been shown to be of importance (Turner 1993, Wick 1993).

Nucleic acid immunisation

Nucleic acid immunisation, also referred to as genetic or naked DNA (or RNA) immunisation, is a novel immunisation strategy that shows considerable promise for vaccine development. It involves the induction of a specific immune response to an antigen expressed *in vivo* following the introduction of its encoding polynucleotide (DNA or RNA) (for a review see Shiver 1996). Because the foreign antigen is synthesised by the host cells, it is able to induce specific MHC class I restricted CTL responses, but without the risk of inadvertent infection. The heat stability and the relative simple quality control are other advantages of nucleic acid vaccines over other types of vaccines. Nucleic acid immunisation against influenza has induced broad cross-protection with antigenically distinct

SHIV challenge has been obtained with a nucleic acid vaccine that induced neutralising antibodies and specific CTL responses (Boyer 1996). A theoretical risk with nucleic acid vaccines is tumour induction as a result of DNA integration into the genome, but so far studies have failed to demonstrate DNA integration.

Specific problems in lentivirus vaccine development

A number of lentivirus characteristics hinder the development of safe and effective vaccines against these viruses. Lentiviruses have a high mutation rate due to low fidelity of the lentivirus reverse transcriptase and the absence of proofreading activity. This results in the constant generation of mutants that escape from surveillance either by VN antibodies or specific T-cells. Following integration of the proviral DNA into the host genome lentiviruses can remain in a quiescent state, invisible for the immune system. The initial site of infection is usually at the mucosae of the genital tracts or the intestine where the virus can replicate in the dendritic cells. To limit viral replication in this initial stage of infection the induction of an effective mucosal immune response will be necessary. Unfortunately most vaccine strategies pursued currently do not induce mucosal immune responses. Another problem in the immunologic control of lentivirus infection is that they destroy or interfere with the functioning of T-helper cells which have a key role in the immune system. This may not be a problem for vaccines inducing sterilising immunity, but it may pose a major obstacle for the development of vaccines that merely limit viral replication.

Outline of this thesis

As described above, attenuated recombinant Salmonella strains have potential as vaccine candidates against lentivirus, but their development has been seriously hindered by problems with instability or poor expression levels of toxic heterologous antigens. Therefore, a major aim of this thesis was to develop a generally applicable expression system for the abundant and stable production of heterologous antigens in *Salmonella* vaccine strains. This expression system should allow constant production of heterologous antigen, even if their levels are toxic for the producing bacteria. The construction of such an expression system and its evaluation using heterologous bacterial, viral and parasitic antigens, are described. Furthermore, it is used to generate a FIV-Gag producing *Salmonella* strain, of which the immunogenicity is evaluated in cats. In a more extended study the immunogenicity and effectiveness of an experimental vaccine based on FIV-Env and Gag producing *Salmonella* strains is tested in the FIV/cat model. To compensate for the lack of glycosilation of bacterially produced proteins, this

vaccine was also tested in combination with an iscom preparation containing glycosilated FIV envelope protein. Finally, the potential of iscom preparations containing glycosilated envelope protein and a bacterially produced b-galactosidase-Env fusion protein mixed with Quil A as an adjuvant, are evaluated as potential FIV vaccine candidates. The results of the studies described in this thesis are summarised and discussed in the last chapter.

REFERENCES

Abreumartin M.T., Targan S.R. (1996). Regulation of immune-responses of the intestinal-mucosa. [Review] *Critical Reviews in Immunology* **16**, 277-309.

Ackley, C.D., Hoover E.A., and Cooper M.D. (1990). Identification of a CD4 homologue in the cat. *Tissue Antigens*. **35**, 92-98

Aggarwal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M. and Sadoff, J. (1990). Oral *Salmonella*: Malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. *J. Exp. Med.*, **172**,1083-1090.

Aldrovandi G.M. and Zack J.A. (1996). Replication and pathogenicity of HIV-1 accessory gene mutants in SCID-hu mice. *J. Virol.* **70**, 1505-1511.

Alkhatib G., Broder C.C. And Berger E.A. (1996). Cell-type-specific fusion cofactors determine human immunodeficiency virus type-1 tropism for T-cell lines versus primary macrophages. *J. Virol.* **70**, 5487-5494.

Alkhatib G., Combadiere C., Broder C.C., Feng Y., Kennedy P.E., Murphy P.M. and Berger E.A. (1996). CC-CKR-5 - a RANTES, MIP-1-alpha, MIP-1-Beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**,1955-1958.

Alter H.J., Eichberg J.W., Masur H., Saxinger W.C., Gallo R., Macher A.M., Lane H.C., Fauci A.S. (1984). Transmission of HTLV-III infection from human-plasma to chimpanzees - an animal-model for AIDS. *Science*, **226**, 549-552

Andrew M.E., Karupiah G., Boyle D.B., Blanden R.V. Mullbacher A., Ramshaw I.A. and Couper B.E.H. (1991). Effects of vaccinia virus-expressed IL-2 on the immune system of sublethally irradiated mice. *Micro. Pathol.* **10**, 363-371.

Arthur L.O., Bess J.W., Waters D.J., Pyle S.W., Kelliher J.C., Nara P.L., Krohn K., Robey W.G., Langlois A.J., Gallo R.C., Fischinger P.J. (1989). Challenge of chimpanzees (*Pan troglodytes*) immunized with human immunodeficiency virus envelope glycoprotein-gp120. *J. Virol.* **63**, 5046-5053.

Arya S.K. Guo C., Josephs S.F., Wongstaal F. (1985) Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science* **229**, 69-73.

Baba, T.W., Jeong, Y.S., Penninck, D., Bronson, R., Greene, M.F., Ruprecht, R.M. 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* **267**, 1820-1825.

Barlough J.E., Ackley C.D., George J.W., Levy N., Acevedo, R., Moore P.F., Rideout B.A., Cooper M.D., and Pederson N.C. 1991. Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections. *J. Acquir. Immune. Defic. Syndr.* **4**, 219-227.

Bergmann K.C. and Waldman R.H. (1988). Stimulation of secretory antibody following oral administration of antigen. *Reviews of Infectious Diseases* **10**, 939-950.

Binley J. and Moore J.P. (1997). The viral mouse trap. *Nature* **387**, 446-448.

Boon, W.H., Liano, D., and Abbas, A.K. (1988). Heterogeneity of helper/inducer T lymphocytes. II. Effects of interleukin-4 and interleukin-2 producing T cell clones on resting B lymphocytes *J. Exp. Med.* **167**,1352-1363.

Boyer, J.D., Wang, B., Ugen, K.E., Agadjanyan, M., Javadian, A., Frost, P., Dang, K.S., Carrano, R.A., Ciccarelli, R., Coney, L., Williams, W.V., Weiner, D.B. (1996). In-vivo protective anti-HIV immune-responses in nonhuman-primates through DNA immunization. *Journal of Medical Primatology* **25**, 242-250.

Brandtzaeg P., Bosnes V., Halstensen T.S., Scott H., Sollid L.M. And Valnes K.N. (1989). T lymphocytes in human gut epithelium preferentially express the a/b antigen receptor and are often CD45/UCH1-positive. *Scand.J.Immunol.* **30**, 123-128.

Brown, M.G., Driscoll, J., and Monaco, J.J. (1991). Structural and serological similarity of MHC-linked LMP and proteosome (multicatalytic proteinase) complexes (1991). *Nature* **353**, 355-357

Burke K.L., Dunn G., Ferguson M., Minor P.D., and Almond J.W. (1988). Antigen chimaeras of poliovirus as potential new vaccines. *Nature* **332**, 81-82.

Cardénas L. and Clements J.D. (1993). Stability, immunogenicity and expression of foreign antigens in bacterial vaccine vectors. *Vaccine* **11**, 126-135.

Cardénas L., Dasgupta U. and Clements J.D. (1994). Influence of strain viability and antigen dose on the use of attenuated mutants of *Salmonella* as vaccine carriers. *Vaccine* **12**, 833-840.

Charbit A., Martineau, P., Ronco, J., Leclerc, C., Lo-Man, R., Michel, V., O'Callaghan, D. and Hofnung, M. (1993). Expression and immunogenicity of the V3 loop from the envelope of human immunodeficiency virus type 1 in an attenuated *aroA* strain of *Salmonella typhimurium* upon genetic coupling to two Escherichia coli carrier proteins. *Vaccine* , **11**,1221-1228

Chatfield S.N., Charles I.G., Makoff A.J., Oxer M.D., Dougan G., Pickard D., Slater D. and Fairweather N.F.. (1992). Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. *Bio/Technology* **10**, 888-892.

Cher, D.J., and Mosman, T.R. (1987). Two types of murine helper cell clones: Delayed-type hypersensitivity is mediated by Th1 clones. *J. Immunol.* **138**, 3688-3694.

Chirmule, N. And Pahwa, S. (1996). Envelope glycoproteins of human-immunodeficiency-virus type-1 - profound influences on immune functions. *Microbiol. Reviews* **60**, 386

Choe H., Farzan M., Sun Y., Sullivan N., Rollins B., Ponath P.D., Wu L.J., Mackay C.R., Larosa G., Newman W., Gerard N., Gerard C., Sodroski J. (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135-1148.

Clarke, B.E., Newton, S.E., Carroll, A.R., Francis, M.J., Appleyard, G., Syred, A.D. and Highfield, P.E. (1987) Impaired immunogenicity of a peptide epitope after fusion to Hepatitis B core protein. *Nature* **330**, 381-384.

Clements, J.D., Lyon, F.L., Lowe, K.L., Farrand, A.L., and El-Morshidy, S. (1986). Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. *Infect. Immun.* , **53**, 685-692.

Cocchi F., Devico A.L., Garzinodemo A., Arya S.K., Gallo R.C. and Lusso P. (1995). Identification of RANTES, MIP-1-alpha, and MIP-1-beta as the major HIV-suppressive factors produced by CD8(+) T-cells. *Science* **270**,

1811-1815.

Cohen, E.A., Dehni G., Sodroski J.G., and Haseltine W.A. (1990). Human immunodeficiency virus *vpr* product is a virion associated regulatory protein. *J. Virol.* **64**, 3097-3099.

Coffman, R.L., Seymour, B.W., Lebman. D.A., Hiraki, D.D., Christiansen, J.A., Shrader, B., Cherwinski, H.M., Savelkoul, H.F.J., Finkelman, F.D., Bond, M.W., Mosmann, T.R. (1988). The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* **102**, 5-28.

Curtiss III, R., Nakayama K., and Kelly S.M. (1989). Recombinant avirulent *Salmonella* vaccine strain with stable maintenance and high level expression of cloned genes *in vivo*. *Immunol. Invest.* **18**, 583-596.

Dahlgren U., Carlsson B., Jalil F., Macdonald R., Mascartlemone F., Nilsson K., Roberton D., Sennhauser F., Wold A., Hanson L.A. (1989). Induction of the mucosal immune-response. [Review] *Current Topics in Microbiology and Immunology* **146**, 155-160.

Daniel, M.D., Kirchhoff, F., Czafak, S.C., Sehgal, P.K. & Desrosiers, R.C. (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. *Science* **258**, 1938-1941.

Debouck, C., Gomiak J.G., Strickler J.E., Meek T.D., Metcalf B.W., Rosenberg M. (1987). Human immunodeficiency virus protease expressed in Escherichia coli exhibits autoprocessing and specific maturation of the gag precursor. *PNAS* **84**, 8903-8906

Dedieu J.F., Ronca J., Vanderwerf S., Hogle J.M., Henin Y. and Girard M. (1992). Poliovirus chimaeras expressing sequences from the principle neutralising domain of HIV type 1 virus. *J. Virol.* **66**, 3161-3167.

Del Prete, G.F., De Carli, M., Mastromauro, C., Biagiotti, R., Macchia, D., Falagiani, P Ricci, M., and Romagnani, S. (1991). Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand *in vitro* human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J. Clin. Invest.* **88**, 346-350.

Del Prete, G.F., Maggi, E. and Romagnani, S. (1994). Biology of disease: Human Th1 and Th2 cells: Functional properties, mechanisms of regulation, and role in disease. *Lab. Invest.* **70**, 299-306.

De Vries, P., Heeney, J.L., Boes, J., Dings, M.E.M., Hulskotte, E.G.J., Dubbes, R., Koornstra, W., Ten Haaft, P., Akkerblom, L., Eriksson, S., Morein, B., Norley, S. and Osterhaus, A.D.M.E. (1994) Protection of rhesus macaques from SIV infection by immunisation with different experimental SIV vaccines. *Vaccine* **12**, 1443-1452.

Deng H.K., Liu R., Ellmeier W., Choe S., Unutmaz D., Burkhart M., Dimarzio P., Marmon S., Sutton R.E., Hill C.M., Davis C.B., Peiper S.C., Schall T.J., Littman D.R., Landau N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661-666.

Dittmer, U., T. Niblein, W. Bodemer, H. Petry, U. Sauermann, C. Stahl-Hennig, and G. Hunsmann. 1995. Cellular immune responses of rhesus monkeys infected with a partially attenuated nef deletion mutant of the Simian immunodeficiency virus. *Virology* **212**, 392-397.

Doranz B.J., Rucker J., Yi Y.J., Smyth R.J., Samson M., Peiper S.C., Parmentier M., Collman R.G. and Doms R.W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149-1158.

Dow, S.W., Poss M.L., and Hoover E.A. (1990). Feline immunodeficiency virus: a neurotropic lentivirus. *J. Acquir. Immune. Defic. Syndr.* **3**, 658-668.

Dragic T., Litwin V., Allaway G.P., Martin S.R., Huang Y.X., Nagashima K.A., Cayanan C., Maddon P.J., Koup R.A., Moore J.P. and Paxton W.A. (1996). HIV-1 entry into CD4(+) cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667-673.

Emerman M., Vazeux R., and Peden K. (1989). The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell* **57**, 1155-1165.

Embretson J., Zupancic M., Ribas J.L., Burke A., Racz P., Tenner-Racz K., and Haase, A.T. (1993). Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* **362**, 359-362.

Evans D.J., McKeating J., Meredith J.M., Burke K.L. Katrak K., John A., Ferguson M., and Minor P. (1989). An engineered poliovirus chimaera elicits broadly reactive HIV-1 neutralising antibodies. *Nature* **339**, 385-388.

Farmerie, W.G., Loeb D.D., Casavant N.C., Hutchison C.A., Edgell M.H. and Swanstrom R. (1987). Expression and processing of the AIDS virus

reverse transcriptase in Escherichia coli. *Science* **236**, 305-308

Farnet C., and Haseltine W. (1990). Integration of immunodeficiency virus type 1 DNA *in vitro*. *PNAS USA* **87**, 4164-4168.

Fauci, A.S. 1993. Mutifactorial nature of human immunodeficiency virus disease: implications for therapy. *Science* **262**,1011-1018.

Fauci, A.S. 1996. Host factors and the pathogenesis of HIV-induced disease. *Nature* **384**, 529-534.

Feiberg, M. B., R.F. Jarrett, A. Aldovini, R.C. Gallo and F. Wong-Staal. 1986, *Cell* **46**, 807-817.

Felber, B.K. Hadzopoulou-Cladaras M., Cladaras C., Copeland T., and Pavlakis G.N. (1989). Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *PNAS USA* **86**, 1495-1499.

Feng Y, Broder C.C., Kennedy P.E. and Berger E.A. (1996). HIV-1 entry cofactor - functional cDNA cloning of a 7-transmembrane, G-protein-coupled receptor. *Science* **272**, 872-877.

Flynn, J.L., Weiss, W.R., Norris, K.A., Seifert, H.S., Kumar, S. and So, M. (1990). Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Mol. Microbiol.* **4**, 2111-2118.

Francis M.J., Hastings G.Z., Brown A.L., Grace K.G., Rowlands D.J., Brown F. And Clarke B.E. (1990). Immunological properties of hepatitis B core antigen fusion proteins. *PNAS USA* **87**, 2545-2549.

Fultz, P.N., McClure H.M., Anderson D.C., Swenson R.B., Anand R., and Srivasan, A. (1986). Isolation of a T-lymphotropic retrovirus from naturally infected soothy mangabey monkeys (*Cercocebus atys*). *PNAS* USA **83**, 5286-5290.

Fultz P.N., McClure H.M., Swenson R.B., McGrath C.R., Brodie A., Getchell J.P., Jensen F.C., Anderson D.C., Broderson J.R. and Francis D.P. (1986). Persistent infection of chimpanzees with human T-lymphotropic virus Type-III lymphadenopathy-associated virus - a potential model for acquired immunodeficiency syndrome. *J. Virol.* **58**, 116-124.

Gao, X.M., Tite, J.P., Lipscombe, M., Rowland-Jones, S., Ferguson, D.J.P. and McMichael, A.J. (1992). Recombinant *Salmonella typhimurium* strains that invade nonphagocytic cells are resistant to recognition by antigen-

specific cytotoxic T lymphocytes. *Infect. Immun.* **60**, 3780-3789

Gay, D., Maddon, P, Sekaly, R., Talle, M.A., Godfrey, M., Long, E., Goldstein, G., Chess, L., and Axel A. (1987). Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature* **328**, 626-629.

Gelderblom, H.R., M. Ozel, G. Pauli. 1989. Morphogenesis and morphology of HIV. Structure-function relations. *Arch. Virol.* **106**, 1

Gibbs, J.S., and R.C. Desrosiers. 1994. Auxiliary proteins of the primate immunodeficiency viruses, p. 137-158. *In* B.R. Cullen (ed.), Frontiers in molecular biology: human retroviruses. Oxford University Press. New York.

Gibbs, J.S., A.A. Lackner, S.M. Lang, M.A. Simon, P.K. Sehgal, M.D. Daniel and R.C. Desrosiers. 1995. Progression to AIDS in the absence of a gene for *vpr* or *vpx*.. *J. Virol.* **69**, 2378-2383.

Giavedoni, L.P., Planelles, V., Haigwood, N.L., Ahmed, S., Kluge, J.D., Martha, M.L., Gardnenr, M.B., Luciw, P.A. and Yilma, T.D. (1993). Immune response of rhesus macaques to recombinant simian immunodeficiency virus gp130 does not protect from challenge infection. *J.Virol.* **67**, 577-583.

Goldberg, A.L., and Rock, K.L. (1992). Proteolysis, proteosomes and antigen presentation. *Nature* **357**, 375-379.

Gotch F., Gallimore A. and McMichael, A. (1996). Cytotoxic T-cells - protection from disease progression - protection from infection. [Review] *Immunology Letters* **51**, 125-128.

Gottlieb M.S., Schroff R., Schanker H.M. Weisman, J.D., Fan P.T., Wolf R.A. amd Saxon A. (1981). Pneumocistis carnii pneumonia and mucosal candidis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N. Eng. J. Med.* **305**, 1425-1431.

Goudsmit J., Debouck C., Meloen R.H., Smit L., Bakker M., Asher D.M., Wolff A.V., Gibbs C.J. and Gajdusek D.C. (1988). Human immunodeficiency virus type-1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees, *PNAS* **85**, 4478-4482.

Harding, C.V., Collins, D.S., Slot, J.W., Geuze, H.J., and Unanue, E.R. (1991). Liposome-encapsulated antigens are processed in lysosomes, recycled and presented to T-cells. *Cell* **64**, 393-401.

Hauber J., Perkins A., Heimer E.P. and Cullen B.R. (1987) Trans-activation of human immunodeficiency virus gene expression is mediated by nuclear events. *PNAS USA* **84**, 6364-6368

Heeg, K., Kuon, W., and Wagner, H. (1991) Vaccination of class I major histocompatibility complex (MHC) restricted murine CD8+ T lymphocytes towards soluble antigens: Immune stimulating ovalbumin complexes enter the class I MHC antigen pathway and allow sensitisation against the immunodominant peptide. *Eur. J. Immunol.* **21**, 1523-1527.

Heeney, J.L., Van Els, C., De Vries, P., Ten Haaft, P., Otting, N., Koornstra, W., Boes J., Dubbes R., Niphuis H., Dings M., Cranage M., Norley S., Jonker M., Bontrop R.E. and Osterhaus A.D.M.E. (1994). Major histocompatibility complex class I associated vaccine protection from Simian immunodeficiency virus-infected peripheral blood cells. *J. Exp. Med.* **180**, 769-774.

Hoffmann-Frezer G., Thum G.J., Ackley C., Herbold M., Mysliwietz J., Thefeld S., Hartmann K., and Kraft W. (1992). Decline in CD4+ cell numbers in cats with naturally acquired feline immunodeficiency virus infection. *J. Virol.* **66**, 1484-1488.

Hoiseth, S.K. and Stocker, B.A.D. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238-239.

Honda, M., Matsuo, K., Nakasone, T., Okamoto, Y., Yoshizaki, H., Kitamura, K., Sugiura, W., Watanabe, K., Fukushima, Y., Haga, S., Katsura, Y, Tasaka, H., Komuro, K., Yamada, T., Asano, T., Yamazaki, A., and Yamazaki, S. (1995) Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin vector candidate vaccine for human immunodeficiency virus type 1 in small animals. *Proc. Natl. Acad. Sci. USA* **92**, 10693-10697.

Hone, D., Attridge S., Van den Bosch L., and Hackett J. (1988). A chromosomal integration system for stabilization of heterologous genes in *Salmonella* based vaccine strains. *Micr. Path.* **5**, 407-418.

Hopper C.D., Sparkes A.H., Gruffydd-Jones T.J., Crispin S.M., Muir P., Harbour D.A., and Stokes C.R. (1989). Clinical and laboratory findings in cats infected with feline immunodeficiency virus. *Vet. Rec.* **125**, 341-346.

Hu, S.-L, K. Abrams, G.N. Barber, P. Moran, J.M. Zarlin, A.J.

Langlois L. Kuller, W.R. Morton and R.E. Benveniste (1992). Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. *Science* **255**, 456-459.

Hulskotte, E.G.J., Geretti, A.M., Siebelink, K.H.J., van Amerongen, G., Cranage, M.P., Rud, E.W., Norley, S.G., de Vries, P. & Osterhaus, A.D.M.E. (1995). Vaccine-induced virus neutralizing antibodies and cytotoxic T cells do not protect macaques from experimental infection with simian immunodeficiency virus SIVmac32H (J5). *Jounal of Virology* **69**, 6289-6296.

Hurtrel M., Ganiere J.P., Guelfi, J.F., Chakrabarti L., Maire M.A., Gray F., Montagnier L., and Hurtrel B. (1992). Comparison of early and late feline immunodeficiency virus encephalopathies. *AIDS* **6**, 399-406.

Hussain L.A. and Lehner T. (1995). Comparative investigation of Langerhans cells and potential receptors for HIV in oral, genitourinary and rectal epithelia. *Immunology* **85**, 475-484.

Ishida T. and Tomoda I. (1990). Clinical staging of feline immunodeficiency virus infection. *Nippon. Juigaku. Zasshi.* **52**, 645-648.

Jacks T., Power M.D., Masiarz F.R., Luciw P.A., Barr P.J. and Varmus H.E. (1988). Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature* **331**, 280-283

Jarquemada, D., Marti, M., and Long, E.O. (1991). An endogenous processing pathway in vaccinia virus-infected cells for presentation of cytoplasmic antigens to class II- restricted T cells. *J. Exp. Med.* **172**, 947-954.

Johnson D., Ghosh-Choudhury, G., Smiley J., Fallis L., and Graham F. (1988). Abundant expression of herpes-simplex virus glycoprotein gB using and adenovirus vector. *Virology* **164**, 1-14.

Kao S.Y., Calman A.F., Luciw P.A. and Peterlin B.M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* **330**, 489-493

Kiyono H., Miller C.J., Lu Y.C., Lehner T., Cranage M., Huang Y.T., Kawabata S., Marthas M., Roberts B., Nedrud J.G., Lamm M.E., Bergmeier L., Brookes R., Tao L., McGhee J.R. (1995). The common mucosal immune-system for the reproductive-tract - Basic principles applied toward an aids vaccine. *Advanced Drug Delivery Reviews*, **18**, 23-52.

Knapp, W., Rieber, P., Dorken, B., Schmidt, R.E. , Stein, H. and

Borne A.E.G. (1989). Towards a better definition of human leukocyte surface molecules. *Immunol. Today* **10**, 253-258.

Kramer R.A., Schaber M.D., Skalka A.M., Ganguly K., Wongstaal F. and Reddy E.P. (1986). HTLV-III Gag protein is processed in yeast cells by the virus pol-protease. *Science* **231**, 1580-1584.

Layton, G.T., Harris, S.J., Gearing,A.J.H., Hill-Perkins, M., Cole, J.S., Griffiths, J.C., Burns,N.R., Kingsman, A.J. and Adams, S.E. (1993) Induction of HIV-specific cytotoxic T lymphocytes in vivo with hybrid HIV-1 V3:Ty-virus-like particles. *J. Immunol.* **151**, 1097-1107.

Lenburg M.E. and Landau N.R. (1993). Vpu-induced degradation of CD4: requirement for specific amino acid residues in the cytoplasmic domain of CD4. *J. Virol.* **67**, 7238-7245.

Letvin N.L., Daniel M.D., Sehgal P.K., Desrosiers R.C., Hunt R.D., Waldron L.M., Mackey J.J., Schmidt D.K., Chalifoux L.V. and King N.W. (1985). Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science* **230**, 71-73.

Lever A., Gottlinger H., Haseltine W.A. and Sodroski J. (1989). Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J. Virol.* **63**, 4085-4087.

Levy, J.A. (1993). Pathogenesis of human immunodeficiency virus infection [Review]. *Microbiol. Rev.* **57**,183-289.

Li, J., Lord. C.I. Haseltine W., Letvin N.L., and Sodroski J. (1992). Infection of cynomolgus macaques with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoprotein. *J. AIDS* **5**, 639-646.

Lifson J.D., Feinberg M.R., Reyes, G.R. Rabin L., Basiripour, B. Chakrabarti S., Moss B., Wong-Staal F., Steimer K.S. and Engelman E.G. (1986). Induction of CD4-dependent cell fusion by HTLV-III/LAV envelope protein. *Nature* **323**, 725-728.

Lusso P., Markham P.D., Ranki A., Earl P., Moss B., Dorner F., Gallo R.C. and Krohn K.J.E. (1988). Cell-mediated immune-response toward viral envelope and core antigens in gibbon apes (*Hylobates lar*) chronically infected with human immunodeficiency virus-1. *J. Immunol.* **141**, 2467-2473.

Malim M.H., Hauber J., Le S.-Y., Maizel J.V. and Cullen B.R. (1989). The HIV-1 Rev trans-activator acts through a structured target sequence to

activate nuclear export of unspliced viral messenger-RNA. *Nature* **338**, 254-257.

Marie C., and Spahr P.I. (1986). Rouse sarcoma virus nucleic acid- binding protein p12 is necessary for viral 70S RNA di-mer formation and packaging. *J. Virol.* **60**, 450-459.

Martinez, C.K., and Monaco, J.J. (1991). Homology of proteasome subunits to a major histocompatibility complex-linked LMP gene. *Nature* **353**, 664-667.

McGhee J.R., Mestecky J., Dertzbaugh M.T., Eldridge J.H., Hirasawa M., and Kiyono H. (1992). The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10**, 75-88.

McCune J.M., Namikawa R, Kaneshima H., Shultz L.D., Lieberman M., and Weissman. I.L. (1988). The SCID-hu mouse: A model for the analysis of human hematolymphoid differentiation and function. *Science* **241**, 1632-1639.

Mellor, J., Malim, M.H., Gull,K., Tuite, , McCready, S., Dibbayawan, T., Kingsman, S.M. and Kingsman, A.J. (1985) Reverse-transcriptase activity and TY RNA are associated with virus-like-particles in yeast. *Nature* **318**, 583-586.

Meyaard L., Schuitemaker, H. and Miedema F. (1993). T-cell dysfunction in HIV infection: anergy due to defective antigen-presenting cell function? *Immunol. Today* **14**, 161-164.

Michetti P., Mahan M.J., Slauch J.M., Mekalanos J.J., Neutra M.R. (1992). Monoclonal secretory immunoglobulin-A protects mice against oral challenge with the invasive pathogen *Salmonella typhimurium*. *Infection and Immunity* **60**,1786-1792.

Montgomery D.L., Shiver, J.W., Leander K.R., Perry H.C., Friedman A., Martinez D., Ulmer J.B., Donnelly J.J., Liu M.A. (1993). Heterologous and homologous protection against influenza-A by DNA vaccination - Optimization of DNA vectors. *DNA and Cell Biology* **12**, 777-783.

Morein, B., Sundquist, B., Höglund S., K. Dalsgaard, K. and Osterhaus, A.D.M.E. (1984). Iscom, a novel structure for antigenic presentation of membrane proteins of enveloped viruses. *Nature* **308**, 457-459.

Morein, B., Ekström,J. and Lövgren, K. (1990). Increased immunogenicity of a non-amphipathic protein (BSA) after inclusion into iscoms.

J. Immunol. Methods **128**, 177-181.

Morrow, C.D., Park, J., Wakefield, J.K. (1994). Viral gene-products and replication of the human immunodeficiency type-1 virus [Review] *Amer. J. Physiol.* **266**, C1135-C1156.

Mosier, D.E., Gulizia R.J., Spector S.A. et al. (1989) *In* Retroviruses of Human AIDS and Related Animal Diseases, p. 173, Pasteur Vaccines

Muster, T., Ferko, B., Klima, A., Purtscher, M., Trkola, A., Schulz, P., Grassauer, A., Engelhardt, O.G., García-Sástre, A., Palese, P. and Katinger, H. (1995). Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. *J. Virol.* , **69**, 6678-6686

Nakayama, K., Kelly S.M. and Curtiss R. (1988). Construction of an *asd*$^+$ expression-cloning vector: Stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**, 693-697.

Norment, A.M., Salter, R.D., Parham, P., Engelhard, V.H. and Littman, D.R. (1988). Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336**, 79-81.

Oroszlan S., and Luftig R.B. (1990). Retroviral proteinases. *In* Current Topics in Immunology, edited by Campans R.W., Cooper M., Koprowski H., Meichers F., Nussenz-Vogt P.K., Wagner H. and Wilson I. Spring-Verlag, Berlin.

Osterhaus, A.D.M.E., De Vries, P., and Heeney, J. 1992. AIDS vaccine developments [letter; comment]. *Nature* **355**, 684-685.

Oyaizu, N. and Pahwa, S. (1995). Role of apoptosis in HIV disease pathogenesis. [Review] *J.Clin. Immun.* **15**, 217-231.

Pantaleo G., Graziosi C., Demarest J.F., Butini L., Montroni M., Fox C.H., Orenstein J.M., Kotler D.P. and Fauci A.S. (1993). HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **263**, 355-358.

Parronchi, P. Macchia, D. Piccinni M.P., Biswas P Simonelli. C., Maggi, E., Ricci, M., Ansari, A.A., and Romagnani, S. (1991). Allergen- and bacterial antigen-specific T cell clones established from atopic donors show a different profile of cytokine production. *Proc. Natl. Acad. Sci. USA* **88**, 4538-4542.

Pedersen N.C., Ho E.W., Brown M.L., and Yamamoto J.K. (1987). Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* **235**, 790-793.

Pedersen N.C., Yamamoto J.K., Ishida, T., and Hansen H. (1989). Feline immunodeficiency virus infection. [Review]. *Vet. Immunol. Immunopathol.* **21**, 111-129.

Peng C., Ho B.K., Chang T.W. and Chang N.T. (1989). Role of the human immunodeficiency virus type 1 specific protease in core protein maturation and viral infectivity. *J. Virol.* **63**, 2550-2556.

Peters , P.J., Neefjes, J.J., Oorschot, V., Ploegh, H.L., and Geuze, H.J., (1991). Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to the lysosomal compartments. *Nature* **349**, 669-676.

Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J. and Harding, C.V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361**, 359-362

Poirier, T.P., Kehoe, M.A. and Beachey, E.H. (1988). Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. *J. Exp. Med.***168,** 25-32.

Radaelli, A., Gimelli M., Cremonesi C., Scarpini C., De Giuli Morghen C. (1994). Humoral and cell-mediated immunity in rabbits immunised with live non-replicating avipox recombinants expressing the HIV-1$_{SF2}$ *env* gene. *Vaccine* **12**, 1110-1116.

Raja. N.U., Vincent M.J. and Abdul-Jabbar M. (1994). Vpu mediated proteolysis of gp160/CD4 chimeric envelope glycoproteins in the endoplasmatic reticulum: requirement of both anchor and cytoplasmic domains of CD4. *Virology* **204**, 357-366.

Ratner, L., Haseltine W., Patarca R. Livak K.J., Starcich B., Josephs S.F., Doran E.R., Rafalski J.A., Whitehorn E.A., Baumeister K., Ivanoff L., Petteway S.R., Pearson M.L. Lautenberger J.A., Papas T.S., Ghrayeb J., Chang N.T., Gallo R.C. and Wongstaal F. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III, *Nature* **313**, 277-284.

Reid, G. (1992). Soluble proteins incorporated into iscoms after covalent attachment of fatty acids. *Vaccine* **10**, 597-602.

Rimmelzwaan, G.F. and Osterhaus A.D.M.E. (1995). A novel generation of viral vaccines based on the ISCOM matrix. *In* Vaccine design: the subunit and adjuvant approach (1995). M.F. Powel and M.J. Newman (ed.), New York, Plenum Press.

Rock, K.L., 1996. A new foreign policy: MHC classI molecules monitor the outside world. *Immunology Today* **17**, 131-137

Ruben S., Perkins A., Purcell R., Joung K., Sia R., Burghoff R., Haseltine W.A. and Rosen C.A. (1989). Structural and functional characterization of human immunodeficiency virus Tat protein. *J. Virol.* **63**, 1-8.

Sadaie M.R., Rappaport J., Benter T., Josephs S.F., Willis R. and Wongstaal F. (1988). Missense mutations in an infectious human immunodeficiency viral genome: Functional mapping of *tat* and the identification of the *rev* splice acceptor. *PNAS* **85**, 9224-9228.

Sadoff, J.C., Ballou, W.R., Baron, L.S., Majarian, W.R., Brey, R.N., Hockmeyer, W.T., Young, J.F., Cryz, S.J., Ou, J., Lowell, G.H. and Chulay, J.D. (1988). Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science* **240**, 336-338.

Sakuragi S., Shibata R., Mukai R., Komatsu T., Fukasawa M., Sakai H., Sakuragi J.-I., Kawamura M., Ibuki K., Hayami M., and Adachi A. (1992). Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus. *J. Gen. Virol.* **73**, 2983-2987.

Sanchez-Pescador R., Power M.D., Barr P.J., Steimer K.S., Stempien M.M., Brown-Shimer S.L., Gee W.W., Renard A., Randolph A., Levy J.A., Dina D. and Luciw P.A. (1985). Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV). *Science* **227**,484-492.

Sattentau, Q.J. (1996).The role of the envelope glycoproteins in HIV-1 transmission and pathogenesis. [Review] *Persp. Drug Disc. Design* **5**, 1-16.

Schlienger, K., Mancini, M., Riviere, Y., Dormont, D., Tiollais, P., Michel, M.L. (1992) Human immunodeficiency virus type 1 major neutralising determinant exposed on hepatitis B surface antigen particles is highly immunogenic in primates. *J. Virol.* **66**, 2570-2576.

Schödel, F., Kelly, S.M., Darrell, L., Milich, D.R. and Curtiss III, R. (1994). Hybrid hepatitis B virus core-pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. *Infect. Immun.* **62**,1669-1676

Schultz A.M., and Hu S.-I. (1993). Primate models for HIV vaccines. *AIDS* **7**, (suppl 1), S161-S170.

Shibata R., Kawamura M., Sakai H., Hayami M., Ishimoto A., and Adachi A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* **65**, 3514-3520.

Shiver J.W., Ulmer J.B., Donnelly J.J., Liu M.A. (1996). Humoral and cellular immunities elicited by DNA vaccines - application to the human-immunodeficiency-virus and influenza. *Advanced Drug Delivery Reviews* **21**, 19-31

Snapper, C. M. and Mond, J.J. (1993). Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today.* **14**, 15-17.

Sodroski J., Patarca R., Rosen C., Wongstaal F. and Haseltine W. (1985). Location of the trans-activating region on the genome of human T-cell lymphotropic virus type III. *Science* **229**, 74-77.

Sparger E.E., Luciw P.A., Elder J.H., Yamamoto J.K., Lowenstine L.J., and Pedersen N.C. (1989). Feline immunodeficiency virus is a lentivirus associated with an AIDS-like disease in cats. [Review]. *AIDS* **3** Suppl 1, S43-49.

Sutjipto S., Pedersen N.C., Miller C.J., Gardner M.B., Hanson C.V., Gettie A., Jennings M., Higgins J., and Marx, P.A. (1990). Inactivated simian immunodeficiency virus vaccine failed to protect rhesus macaques from intravenous or genital mucosal infection but delayed disease in intravenously exposed animals. *J. Virol.* **64**, 2290-2297.

Stahl, S.J. and Murray, K. (1989). Immunogenicity of peptide fusions to the Hepatitis B virus core antigen. *Proc Natl. Acad. Sci. USA* **86**, 6283-6287.

Stanley S.K. and Fauci A.S. (1993). T-cell homeostasis in HIV infection-part of the solution, part of the problem, *J. Acquir. Immun Defic. Syndr.* **6**, 142-143.

Stein B.S., Gowda S.F., Lifson J.D., Penhallow R.C., Bensch K.G. and Engelman E.G. (1987). pH-independent HIV entry into CD4- positive T cells via virus envelope fusion to the plasma membrane. *Cell* **49**, 659-668.

Stott, E.J. (1991). Anti-cell antibody in macaques. *Nature* **353**, 393.

Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal , G.P., Young, J.F., Lee, M.H., Hatfull, G.F., Snapper, S.B., Barletta, R.G., Jacobs, W.R., Jr, and Bloom, B.R. (1991). New use of BCG for recombinant vaccines. *Nature* **351**, 456-460.

Strugnell, R.A., Maskell D., Fairweather N., Pickard D., Cockayne A., Penn C., and Dougan G. (1990). Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium* vaccine strains. *Gene* **88**, 57-63.

Tite, J.P., Gao, X.M., Hughes-Jenkins, C.M., Lipscombe, M., O'Callaghan, D. and Dougan, G. (1990). Antiviral immunity induced by recombinant nucleoprotein of influenza-A virus. III. Delivery of recombinant nucleoprotein to the immune system using attenuated *Salmonella typhimurium* as a live carrier. *Immunol.* **70**, 540-546

Torres B.A., Tanabe T and Johnson H.M. (1996). Characterization of Nef-induced CD4 T-cell proliferation. *Biochemical and Biophysical Research Communications* **225**, 54-61.

Torres, B.A., Tanabe T., Yamamoto J.K. and Johnson H.M. (1996). HIV encodes for its own CD4 T-cell superantigen mitogen. *Biochemical and Biophysical Research Communications* **225**, 672-678.

Torten M., Franchini M., Barlough J.E., George J.W., Mozes E., Lutz H., and Pedersen N.C. (1991). Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus. *J. Virol.* **65**, 2225-2230.

Townsend, A., Bastin, J. Bodmer,H, Brownlee, G., Davey, J., Gothch, F., Gould, K., Jones.,and McMichael. A.J. (1989). Recognition of influenza virus proteins by cytotoxic T lymphocytes. *Phil. Trans. R. Soc. Lond.* **323**, 527-533.

Tristen M., C. Marshall, A. Karpas, and F. Hill. 1992. Evolution of the primate lentiviruses: evidence from *vpx* and *vpr*. *EMBO J.* **11**, 3405-3412.

Tristen M., C. Marshall, A. Karpas, J. Patrik and F. Hill. 1990. Origin of *vpx* in lentiviruses. *Nature* (London) **347**, 341-342. (Letter)

Trkola A., Dragic T., Arthos J., Binley J.M., Olson W.C., Allaway G.P. Chengmayer C., Robinson J., Maddon P.J. and Moore J.P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its coreceptor CCR-5. *Nature* **384**,184-187.

Turner, S.J., Carbone, F.R. and Strugnell, R.A. (1993). *Salmonella typhimurium delta aroA delta aroD* mutants expressing a foreign recombinant protein induce specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes in mice. *Infect. Immun.* **61**, 5374-5380

Valentine, P.J., Meyer, K., Rivera, M.M., Lipps, C., Pauza, D., Maziarz, R.T., So, M. & Heffron, F. (1996). Induction of SIV capsid-specific CTL and mucosal sIgA in mice immunized with a recombinant *S. typhimurium aroA* mutant. *Vaccine* **14**, 138-146.

Van Binnendijk, R.S., Van Baalen, C.A., Poelen, M.C.M., De Vries, P., Boes, J., Cerundolo, V., Osterhaus, A.D.M.E. and UytdeHaag, F.G.C.M. (1992). Measles virus transmembrane fusion protein synthesized *de novo* or presented in iscom is endogenously processed for HLA class I and class II restricted cytotoxic T cell recognition . *J. Exp. Med.* **176**, 119-128.

Van Noort, J.M., Boon, J., Van der Drift, A.C.M., Wagenaar, J.P.A., Boot, A.M.H., and Goog, C.J.P., (1991). Antigen processing by endosomal proteases determines which sites on sperm-whale myoglobin are eventually recognized by T cells. *Eur. J. Immunol.* **21**, 1989-1996.

Varmus H.E., and Swanstrom R. (1985). Replication of retroviruses. *In* RNA Tumor Viruses, edited by R. Weiss, N.Teich, H. Varmus and J Coffin, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, pp. 75-134.

Veronese P.D.M., Copeland T.D., Oroszlan S., Gallo R.C., and Sarngadharan M.G. (1988). Biochemical and immunological analysis of human immunodeficiency virus *gag* gene products p17 and p24. *J. Virol.* **62**, 795-801.

Vidard, L., Rock, K.L., and Benacerraf, B. (1991). The generation of immunogenic peptides can be selectively increased or decreased by proteolytic enzyme inhibitors. *J. Immunol.* **21**, 1989-1996.

Von Brunn, A., Brand, M., Reichhuber, C., Morys wortman, C., Deinhardt, F And Schodel, F. (1993). Principal neutralising domain of HIV-1 is highly immunogenic when expressed on the surface of heptatis B core particles. *Vaccine* **11**, 817-824.

Walker M.J., Rohde M., Timmis K.N., and Guzman C.A. (1992). Specific lung mucosal and systemic immune-responses after oral immunization of mice with *Salmonella-typhimurium-aroA*, *Salmonella-typhi Ty21a*, and invasive *Escherichia-coli* expressing recombinant *pertussis* toxin S1 subunit. *Infection and Immunity* **60**, 4260-4268.

Weissenhorn W., Dessen A., Harrison S.C., Skehel J.J., and Wiley D.C. (1997). Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**, 426-430.

Wick, M.J., Pfeifer J.D., Findlay K.A., Harding C.V., and Normark S.J. (1993). Compartmentalization of defined epitopes expressed in *Escherichia coli* has only a minor influence on efficiency of phagocytic processing for presentation by class I and class II major histocompatibility complex molecules to T cells. *Infect. Immun.* **161**, 4848-4856.

Yamamoto J.K., Sparger E., Ho E.W., Andersen P.R., O'Connor T.P., Mandell C.P., Lowenstine L., Munn R., and Pedersen N.C. (1988). Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. *Am. J. Vet. Res.* **49**, 1246-1258.

Yamamoto, J.K., Okuda, T., Ackley, C.D., Louie, H., Pembroke, E., Zochlinski, H., Munn, R.J. and Gardner, M.B. (1991). Experimental vaccine protection against feline immunodeficiency virus. *AIDS Res. Hum. Retrov.* **7**, 911-922.

Yamamoto, J.K., Hohdatsu, T., Olmsted, R.A., Pu, R., Louie, H., Zochlinski, H.A., Acevedo, V., Johnson, H.M., Soulds, G.A. and Gardner, M.B. (1993). Experimental vaccine protection against homologous and heterologous strains of feline immunodeficiency virus. *J. Virol.* **67**, 601-605.

Yang, D.M., Fairweather, N., Button, L.L., McMaster, W.R., Kahl, L.P. and Liew, F.Y. (1990). Oral *Salmonella typhimurium* (AroA-) vaccine expressing a major Leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against Leishmaniasis. *J. Immunol.* , **145**, 2281-2285

CHAPTER 2

Construction and evaluation of an expression vector allowing the stable expression of foreign antigens in a *Salmonella typhimurium* vaccine strain

Vaccine 12:1004-1011 (1994)

Construction and evaluation of an expression vector allowing the stable expression of foreign antigens in a *Salmonella typhimurium* vaccine strain

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Salmonella *strains have great potential as live carriers of heterologous antigens to induce immunity against a variety of infectious diseases. However, the amount of heterologous antigen required to induce an adequate immune response may be toxic for the bacterium and result in cell death, overattenuation or loss of expression of the heterologous antigen. To solve this problem an expression vector was developed with a strong promoter located on a DNA fragment which is inverted at random. Antigen is only expressed in one particular orientation of the promoter. Thus a bacterial population harbouring the plasmid will consist of a subpopulation which does not produce heterologous antigen, and is therefore not affected in growth, persistence and dissemination within the host. Further, this non-producing population will continuously segregate antigen-producing bacteria. To evaluate the system, CtxB was used as a model antigen. Analysis of the plasmid DNA isolated from* Salmonella *revealed a selection against the promoter orientation that directs transcription of the* ctxB *gene. In spite of this, the vector was stably maintained* in vivo *and induced CtxB-specific IgA and IgG in mice. These results indicate that this kind of expression vector may offer a solution to the problem of unstable expression of foreign antigens in live bacterial vaccine strains.*

Keywords: *Salmonella typhimurium*; plasmid stability; invertible promoter; CtxB

Attenuated *Salmonella* strains expressing heterologous antigens have been shown to be able to induce mucosal, humoral and cell-mediated immunity against these antigens[1]. With respect to cell-mediated immunity, both class I and II MHC-restricted cytotoxic T cells directed against heterologous antigens have been detected[2–4]. Furthermore, in animal models protective immunity has been induced with a number of *Salmonella*-recombinant strains against bacterial, viral and protozoal infections[4–6].

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Live *Salmonella* vaccine strains have been accepted for use in humans. An oral human typhoid vaccine based on *Salmonella typhi* strain Ty21a, a *galE* mutant, has been licensed in many countries after it was shown to be efficacious in field trials[7]. Other more defined attenuated *Salmonella* strains have been constructed, such as *aroA* and *cya, crp* mutants. These strains have been shown to be safe and immunogenic in a number of animal models. *Salmonella* strains attenuated by *aro* mutations have been shown to be safe even in immune-suppressed animals[1]. *S. typhi aroA, purA* and *S. typhi aroC,D* strains have undergone phase I clinical trials in humans[8,9].

Clearly, *Salmonella* strains have great potential as live carriers to induce immunity against a variety of infectious diseases. However, the amount of heterologous antigen required to obtain an adequate immune response is often toxic for the bacterium, and this forms a major obstacle to the use of recombinant *Salmonella* strains. In order to solve this problem, an expression system was developed, based on an invertible promoter, which results in high-level expression of the antigen of interest in only a minor part of the bacterial population[10]. If the amount of antigen produced is toxic for the bacterium, it will stop growing, but the non-producing part of the

population will continue to grow and segregate new antigen producers. Here we describe the construction of this expression vector and its evaluation in a mouse model using the B subunit of cholera toxin (CtxB) as a model antigen.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Escherichia coli strain DH5α (BRL Life Technologies, Breda, The Netherlands) was used as a host for all plasmid constructions. Before introduction into the *Salmonella typhimurium* vaccination strain SL3261[11], plasmids were passaged through the restriction-deficient, modification-proficient *S. typhimurium* strain SL5283. All bacteria were grown in NZCYM[12] aerobically at 37°C, unless indicated otherwise. Ampicillin was used at 100 $\mu g\,ml^{-1}$.

DNA manipulations

Isolation of plasmid DNA, preparation of DNA fragments and ligations were carried out using standard methods[12]. Plasmids were introduced into bacteria by electroporation using the Biorad Gene Pulser (Bio-Rad, Richmond, CA, USA) as follows: overnight cultures were diluted 1:100 in NZCYM and grown aerobically at 37°C to mid-log phase. The cells were subsequently harvested by centrifugation and washed twice with ice-cold distilled water. Finally, the cells were resuspended to 10^{10} cells ml^{-1} in distilled water containing 10% (w/v) glycerol, frozen in dry ice and stored at −70°C until use. For electroporation, DNA was precipitated, resuspended in distilled water and subsequently mixed with 10^9 cells in an ice-cooled cuvette and electroporated at 2.5 kV, 25 μF and 200 Ω. Immediately after electroporation 1 ml of NZCYM was added and the cells were subsequently incubated aerobically for 30 min at 37°C. Aliquots were plated on NZCYM–agar plates containing ampicillin and incubated at 37°C.

Immunization of mice and *in vivo* stability of pYZ17

For oral immunization, bacteria from logarithmically growing cultures were harvested by centrifugation, washed in phosphate-buffered saline (PBS) and resuspended to a cell concentration of 2×10^{10} cells ml^{-1} in 10% $NaHCO_3$. Subsequently, 6-week-old female Balb/c mice received two successive oral immunizations with 10^{10} bacteria at days 1 and 4 followed by two successive booster immunizations with the same number of bacteria at days 32 and 36. At days 11, 18, 25, 32 and 60 two mice per group were bled and their spleens and guts were removed. Gut-washes were obtained by washing the complete gut-segment distal from the stomach with 1 ml PBS, centrifuging the samples for 10 min at 10000 rev min^{-1} and collecting the supernatants. Gut-washes and sera were stored at −20°C until tested by ELISA. The spleens were homogenized and the stability of pYZ17 was determined by comparing the number of viable bacteria that could be recovered on McConkey agar plates with and without ampicillin (100 $\mu g\,ml^{-1}$). Essentially the same procedure was followed for intraperitoneal (i.p.) immunization, except that bacteria were suspended in PBS to a cell concentration of 2×10^6 cells ml^{-1}, and mice were immunized with 10^6 bacteria intraperitoneally (i.p.) at days 4 and 32.

ELISA

β-lactamase ELISA. β-lactamase (TEM R^+, Boehringer Mannheim, Germany) was diluted in 0.1 M phosphate buffer, pH 8.1, to a protein concentration of 2.5 $\mu g\,ml^{-1}$ and adsorbed onto 96-well high-binding microtitre plates (Costar E.I.A./R.I.A. plate HB, Cambridge, USA) by overnight incubation at 4°C. After washing with demineralized water containing 0.05% Tween-80, plates were blocked with PBS containing 5% fetal calf serum (FCS), 5% NaCl and 0.1% Tween-20. Plates were washed with 0.05% Tween-80 containing demineralized water and each well was incubated with 100 μl dilutions of the sera or gut-washes. After incubation for 1 h at 37°C, the plates were washed twice and the wells were incubated with goat anti-mouse IgG or IgA HRP-conjugates (Southern Biotechnology Inc., Birmingham, UK) diluted 8000 times. Binding of the conjugate was detected by addition of 100 μl of a TMB/DONS-based substrate solution to each well. After 10 min at room temperature, 100 μl 2 M H_2SO_4 was added to stop the colour reaction. The absorbance at 450 nm was determined in a Titertek Multiscan (Titertek, ICN Biomedicals, Amsterdam, The Netherlands). The titre was defined as the highest dilution of test samples at which the A_{450} was higher than the mean A_{450} + three times s.d. of the corresponding samples from Balb/c mice immunized identically with SL3261 without pYZ17.

LPS-ELISA. The ELISA for LPS differed from the β-lactamase ELISA only in the coating step, which was performed in 0.11 M acetate buffer, pH 5.5, containing 5.0 $\mu g\,ml^{-1}$ *S. typhimurium* LPS (Sigma, cat. no. L6511, Axel, The Netherlands) and the anti-IgG and IgA HRP-conjugate dilutions employed (1:4000). The titre was defined as the highest serum dilution at which the A_{450} was higher than the mean A_{450} + three times standard deviation (s.d.) of preimmune sera.

CtxB-ELISA. CtxB (List Biological Labs, Campbell, CA, USA) was diluted in PBS to a concentration of 5.0 $\mu g\,ml^{-1}$ and adsorbed to the microtitre plates. The used anti-IgG and IgA conjugates were diluted 10000 and 4000 times respectively. All the other conditions were as described for the β-lactamase ELISA.

Western blot analysis of recombinant bacteria

Overnight cultures were diluted ten times in NZCYM and incubated aerobically until the absorbance of the culture at 550 nm reached 1.0. Aliquots of bacteria were pelleted, resuspended in 100 μl sample buffer[13] and boiled for 20 min. Samples of 10 μl were separated on 15% SDS–PAGE and subsequently transferred to nitrocellulose. After blocking with 0.5% Tween-80 in PBS for 30 min the blots were incubated at room temperature with a goat-anti-CtxB serum (List Biological Labs, Campbell, CA, USA) diluted 500 times in PBS containing 0.5% Tween-80 and 0.5% low-fat milk powder. After washing three times over a period of 30 min in PBS containing 0.5% Tween-80, the blot was incubated for 1 h at room temperature with horseradish peroxidase-conjugated protein A (Amersham, Den Bosch, The Netherlands) diluted 2500 times with 0.5% Tween-80 in PBS. After three washings, the blot was developed in a TMB/DONS-based substrate.

RESULTS

Construction and characterization of pYZ17

Plasmid pYZ17 is an expression plasmid with an invertible promoter based on the bacteriophage Mu Gin system. The Gin invertase of phage Mu initiates spontaneous G segment inversion on phage Mu genome, which takes place between two 34 bp inverted repeat (IR) sequences[14]. The size of the invertible segment is not a crucial factor in the inversion process, a G segment as small as 40 bp is still inverted, and the left (L) and right (R) IR sequences are equivalent in terms of their roles in the recombination process and can therefore substitute for each other[15]. Based on these observations, an invertible promoter can be constructed by inserting a promoter fragment between two identical IR sequences. Plasmid pYZ17 is a derivative of pYZ16a (Yan and Meyer, manuscript in preparation), which has the following features; a promoterless DNA fragment coding for phage Mu Gin invertase, a clts857 gene fragment coding for temperature-sensitive lambda cI repressor, a fd transcriptional terminator downstream and an rrnB T1 transcriptional terminator upstream of clts857 fragment, a promoterless cholera toxin subunit B fragment sequence, origin of replication and ampicillin resistance gene from pBR328. Plasmid pYZ17 (*Figure 1*) was constructed by introducing an *Xho*I IR-P_L-IR module into the single *Xho*I site on pYZ16a. To construct the IR-P_L-IR module, the lambda leftward promoter P_L was isolated from pLC2833[16] as an ~250 bp *Xho*II fragment and ligated at both ends with a synthetic IR sequence flanked by *Bcl*I/*Xho*I sites: 5'-GATCATTTA-CCGTTTCCTGTAAACCGAGGTTTTGGATAAC-3'. After ligation, the IR-P_L-IR module was inserted into pYZ16a. In the resulting plasmid, pYZ17, antigen (CtxB) expression can only occur in one orientation of the P_L promoter (designated the 'ON' orientation). Besides a correct orientation of the P_L promoter, expression of CtxB requires the bacteria containing pYZ17 to be grown at temperatures higher than 28°C to inactivate the thermosensitive repressor of P_L.

Expression of CtxB was analysed on Western blots, after growing strain SL3261 harbouring pYZ17 at 28 and 37°C. As expected, CtxB could not be detected when bacteria were grown at 28°C, but was clearly induced at 37°C (*Figure 2*).

If there is no selection against a particular orientation of the P_L promoter, one expects that in a bacterial population habouring pYZ17, 50% of the plasmids will contain the P_L promoter in the 'ON' orientation. However, if transcription driven by the P_L promoter in a particular orientation (most probably the ON orientation) is detrimental, the percentage of plasmids with this orientation will be smaller than 50%. To estimate the fraction of pYZ17 molecules in the bacterial population which contained P_L in the ON orientation, plasmid DNA was isolated from bacteria grown at 28 and 37°C, and subsequently digested with *Eco*RI. Plasmid pYZ17 contains two *Eco*RI restriction sites (*Figure 1*), one of which is located between the two inverted repeats (IR) just downstream of the P_L promoter. Digestion of pYZ17 with *Eco*RI should result in fragments of 5.0 and 1.9 kb for plasmids containing the P_L promoter in the 'OFF' orientation and 4.7 and 2.2 kb for the ON orientation. Analysis of *Eco*RI-digested pYZ17 DNA derived from bacteria grown at 37°C revealed that a small fraction of

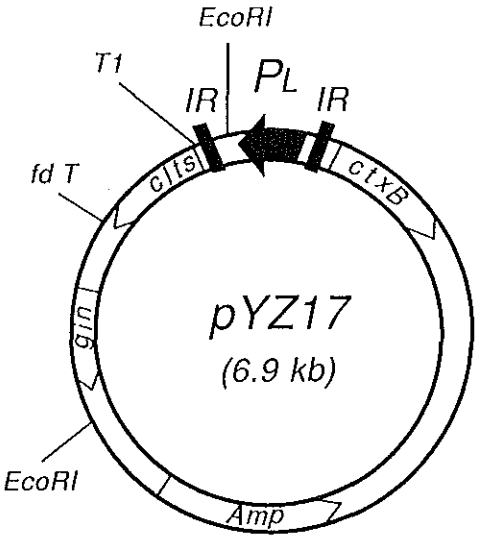

Figure 1 Plasmid pYZ17 containing an invertible promoter for CtxB expression. P_L, leftward promoter of bacteriophage lambda; IR, inverted repeat; T1, rrnB T1 transcription terminator; fd T, transcription terminator fd T; *amp*, ampicillin resistance gene; *gin*, invertase gene; *clts*, phage lambda temperature-sensitive repressor protein gene; *ctxB* cholera toxin B subunit gene. The two *Eco*RI sites which can be used to determine the orientation of the P_L promoter are indicated. Shown is pYZ17 with the P_L promoter in the OFF orientation

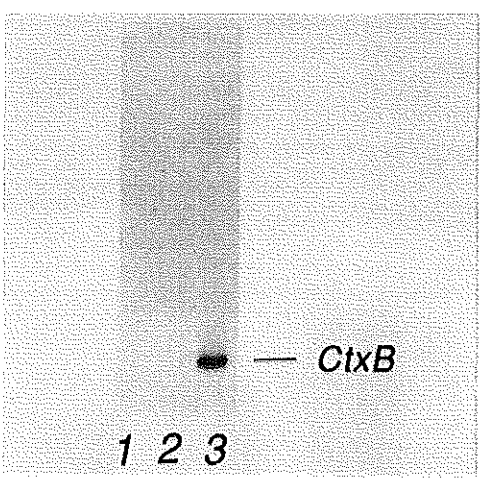

Figure 2 CtxB expression by SL3261(pYZ17) at 28 and 37°C. Total cell lysates containing equal quantities of bacteria were subjected to SDS-PAGE, followed by Western blotting using a CtxB-specific serum. Lane 1, SL3261 grown at 37°C; lane 2, SL3261(pYZ17) grown at 28°C; lane 3, SL3261(pYZ17) grown at 37°C

pYZ17 plasmids contained P_L in the ON orientation, suggesting that P_L-driven transcription towards the *ctxB* gene is selected against (*Figure 3*). Unexpectedly, when cells were grown at 28°C most pYZ17 molecules were also found to contain the P_L promoter in the OFF orientation. This suggests that even the low transcription

level from the P_L promoter at 28°C is sufficient to select against the ON-form of pYZ17.

Immunogenicity and stability of SL3261 (pYZ17)

To determine whether pYZ17 was stably maintained *in vivo*, mice were infected i.p. with SL3261(pYZ17), and bacteria were recovered from the spleens after 1, 2 and 3 weeks to determine the fraction of SL3261 cells still containing pYZ17 (*Figure 4*). During this period, pYZ17 appeared to be completely stable. Five bacterial colonies recovered from the spleens were tested for expression of CtxB, and turned out to produce the same amount of CtxB as the original strain used for infection (results not shown).

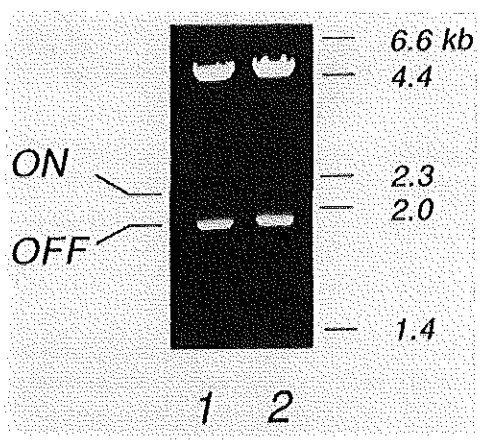

Figure 3 Restriction analysis of pYZ17, derived from strains grown at 28 or 37°C. Plasmid DNA was isolated from SL5283(pYZ17) grown at 28 or 37°C, digested with *Eco*RI and subjected to electrophoresis on a 1% agarose gel. Fragments characteristic for plasmids containing the P_L promoter in ON and OFF orientation are indicated

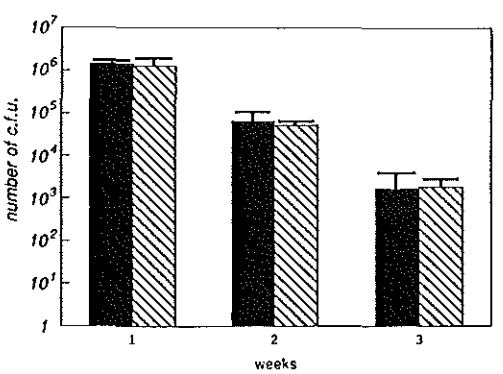

Figure 4 Stability of pYZ17 *in vivo*; 10^5 cells of strain SL3261(pYZ17) were administered i.p. to Balb/c mice. After 1, 2 and 3 weeks, mice were killed and the number of bacteria present in the spleen still containing pYZ17 was compared with the total number of bacteria by plating on McConkey agar plates with (hatched bars) and without (filled bars) ampicillin, respectively. Represented are the geometric means (bars) and the corresponding maximum numbers of colony-forming units

The immunogenicity of SL3261(pYZ17) was determined in mice after oral and i.p. immunization. After oral immunization, CtxB-specific IgG and IgA could be detected in serum (*Figure 5a* and *b*, respectively). Furthermore, IgA could also be detected in gut washes (*Figure 6a*), indicating that, besides a systemic immune response, a mucosal immune response was induced. The IgG titres remained high for up to 4 weeks after immunization. The IgA titres reached their maximum value within 1 week after immunization. During the following weeks these titres decreased, but they were restored by a booster at week 4.

In contrast to the oral immunization, no CtxB-specific antibodies could be detected after i.p. immunization (*Figure 5*). The difference in immune response against CtxB observed after oral and i.p. immunization could be due to the nature of CtxB, which is known to be a very effective mucosal immunogen[17,18]. The immunization with CtxB by the oral route may be inherently more effective than immunization by the i.p. route. Alternatively the i.p. immunization could have been less effective because a lower number of cells was administered than in oral immunization. To discriminate between these possibilities, the antibody responses of the orally and i.p. immunized mice against two other bacterial antigens were compared. These antigens were lipopolysaccharide (LPS) and β-lactamase, which are expressed on the surface and in the periplasmic space of SL3261(pYZ17), respectively. Both oral and i.p. immunization resulted in the appearance of LPS-specific IgG and IgA in serum (*Figure 7a* and *b*, respectively). Furthermore, both routes of immunization induced a LPS-specific secretory IgA response (*Figure 6b*). Both oral and i.p. immunization resulted in a β-lactamase-specific IgG response in serum (*Figure 8*), although the i.p. route was clearly more effective than the oral route. Specific IgA against β-lactamase could not be detected in the sera or in the gut washes after oral or i.p. immunization (results not shown).

These results indicate that for LPS and β-lactamase i.p. immunization was at least as effective as oral immunization. Thus the difference between the response against CtxB after oral and i.p. immunization is most probably due to the fact that CtxB is much more immunogenic when presented orally than when presented parentally.

DISCUSSION

Attenuated *Salmonella* strains have great potential as carriers for the delivery of heterologous antigens to the immune system. However, construction of *Salmonella*-recombinant strains expressing the heterologous antigen at levels high enough to induce protective immunity without affecting the ability of the strain to invade and persist in the host, is a major problem. Expression of (high levels of) heterologous antigens may be detrimental or toxic for the bacteria, resulting in loss of expression *in vivo*[19,20] or overattenuation. In particular, loss of expression due to plasmid segregation has been a problem[21]. For this reason much effort has been put into developing systems which increase the stability of the DNA expressing the heterologous antigen. Curtiss *et al.*[22–24] have developed a host–vector system in which the antigen expressing DNA is located on a plasmid which contains a gene, coding for aspartate

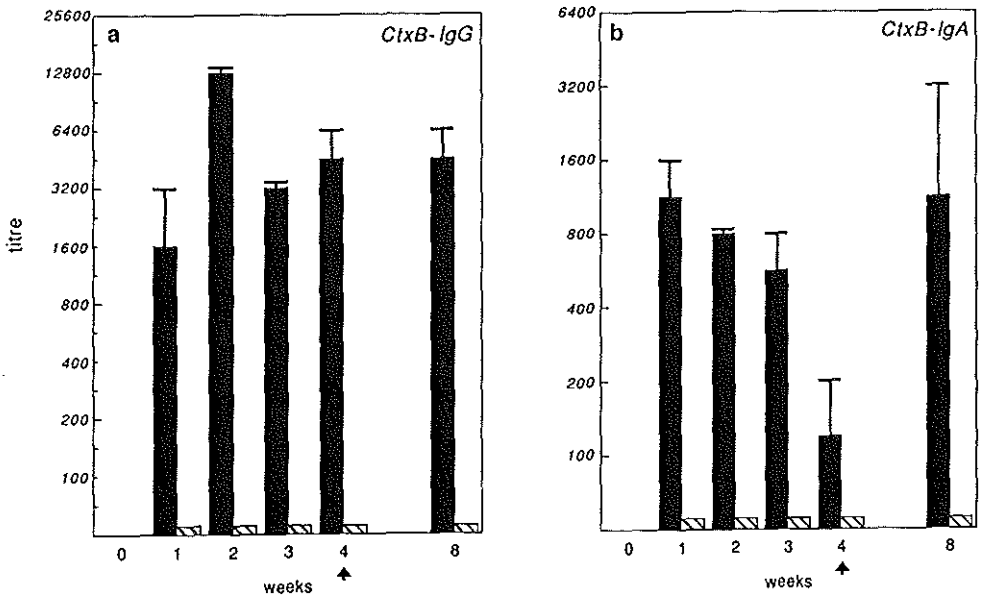

Figure 5 Antibody response to CtxB in sera after oral (filled bars) and i.p. (hatched bars) immunization of mice with SL3261(pYZ17). SL3261(pYZ17) was administered to Balb/c mice at weeks 0 and 4. Every week, two mice of both groups were bled and their anti-CtxB serum titres were determined. Represented are geometric means (bars) and the corresponding maximum titres. The arrow indicates the booster immunization. (a) Anti-CtxB IgG response; (b) anti-CtxB IgA response

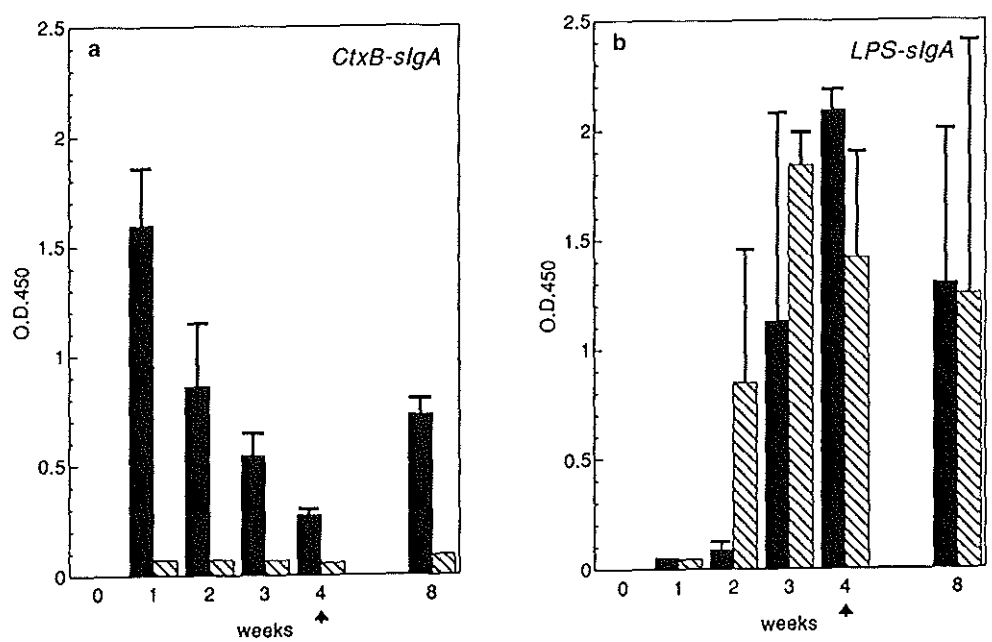

Figure 6 IgA response of four times diluted gut-washes after oral (filled bars) and i.p. (hatched bars) immunization of mice with SL3261(pYZ17). SL3261(pYZ17) was administered to Balb/c mice at weeks 0 and 4. Every week, two mice of both groups were killed and their gut washes were tested in ELISA. Represented are geometric means (bars) and the corresponding maximum A_{450} values. The arrow indicates the booster immunization. (a) Anti-CtxB response; (b) anti-LPS response

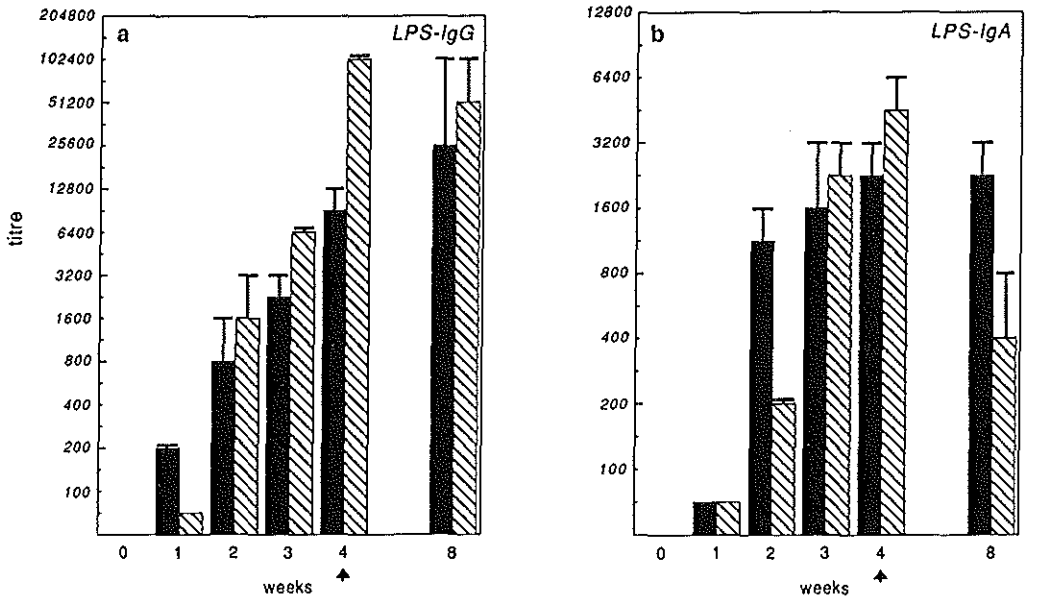

Figure 7 Antibody response to LPS in sera after oral (filled bars) and i.p. (hatched bars) immunization of mice with SL3261(pYZ17). SL3261(pYZ17) was administered to Balb/c mice at weeks 0 and 4. Every week, two mice of both groups were bled and their anti-LPS serum titres were determined. Represented are geometric means (bars) and the corresponding maximum titres. The arrow indicates the booster immunization. (a) Anti-LPS IgG response; (b) anti-LPS IgA response

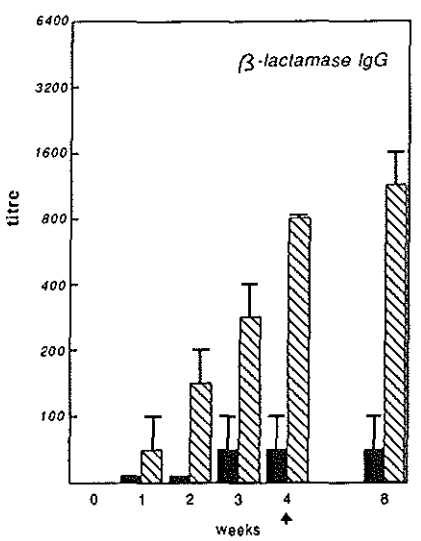

Figure 8 IgG response to β-lactamase in sera after oral (filled bars) and i.p. (hatched bars) immunization of mice with SL3261(pYZ17). SL3261(pYZ17) was administered to Balb/c mice at weeks 0 and 4. Every week, two mice of both groups were bled and their anti-β-lactamase serum titres were determined. Values shown are geometric means with their maximum titres. The arrow indicates the booster immunization

β-semialdehyde dehydrogenase, which is essential for growth of the host strain. The stability of the antigen-expressing DNA may also be increased by integrating it into the chromosome[3,25,26]. Although these systems have proven to be very useful for the stable expression of a number of antigens, they do not allow expression of antigens at levels which inhibit cell growth, because this will inevitably result in mutational alterations to reduce expression[25]. This is a major problem, because many heterologous antigens are toxic for *S. typhimurium* due to different codon usage or the presence of toxic (e.g. hydrophobic) sequences.

Deliberate reduction of the expression to a level high enough to induce an adequate immune response and low enough not to affect the fitness of the carrier strain is another strategy to obtain stable antigen expression. However, this balance between the immunogenicity and toxicity will depend on the nature of the antigen and will have to be determined anew every time another antigen is used. For some antigens this approach will be ineffective because, to induce an adequate immune response, they require expression at levels that are toxic to the producing vaccine strain.

We have developed a vector for stable expression of antigens, based on an invertible P_L promoter. The essence of the vector is that it directs expression of an antigen in only a minor part of the bacterial population. If production of the antigen is lethal, the antigen-producing bacteria will stop dividing. However, the non-producing part of the population will continue to grow and segregate antigen-producing cells due to inversion of the P_L promoter. In this way, dissemination and invasion of the total bacterial population will not be affected by antigen production (*Figure 9*).

To evaluate our system, we used CtxB as a model antigen. A plasmid, designated pYZ17, was constructed in which expression of the *ctxB* gene is controlled by an invertible P_L promoter. It appeared that pYZ17 was stably maintained *in vitro* and *in vivo*. Analysis of pYZ17 isolated from SL5283 revealed that the copy number of

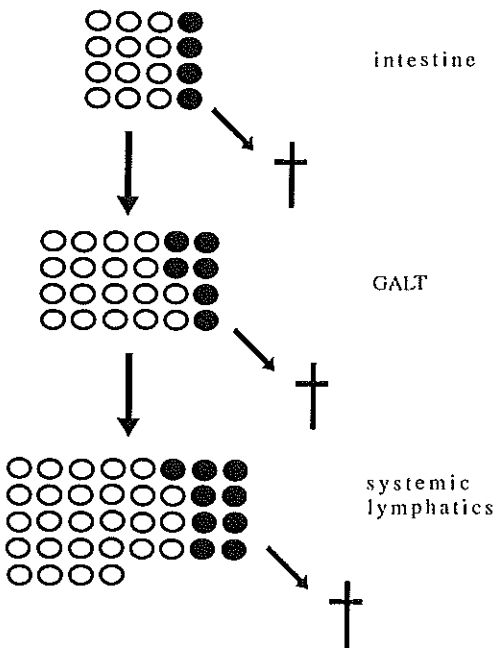

Figure 9 Model illustrating the course of an infection with SL3261 expressing foreign antigens due to promoter inversions. Circles indicate bacteria that do (●) and do not (○) produce antigen. In this figure it is assumed that in every generation, 25% of the bacteria will produce toxic amounts of antigen due to inversion of the P_L promoter and stop growing. After oral administration the bacteria will start to colonize the gut-associated lymphoid tissue (GALT). The bacteria that produce the foreign antigen will stop growing and may even lyse. The non-producing bacteria will continue the infection and may colonize the lymph nodes, spleen and liver without being affected by production of the antigen. During the infection antigen-producing cells will constantly be generated due to inversion of the P_L promoter

the ON plasmids was substantially lower than the copy number of the OFF plasmids. The apparent low copy number of pYZ17-ON may be caused by effects at the level of cell growth and/or plasmid replication. If the production of CtxB inhibits cell growth, bacteria containing higher numbers of pYZ17-ON will be outgrown by bacteria containing lower numbers of this plasmid. However, the observation that the ON/OFF ratio is not affected by the large difference in CtxB production at 28 and 37°C, nor by deletion of the *ctxB* gene (result not shown) indicates that this effect is of minor importance. The alternative explanation for the low copy number of pYZ17-ON, i.e. an effect on plasmid replication, seems more likely. For example, transcription from the P_L promoter in the ON orientation may interfere with plasmid replication. A similar phenomenon has been described by Strueber and Bujard[27]. The observation that the growth temperature does not affect the ON/OFF ratio suggests that even the leaky transcription at 28°C is sufficient to interfere with plasmid replication. We are currently addressing these questions.

In spite of the low copy number of pYZ17-ON, strains isolated from the spleens of mice still produced CtxB. Apparently pYZ17-ON is stably maintained in the bacterial population due to continuous replenishment from pYZ17-OFF. Furthermore, strain SL3261(pYZ17) was able to induce an immune response against CtxB. The induced CtxB-specific IgG and IgA titres in the serum and the presence of IgA in gut washes demonstrated that CtxB was efficiently presented in the immune system after oral administration of the *S. typhimurium* strain SL3261 harbouring pYZ17 to Balb/c mice. The i.p. route turned out to be ineffective to induce an immune response against CtxB. This phenomenon does not generally apply to other antigens as demonstrated by the fact that antibodies to LPS and the pYZ17-encoded β-lactamase were induced more efficiently by the i.p. than by the oral route. Others have also found that i.p. immunization in general induces stronger immune responses than oral administration of recombinant *Salmonella* strains[28]. The CtxB-pentamer binds specifically to the GM1 gangliosides that are abundantly present on mucosal epithelial cells[17,18]. This results in a very efficient presentation of CtxB to the immune system by the oral route. We speculate that the amount of CtxB expressed by SL3261(pYZ17) was too low to induce humoral responses after i.p. immunization. In fact, using an improved invertible promoter system that results in much higher CtxB levels, specific antibodies were also induced by i.p. immunization (manuscript in preparation).

At present we are evaluating the immune responses to a number of HIV-1 and FIV-antigens expressed in SL3261 by this improved invertible promoter system.

ACKNOWLEDGEMENTS

The authors are grateful to Geert van Amerongen, Herman Näring, Nico Schmidt and Ab van Arnhem of the Animal Facilities Department for animal handling, and to Conny Kruyssen for preparing the manuscript. This investigation was supported by a grant of the 'Raad voor Gezondheidsonderzoek', The Hague, The Netherlands (grant number 88-108/89027).

REFERENCES

1 Dougan, G. and Maskell, C.E. Live attenuated *Salmonella* vaccines as carriers of antigens to the secretory and systemic immune system. In: *Vaccination Strategies of Tropical Diseases* (Ed. Liew, F.Y.) CRC Press, Boca Raton, FA, 1991, pp. 48–64

2 Aggarwal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M. and Sadoff, J. Oral *Salmonella*: Malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. *J. Exp. Med.* 1990, **172**, 1083–1090

3 Flynn, J.L., Weiss, W.R., Norris, K.A., Siefert, H.S., Kumar, S. and So, M. Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Mol. Microbiol.* 1990, **4**, 2111–2118

4 Tite, J.P., Gao, X.M., Hughes-Jenkins, C.M., Lipscombe, M., O'Callaghan, D. and Dougan, G. Antiviral immunity induced by recombinant nucleoprotein of influenza-A virus. III. Delivery of recombinant nucleoprotein to the immune system using attenuated *Salmonella typhimurium* as a live carrier. *Immunology* 1990, **70**, 540–546

5 Poirier, T.P., Kehoe, M.A. and Beachey, E.H. Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. *J. Exp. Med.* 1988, **168**, 25–32

6 Sadoff, J.C., Ballou, W.R., Baron, L.S., Majarian, W.R., Brey, R.N., Hockmeyer, W.T. *et al.* Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science* 1988, **240**, 336–338

7 Wadhan, M.H., Sérié, C., Cerisier, Y., Sallam, S. and Germanier, R. A controlled field trial of live *Salmonella typhi* strain Ty21a oral vaccine against typhoid: three-year results. *J. Infect. Dis.* 1982, **145**, 292–295

8 Levine, M.M., Ferreccio, C., Cryz, S. and Ortiz, E. Comparison of enteric-coated capsules and liquid formulation of Ty21A typhoid vaccine in randomised controlled field trial. *Lancet* 1990, **336**, 891–894
9 Tacket, C.O., Hone, D.M., Curtiss, R. III, Kelly, S.M., Losonsky, G., Guers, L. *et al.* Comparison of the safety and immunogenicity of delta aroC delta aroD and delta cya delta crp *Salmonella typhi* strains in adult volunteers. *Infect. Immun.* 1992, **60**, 536–541
10 Yan, Z.X., Reuss, F. and Meyer, T.F. Construction of an invertible DNA segment for improved antigen expression by a hybrid *Salmonella* vaccine strain. *Res. Microbiol.* 1990, **141**, 1003–1004
11 Hoiseth, S.K. and Stocker, B.A.D. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 1981, **291**, 238–239
12 Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 2nd edn, 1989
13 Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, **227**, 680–685
14 Plasterk, R.H.A., Kanaar, R. and Van de Putte, P. A genetic switch *in vitro*: DNA inversion by Gin protein of phage Mu. *Proc. Natl Acad. Sci. USA* 1984, **81**, 2689–2692
15 Kahmann, R., Rudt, F. and Mertens, G. Substrate and enzyme requirements for *in vitro* site specific recombination in bacteriophage Mu. *Cold Spring Harbor Symp. Quant. Biol.* 1984, **49**, 285–294
16 Remaut, E., Stanssens, P. and Fiers, W. Plasmid vectors for high-efficiency expression controlled by the P_L promoter of coliphage lambda. *Gene* 1981, **15**, 81–93
17 Holmgren, J. Actions of cholera toxin and the prevention and treatment of cholera. *Nature* 1981, **292**, 413–417
18 Elson, C.O. and Ealding, W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.* 1984, **132**, 2736–2741
19 Molina, C.N. and Parker, C.D. Murine antibody response to oral infection with live *aroA* recombinant *Salmonella dublin* vaccine strains expressing filamentous hemagglutinin antigen from *Bordetella pertussis*. *Infect. Immun.* 1990, **58**, 2523–2528
20 Maskell, D.J., Sweeney, K., O'Callaghan, D., Hormaeche, C.E., Liew, F.H. and Dougan, G. *Salmonella typhimurium aroA* mutants as carriers of the *Escherichia coli* heat-labile enterotoxin B subunit to the murine secretory and systemic immune systems. *Microb. Pathog.* 1987, **2**, 211–221
21 Connell, N., Stover, C.K. and Jacobs, W.R. Old microbes with new faces: molecular biology and the design of new vaccines. *Curr. Opin. Immunol.* 1992, **4**, 442–448
22 Curtiss, R. III, Kelly, S.M., Gulig, P.A. and Nakayama, K. Selective delivery of antigens by recombinant bacteria. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 35–49
23 Curtiss, R. III, Nakayama, K. and Kelly, S.M. Recombinant avirulent *Salmonella* vaccine strain with stable maintenance and high level expression of cloned genes *in vivo*. *Immunol. Invest.* 1989, **18**, 583–596
24 Nakayama, K., Kelly, S.M. and Curtiss, R. Construction of an *asd*$^+$ expression-cloning vector: Stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. *Biotechnology* 1988, **6**, 693
25 Hone, D., Attridge, S., Van den Bosch, L. and Hackett, J. A chromosomal integration system for stabilization of heterologous genes in *Salmonella* based vaccine strains. *Microb. Pathog.* 1988, **5**, 407–418
26 Strugnell, R.A., Maskell, D., Fairweather, N., Pickard, D., Cockayne, A., Penn, C. and Dougan, G. Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium* vaccine strains. *Gene* 1990, **88**, 57–63
27 Stueber, D. and Bujard, H. Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes. *EMBO J.* 1982, **1**, 1399–1404
28 Mitov, I., Denchev, V. and Linde, K. Humoral and cell-mediated immunity in mice after immunization with live oral vaccines of *Salmonella typhimurium*: auxotrophic mutants with two attenuating markers. *Vaccine* 1992, **10**, 61–66

CHAPTER 3

Continuous high level production of heterologous antigens in a *Salmonella* vaccine strain for the induction of local and systemic immune responses

Submitted for publication

Continuous high level production of heterologous antigens in a *Salmonella* vaccine strain for the induction of local and systemic immune responses

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ABSTRACT

In this report we describe a system that allows the stable production of heterologous antigens in Salmonella *strains at levels that are toxic for the producing bacteria. The system is based on two plasmids. One plasmid contains the T7 RNA polymerase gene under the control of a strong promoter that is located on a DNA fragment which is inverted at random. When the promoter inverts to the ON position it directs expression of T7 RNA polymerase which in turn directs expression of a gene under the control of a T7 promoter, present on a second plasmid in the same bacterium. The essence of the system is, that the promoter that controls expression of the T7 RNA polymerase gene, inverts with a frequency that results in antigen production at high, toxic, levels in a minor part of the bacterial population. Therefore, the major part of the population will not be affected by production of the heterologous antigen and will continuously segregate new antigen-producing bacteria.*

Using this two plasmid system much higher levels of the Cholera toxin B subunit (CtxB) were expressed compared to a one plasmid system, in which ctxB *expression was under direct control of the invertible promoter. The higher expression resulted in enhanced CtxB specific IgG and IgA responses in mice.*

A series of invertible promoter plasmids was constructed which revealed different expression levels of genes under control of a T7 promoter. Although expression of an HIV-1 env *gene fragment was toxic for the producing bacteria, most of the plasmid combinations were stably maintained* in vivo. *Only the combination that resulted in the highest level of antigen production was unstable. Plasmid combinations which were found to be stable* in vivo *were also stably maintained after invasion of a cell line by* S.typhimurium. Salmonella *strains in which production of the maltose binding protein (MBP) was controlled by the invertible promoter system, induced much higher MBP-specific antibody titers after oral administration to mice, compared to strains in which MBP production was dependent on a fixed promoter.*

INTRODUCTION

Attenuated *Salmonella* strains have been used successfully to present heterologous bacterial (8, 9, 18, 22), viral (6, 26, 33, 36) and protozoal (1, 40) antigens to the immune system. Mucosal, humoral and cellular responses against such antigens have been demonstrated. The potential of *Salmonella* strains to induce class I restricted cytotoxic T lymphocytes (CTL) to the heterologous antigens has been demonstrated *in vitro* (18, 19, 37) as well as *in vivo* (1, 12, 13, 14, 35). Clearly, recombinant *Salmonella* strains have great potential as live vaccines against infections diseases. However, construction of *Salmonella*-recombinant strains expressing the heterologous antigen at levels high enough to induce protective immunity without affecting the ability of the strain to invade and persist in the host, is a major problem. Production of (high levels of) heterologous antigens may be detrimental or toxic for the bacteria, resulting in over-attenuation or instability of the expression vector *in vivo*. Especially loss of expression due to plasmid segregation has been a problem (17). To solve these problems we have devised a phase variable expression system, which causes a vaccine strain to split up spontaneously into two subpopulations: antigen and non-antigen producers. While antigen production may have negative effects on plasmid stability and the fitness of the host strain, these effects do not occur in the non-producing population. Unhindered by antigen production, the non-producing population may disseminate within the host, and segregate antigen-producers so that antigen may be presented to the immune system in different tissues. This expression system is based on the G DNA segment inversion system of bacteriophage Mu, that controls phage infectivity (16, 20). In phage Mu the inversion of the G gene segment results from a site-specific recombination between two short inverted repeats mediated by the invertase Gin. One possibility to establish a phase variable expression system, was to replace the G segment between the two short inverted repeats by a promoter, making this promoter invertible. When this invertible promoter is positioned in front of the gene of interest, it will only direct expression in one orientation.

Using the Cholera toxin B subunit (CtxB) as a model antigen, we demonstrated that a *Salmonella typhimurium aroA* strain harbouring the invertible promoter plasmid pYZ17, stably expressed the *ctxB* gene and was able to induce high antibody titers to CtxB in mice after oral administration (31, 38, 39). However, no antibodies to CtxB were induced after intraperitonial (i.p.) administration. We hypothesized that the production of CtxB in *S.typhimurium* was too low to induce specific antibodies when the bacteria were administered by the i.p. route.

Here we describe an improved expression system that combines the ability of the invertible promoter plasmids to express toxic antigens with the high expression that can be obtained by the T7 RNA polymerase system (23, 28, 29, 30). Using this improved expression system, much higher levels of CtxB could be produced, resulting in enhanced immune responses. Furthermore, using the *malE* gene, we demonstrate that the system is able to induce much higher specific antibody responses than a comparable system that uses a fixed promoter.

MATERIAL AND METHODS

Bacterial strains, plasmids and media

Escherichia coli strain DH5α (BRL Life technologies, Breda, The Netherlands) was used as a host for all plasmid constructions. Before introduction into the *S.typhimurium* vaccination strain SL3261 (15), plasmids were passaged through the restriction deficient, modification proficient *S.typhimurium* strain SL5283, a *galE503* derivative of LB5000 (3, 25). All bacteria were grown in LB aerobically at 37°C, unless indicated otherwise. Ampicillin was used at 100 µg/ml in LB agar plates and 200 µg/ml in liquid media. Kanamycin was used at a concentration of 50 µg/ml in agar plates and liquid media.

DNA manipulations

Isolation of plasmid DNA, preparation of DNA fragments and ligations were carried out using standard methods (24). Plasmids were introduced into bacteria by electroporation as previously described (31).

Construction of plasmids

The vectors used in this study are represented in Figure 1. Plasmid pYZ17 has been described previously (31, 38). Relevant features are; the invertible P_L promoter flanked by two inverted repeats, *gin* encoding the invertase which binds to the inverted repeats and drives the inversion of the DNA fragment with the P_L promoter, and the *cI857* gene, encoding the temperature sensitive repressor for P_L. Finally, pYZ17 contains the cholera toxin B subunit encoding *ctxB* gene, the transcription of which is dependent on the orientation of P_L. Vector pYZ17GP (38) (this plasmid was previously designated pIP1 (32)) is similar to pYZ17, except that it contains a different *ori* and that *ctxB* has been replaced by the gene coding for T7 RNA polymerase. The vectors pYZ17GP and pYZ27bGP (previously designated pIP3 and pIP2, respectively (32)) are identical to pYZ17GP except for the orientation of *gin* and *cI857*. The vector pYZ27bGP differs only from pYZ27GP by an additional nucleotide between the

P_L promoter and the GTG start codon for *gin* (Yan, unpublished). The vector pYZ17 inverts its P_L promoter with a higher frequency than pYZ27b (38), the plasmid from which pYZ17bGP was derived.

To construct derivatives of pYZ27bGP and pYZ27GP that would be maintained at lower copy numbers, the p15A *ori*, which results in an intermediate copy number, was replaced by the *ori* of the low copy number plasmid pSC101. Therefore the part of pSC101 located between nucleotides 4581 and 6240 (numbering according to Bernardi and Bernardi (2)), which contains the *ori* and the partitioning (*par*) locus, was amplified by PCR using the primers 5'-CAGGATCCAGTCTGAATGACCTG-3' and 5'-ACCGGATCCAAGAGCCA TAAGA-3'. After treatment of the PCR fragment with Klenow fragment it was inserted into the *Sma*I site of pUC19 to generate pUCSC. This vector was digested with *Eco*RI followed by filling-in with Klenow fragment and *Pst*I digestion to excise the fragment containing the pSC101 *ori*. Finally, the fragment was inserted in the *Pst*I site and Klenow fragment filled-in *Nhe*I site of pYZ27bGP and pYZ27GP to generate pIP4 and pIP5, respectively.

The pYZ27GP, pYZ27bGP and pIP-plasmids were used in combination with plasmids that contain the gene for a model antigen under the control of a T7 promoter. Two of such plasmid, pT7ctxB and pET3xa-env (Fig. 1), were constructed for this study. Plasmid pT7ctxB was obtained by cloning the *Bgl*II-*Sal*I fragment of pYZ17, containing the promoterless *ctxB* gene, into the *Bam*HI and *Sal*I site of pT7-5 (30). Plasmid pET3xa-env was obtained by cloning the *Bgl*II-*Bgl*II fragment of the *env* gene of HIV-1 IIIB, coding for amino acids 279 to 473, into the *Bam*HI site of pET3xa (28). This resulted in a fusion between *gene-10* and *env* sequences, providing efficient translation signals for *env*. Construction of pETMALp, which contains the *malE* gene under control of a T7 promoter, has been described previously (32).

Effect of different plasmids on host strain viability at 37°C

At 37 °C, but not at 28 °C, the cI857 repressor is inactive, resulting in strong transcription from the P_L promoter which will result in the high level expression of the model antigen. To assess how this may affect cell viability, a single colony of a particular strains was spread on a plate containing the appropriate antibiotics and grown at 25 °C for 16 hours. Subsequently, the bacteria were harvested and resuspended in PBS to a concentration of about 100 to 500 bacteria/150 µl. Aliquots of 150 µl were plated in duplicate on plates containing the appropriate antibiotics. One half of the plates was incubated at 28

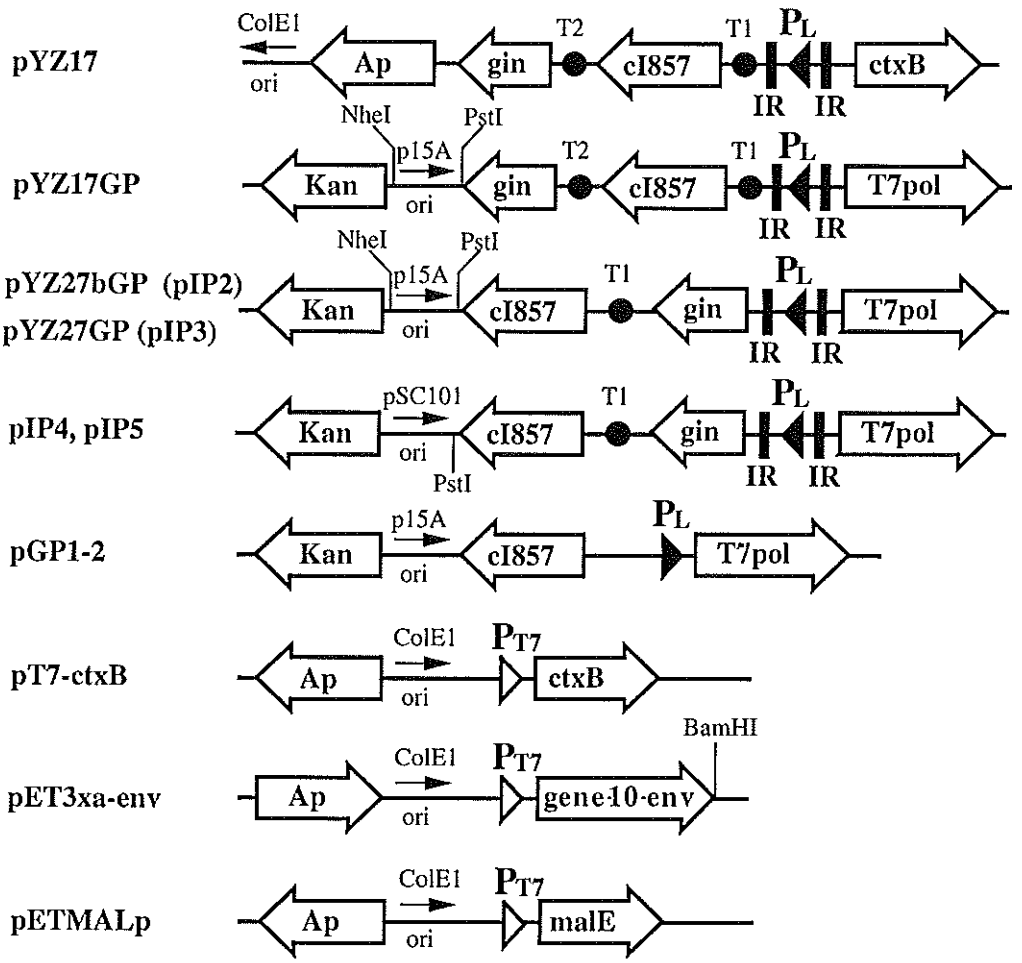

Fig. 1. Schematic representation of the plasmids used in this study. Transcription of the *ctxB* gene in pYZ17 is under direct control of the invertible P_L promoter. In the pIP-vectors the invertible promoter controls transcription of the T7 RNA polymerase gene, which in its turn directs transcription of a gene located on a second plasmid under control of the P_{T7} promoter (e.g. pT7-ctxB or pET3xa-env). The P_L promoter of pGP1-2 that controls the expression of T7 RNA polymerase is fixed. Plasmids pYZ27bGP and pYZ27GP have previously also been designated pIP2 and pIP3, respectively. Vectors pYZ27GP and pIP5 are identical to pYZ27bGP and pIP4, respectively, except for an additional adenine about 50 nucleotides in front of the GTG start codon for *gin*. Arrows indicate the transcriptional orientation. Abbreviations: P_L, leftward promoter of bacteriophage lambda; IR, inverted repeat; T1, rrnB T1 transcription terminator; T2, transcription terminator fd T; gin, invertase gene; cI857, phage lambda temperature sensitive repressor protein gene; Ap, ampicillin resistance gene; Kan, kanamycin resistance gene; *ctxB*, cholera toxin B subunit gene; T7pol, T7 RNA polymerase gene; P_{T7}, gene-10 promoter of bacteriophage T7; ColE1, ColE1 origin of replication; p15A, p15A origin of replication.

°C and the other half at 37 °C. The viability at 37 °C was defined as: (CFU at 37 °C / CFU at 28 °C) x 100 %.

Plasmid stability in media without selective antibiotics.
For each strain a single colony was plated on plates containing the appropriate antibiotics. After growth at 25 °C for 16 hours bacteria were harvested and resuspended in LB without antibiotics. The cultures were diluted to an OD600 of 0.1, and subsequently duplicate samples were grown aerobically at 37 °C. During the experiment the bacteria were kept in logarithmic growth phase by dilution with medium of 37 °C. At several time points samples were plated on media with or without the appropriate antibiotics, followed by incubation at 28 °C. The plasmid stability of the recovered bacteria was defined as: (CFU on plates with antibiotics / CFU on plates without antibiotics) x 100 %.

Immunisation of mice and in vivo plasmid stability.
For immunisation, bacteria producing CtxB or the Env-fusion protein were grown at 37°C in the presence of ampicillin and kanamycin. Bacteria were harvested by centrifugation at an OD600 of 0.6 to 0.8, washed in PBS and resuspended to a cell concentration of 10^{10} cells/ml in 10% $NaHCO_3$ for intragastric intubation or $2x10^7$ cells/ml in PBS for intraperitonial (i.p.) immunisation. Subsequently, 0.5 ml of the bacterial suspensions were administered by the oral or i.p. route to six week old female Balb/c mice. An identical booster immunisation was given at four weeks after the primary immunisation. Blood samples were obtained by retro-orbita puncture of anaesthetized mice. Gut-washes were obtained as described previously (31). Sera and gut-washes were stored at -20 °C until tested in an ELISA.

Immunization with bacteria producing MBP was performed essentially as described above, except that bacteria containing the fixed promoter plasmid pGP1-2 were grown at 28 °C to repress MBP production and mice received $3x10^8$ bacteria in a volume of 0.3 ml of PBS via intragastric intubation.

To determine plasmid stability *in vivo*, mice were injected i.p. with 0.5 ml PBS containing 10^7 bacteria. Each construct was tested in two mice. One week after administration the mice were sacrificed and their spleens were removed. The spleens were homogenized and plasmid stability was determined by comparing the number of CFU that could be recovered on McConkey agar plates with and without ampicillin + kanamycin.

Assay for plasmid stability of bacteria grown in eukaryotic cells

The mouse cell line P815 (American Tissue Culture Collection) was cultured in RPMI 1640 (Gibco) supplemented with 10 % (v/v) FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C. Prior to infection P815 cells were washed two times in RPMI 1640 without any additives. Bacteria from logarithmical growth cultures were harvested by centrifugation and washed two times with RPMI 1640. About 10^8 bacteria were added to 10^6 P815 cells in a total volume of 1 ml RPMI 1640. Subsequently, cells were incubated for 2 hours at 37 °C, during which period they were resuspended every 20 minutes. Finally the cells were washed three times in RPMI and suspended in 10 ml culture media supplemented with gentamicin (50 µg/ml) to kill extracellular bacteria. After incubation for 1 and 3 days at 37 °C, cells from 1 ml of culture were harvested, washed two times in PBS and lysed in 350 µl PBS containing 1% Triton x-100 to release intracellular bacteria. The bacteria were plated on media without antibiotics and colonies were allowed to form at 28 °C. Subsequently, about 50 colonies of each sample were tested for resistance on plates containing the appropriate antibiotics. The plasmid stability was expressed as the percentage bacteria that was still resistant to the relevant antibiotics.

ELISA

The CtxB and LPS ELISA's were performed as previously described (31). The MBP ELISA was essentially performed as described previously (32), but mice antibodies were detected with anti-mouse Ig HRP linked $F(Ab')_2$ fragment from sheep (Amersham, Cat. No. NA9310) diluted 1:2000.

SDS-PAGE

For SDS-PAGE, overnight bacterial cultures were diluted 20 times in LB containing the appropriate antibiotics and incubated aerobically until they reached an $O.D._{600}$ of 0.6. Aliquots of bacteria were pelleted, resuspended to an $O.D._{600}$ of 10 in sample buffer and boiled for 20 min. Samples were subjected to SDS-PAGE, followed by fixation and Coomassie brilliant blue staining.

Western blotting

Detection of CtxB by Western blotting was performed as previously described (31). For detection of Env fusion protein by Western blot analysis, SDS-PAGE was immediately followed by transfer of the proteins to nitrocellulose. After blocking with PBS containing 0.5 % Tween-80 and 0.5 % low fat milk powder, the blots were incubated for one hour at room temperature with a biotinylated

mouse monoclonal antibody directed to the hypervariable V3-loop of gp120 of HIV-1 strain IIIB, diluted in the same buffer. After washing 3 times in PBS containing 0.5 % Tween-80 during a period of 30 min., the blot was incubated for 1 hour at room temperature with a HRP-streptavidin conjugate diluted 2000 times in PBS containing 0.5 % Tween-80. After 3 washings, the blot was developed in a TMB/DONS based substrate.

RESULTS

Construction and evaluation of an improved phase variable expression system.

In pYZ17 (Fig. 1) transcription of *ctxB* is under direct control of the invertible P_L promoter. On average, in a single CtxB producing cell only a fraction of the P_L promoters will be in the plus orientation and thus only a fraction of the *ctxB* copies will be expressed. We hypothesized that production of CtxB could be increased if a single promoter inversion in a particular cell would result in transcription of all *ctxB* copies within that cell. To obtain this situation, we combined the invertible promoter system with the efficient T7 RNA polymerase/promoter expression system (28, 29, 30). The bacteriophage T7 RNA polymerase is about 8 times more active than *E.coli* RNA polymerase (5) and less stringent with respect to template structure (11). It directs highly specific transcription from a conserved 23-bp sequence (4), which is not recognized by polymerases of *E. coli* or *S.typhimurium* strains.

We cloned the *ctxB* gene downstream of the T7 promoter, so that production of CtxB became dependent on the presence of T7 RNA polymerase. The resulting plasmid, pT7ctxB (Fig. 1), was used in combination with pYZ27bGP (Fig. 1), which contains the T7 RNA polymerase gene under control of the invertible P_L promoter. In this configuration a single promoter inversion would result in production of T7 RNA polymerase, which in turn would direct transcription of all the *ctxB* genes present in a particular bacterium.

The production of CtxB in *S.typhimurium* strain SL3261 harbouring pYZ17 or the two-plasmid system, was compared (Fig. 2). As anticipated, much higher levels of CtxB were produced by SL3261 harbouring the two-plasmid system compared to SL3261(pYZ17).

Immune responses against CtxB expressed by SL3261 carrying the one or two plasmid system.

To investigate whether the higher CtxB-production obtained by the two-plasmid system resulted in the induction of higher titers of CtxB-specific antibodies, mice

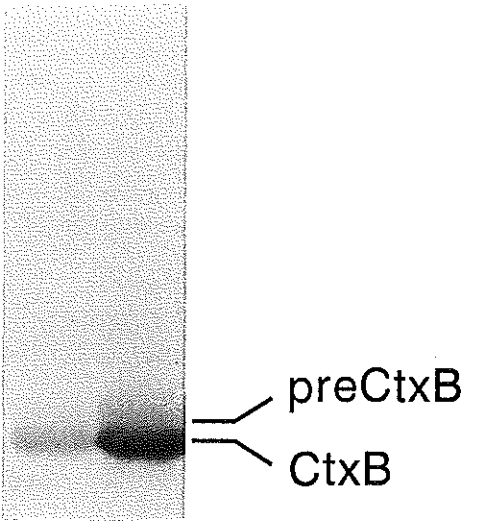

Fig. 2 CtxB Production by the single- and two-plasmid system. Bacteria were grown at 37°C and equal amounts of cells were subjected to SDS-PAGE. Subsequently CtxB was detected by Western blotting. Lane 1, SL3261(pYZ17); Lane 2, SL3261(pT7ctxB + pYZ27bGP).

were immunised orally or i.p. with SL3261(pYZ17) or SL3261(pT7ctxB + pYZ27bGP). Both strains were able to induce CtxB-specific serum IgG and IgA after oral administration (Fig. 3A and B), but the titers were much higher in mice immunised with SL3261(pT7ctxB + pYZ27bGP). This strain also induced the highest mean level of CtxB-specific sIgA in gut washes after oral administration (Fig. 3C). Intraperitoneal administration of SL3261(pYZ17). induced a considerable CtxB-specific IgG response in a few mice, but most mice developed only low or undetectable (<100) antibody titers. In contrast SL3261(pT7CtxB + pYZ27bGP) induced considerable anti-CtxB IgG responses in almost all i.p. immunised mice (Fig. 3A). As expected for the i.p. route, the mean serum IgA titers and especially the mucosal IgA responses were very low in mice (Fig. 3B and C), irrespective of the used bacterial strain.

Thus in all cases cells harbouring the two-plasmid system were able to induce higher levels of CtxB-specific antibodies compared to cells harbouring the one-plasmid system.

Construction of plasmids with different inversion frequencies

When a toxic level of antigen is defined as the level that prevents cell division, a bacterial population containing the two-plasmid system can only increase in size if the percentage of bacteria expressing toxic level of antigen is smaller than

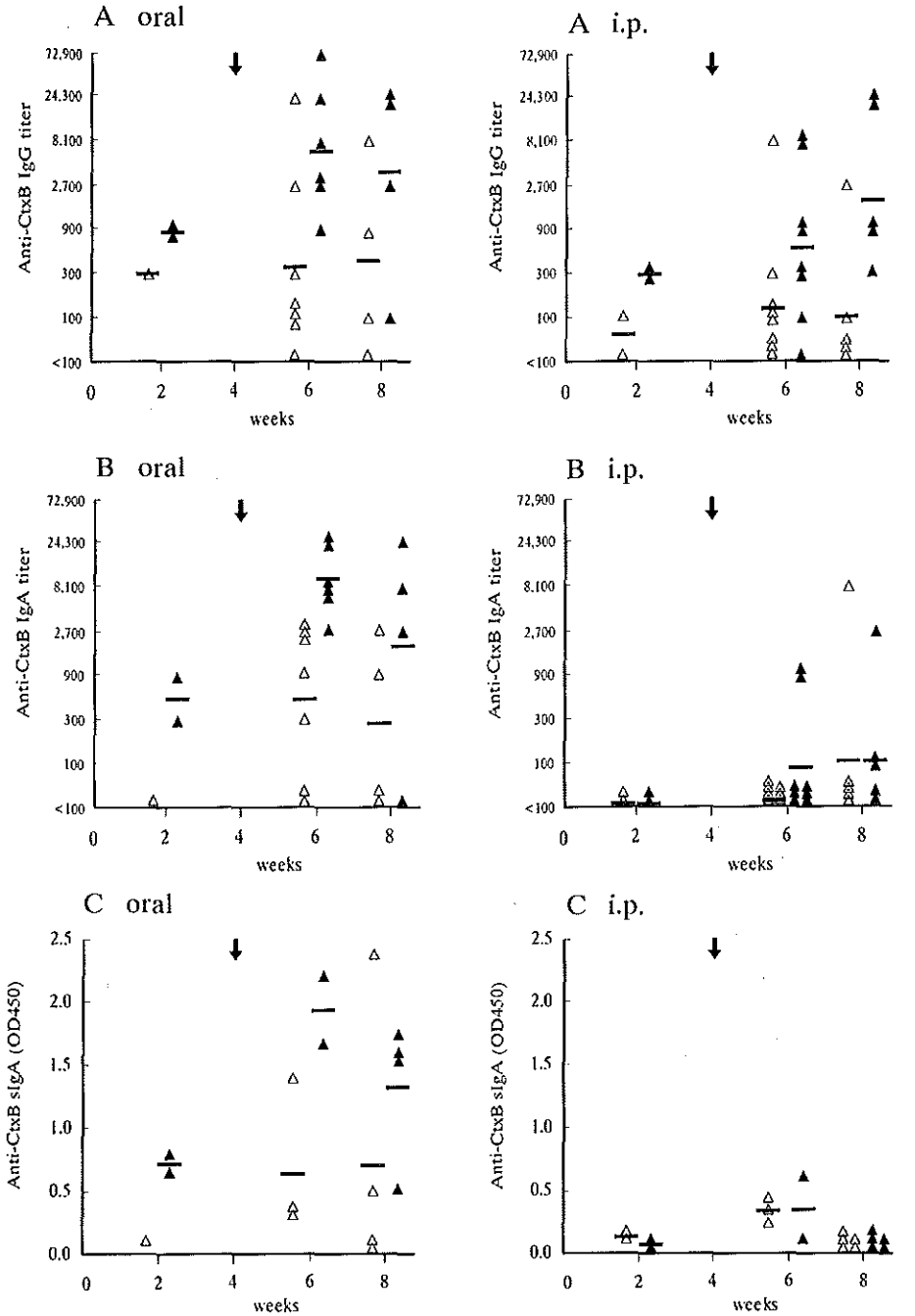

Fig. 3 CtxB-specific antibodies after immunization with SL3261(pYZ17) (open triangles) or SL3261(pT7ctxB + pYZ27bGP) (filled triangles). Mice were immunized orally or intraperitoneally in week 0 and received a booster at week 4 (indicated by the arrow). Individual responses are indicated by triangles, with the mean antibody response of each group represented by a horizontal bar. A. Serum IgG titer; B. Serum IgA titer; C. Secretory IgA response. Titers in panel A and B were determined as described in Materials and Methods, In panel C responses are presented as the OD450 value of 16 times diluted gut washes.

50%. The percentage of bacteria producing a toxic level of antigen is determined by the inversion frequency, defined as the number of inversions of the P_L promoter per cell per generation, and by the toxicity of the expressed antigen. Thus the inversion frequency may have to be adapted to different antigens. Therefore a series of invertible promoter vectors were constructed that were expected to reveal different inversion frequencies (Fig. 1). Based on their derivation, it was expected that the inversion frequency of the PL promoter in pYZ17GP, pYZ27bGP and pYZ27GP would decrease in the order pYZ17GP, pYZ27bGP, pYZ27GP. The different inversion frequencies of these plasmids is probably due to the different levels of transcription of *gin*. Another way to affect the invertion frequency is by reducing the plasmid copy number. Plasmid copy number may affect the inversion frequencies in at least two ways. First, because of the reduced number of invertible promoter fragments present in a single cell. Secondly, because the production of Gin will probably be less, due to a gene-dose effect, and lower levels of Gin have been shown to result in a reduced inversion frequency (21). A lower copy number of the plasmids with the invertible PL promoter was achieved by replacing the p15A *ori* in pYZ27GP and pYZ27bGP by the *ori* and *par* region of the low copy number plasmid pSC101. This resulted in a copy number that is two to three times lower than that of the parental plasmids (results not shown). The *par* region of pSC101 ensures equal distribution of the plasmid copies over the daughter cells during cell division (34).

In previous experiments (unpublished) we used vectors in which expression of a 580 bp *env* fragment from HIV-1 was under direct control of an inducible, fixed, promoter. Induction of expression appeared to be toxic for the producing bacteria, resulting in rapid plasmid loss. We wanted to determine whether high level, stable expression of this gene fragment could be accomplished with the two-plasmid system. Therefore pET3xa-env (Fig. 1) was constructed. This plasmid contains a *gene-10-env* fusion under control of a T7 promoter and is compatible with the plasmids that contain the T7 RNA polymerase gene under control of the invertible P_L promoter.

Strain SL5283 containing pYZ27bGP and pET3xa expressed high levels of gene-10 protein (Fig. 4A). The production level of the gene-10-env fusion protein was lower, but a band corresponding to the predicted molecular mass of 53 kD was clearly visible on a Coomassie stained SDS-PAGE gel of total bacterial lysates (Fig. 4B). The identity of the protein band was confirmed by Western blotting using a monoclonal antibody specific for the V3-loop of gp120 (result not shown).

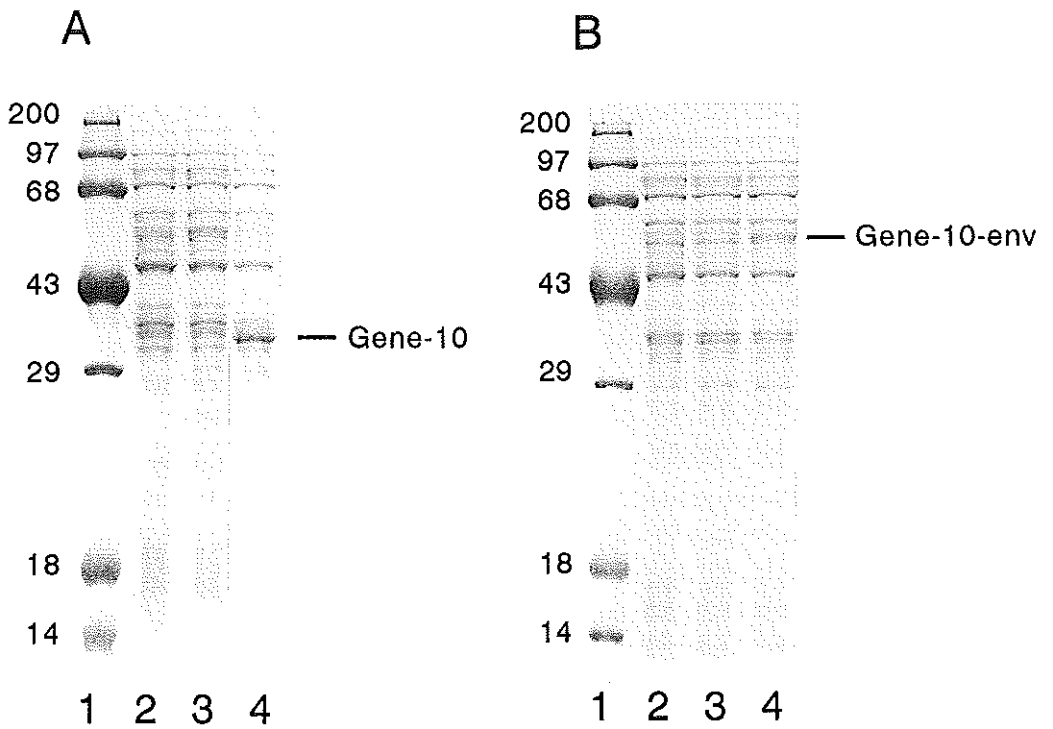

Fig. 4. Production of gene-10 and the gene-10-env fusion protein by SL5283 carrying the two-plasmid system. Total cell lysates of bacteria grown at 37°C were subjected to SDS-PAGE followed by fixation and staining of the gels. A. Lane 1, molecular weight markers; Lane 2, SL5283; Lane 3, SL5283(pET3xa); Lane 4, SL5283(pET3xa + pYZ27bGP); B. Lane 1, molecular weight markers; Lane 2, SL5283; Lane 3, SL5283(pET3xa-env); Lane 4, SL5283(pET3xa-env + pYZ27bGP).

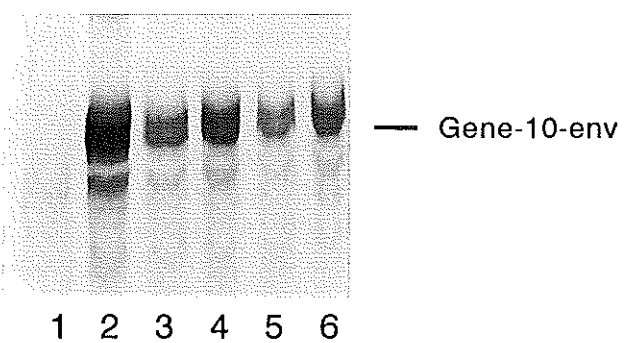

Fig. 5. Production of a gene-10-env fusion protein by SL3261 strains carrying pET3xa-env in combination with different invertible promoter vectors. Strains were grown at 37°C in the presence of appropriate antibiotics. Subsequently, equal amounts of bacteria were subjected to SDS-PAGE followed by Western blotting using a monoclonal antibody specific for the V3-loop of Env. Lane 1, SL3261(pET3xa-env); Lane 2, SL3261(pET3xa-env + pYZ17GP); Lane 3, SL3261(pET3xa-env + pYZ27GP); Lane 4, SL3261(pET3xa-env + pYZ27bGP); Lane 5, SL3261(pET3xa-env + pIP5); Lane 6, SL3261(pET3xa-env + pIP4).

A higher inversion frequency will result in a higher percentage of antigen producing cells in the bacterial population and this will be reflected by a higher amount of antigen produced by the total population. Production of the gene-10-env fusion protein by strain SL3261 containing pET3xa-env in combination with the different invertible promoter vectors, was compared on Western blot (Fig. 5). Of the vectors containing a p15A *ori*, the highest production of the fusion protein was obtained by pYZ17GP, followed by pYZ27bGP and finally pYZ27GP. As expected pIP4 exhibited a higher production level of the fusion protein than pIP5 and both plasmids revealed lower production levels than their higher copy number counterparts pYZ27bGP and pYZ27GP, respectively.

Stable antigen production with the two-plasmid system

To asses the importance of the invertible promoter system for stable antigen production, the effect of expression of the gene-10-Env fusion protein by the invertible promoter plasmids was compared with a fixed promoter. The viability of strains with pET3xa-env in combination with pGP1-2, which has a fixed promoter, or one of the invertible promoter plasmids, was determined by comparing plating efficiencies at 28 °C (to repress the P_L promoter) and 37 °C (Table 1). The viability of strain SL5283 with pET3xa-env or pGP1-2 only, was slightly affected (i.e. respectively 92% and 86%). However bacteria containing both plasmids could only be recovered at 28 °C, demonstrating that expression of the *gene-10-env* gene fragment is toxic for the producing bacteria. When pET3xa-env was combined with an invertible promoter vector (i.e. pYZ17bGP), viability at 37 °C was comparable to strains harboring either one of these plasmids (i.e. ~ 90%). In contrast to strain SL5283(pGP1-2), strain SL3261(pGP1-2) was not able to grow at 37 °C. At present we are unable to explain the different behaviours of these strains. Comparable amounts of colonies were recovered at both temperatures from SL3261 containing pET3xa-env in combination with each of the pIP-vectors. Clearly the pIP system can be used to produce antigens at toxic levels in a bacterial population.

Assessment of stability *in vivo*.

Mice were infected by the i.p. route, and after one week the percentage of bacteria in the spleens that had stably maintained their plasmid combination, was determined (Table 2). The combination of pET3xa-env and pYZ17GP was unstable. Only 7 % of the bacterial colonies isolated from mice to which SL3261(pET3xa-env + pYZ17GP) was administered contained both plasmids. Moreover, 10 of these colonies were tested and all failed to express the gene-10-

Table 1. Effect of different plasmids on the host strain viability at 37°C.

Strain	Viability at 37°C[a]
SL5283(pET3xa-env)	92± 6%
SL5283(pGP1-2)	86 ± 11%
SL5283(pET3xa-env + pGP1-2)	0%
SL5283(pET3xa-env + pYZ27bGP)	90 ± 26%
SL3261(pGP1-2)	0%
SL3261(pET3xa-env + pGP1-2)	0%
SL3261(pET3xa-env + pYZ17GP)	69 ± 10%
SL3261(pET3xa-env + pYZ27bGP)	75 ± 14%
SL3261(pET3xa-env + pYZ27GP)	98 ± 8%
SL3261(pET3xa-env + pIP4)	85 ± 7%
SL3261(pET3xa-env + pIP5)	102 ± 13%

[a] Equal amounts of bacteria grown at 28°C in the presence of the appropriate antibiotics were plated in duplicate on plates containing the appropriate antibiotics. One half of the plates was incubated at 28°C and the other half at 37°C. The viability is defined as: [(CFU at 37°C / CFU at 28°C) x 100%]. Represented is the mean viability at 37°C ± SD of several independent experiments.

Env fusion (Table 2). All the other invertible promoter vectors were essentially stable in combination with pET3xa-env and all the recovered bacteria that were tested still produced the fusion protein at equal levels compared to the original strains (not shown).

Growth of bacteria within eukaryotic cells may resemble the natural growth conditions of *S.typhimurium* in mammals more closely than growth in liquid cultures. Therefore, the plasmid stability of the *Salmonella* strains with the different plasmid combinations was determined after intracellular growth in P815 cells. In this test all the combinations with pET3xa-env were stably maintained in SL3261 except for the combination of pYZ17GP with pET3xa-env (Table 3). So, the plasmid stability obtained by this test correlates with the stability found in mice (Table 2).

Immunogenicity of strains with an invertible or fixed P_L promoter

The ability of strains harboring the two-plasmid system with an invertible or a

Table 2. Stability of plasmids *in vivo*.

Strain	Stability	
	plasmids[a]	expression[b]
SL3261(pET3xa-env + pYZ17GP)	7%[c]	0/10
SL3261(pET3xa-env + pYZ27bGP)	95% ± 5%	9/9
SL3261(pET3xa-env + pYZ27GP)	103% ± 7%	5/5
SL3261(pET3xa-env + pIP4)	101% ± 16%	5/5
SL3261(pET3xa-env + pIP5)	102% ± 4%	5/5

[a] Mice were infected intraperitonially with the indicated strains, and after one week the percentage bacteria that was still resistant to both ampicillin and kanamycin was determined in the spleens of two mice for each strain. Represented is the mean plasmid stability ± the difference with individual values. [b] Gene-10-env expression of individual ampicillin and kanamycin resistant colonies was tested by Western blot analysis using a monoclonal antibody specific for the V3-loop of Env. Indicated is the number of antigen-expressing colonies/number of tested colonies. [c] From only one of the two mice inoculated with this strain enough colonies were recovered on plates containing both ampicillin and kanamycin to allow a reliable determination of plasmid stability.

Table 3. Plasmid stability in P815 cells

Strain	Plasmid stability[a]	
	1 day	3 days
SL3261(pET3xa-env + pYZ17GP)	2 ± 2%	2 ± 3%
SL3261(pET3xa-env + pYZ27bGP)	95 ± 8%	82 ± 5%
SL3261(pET3xa-env + pYZ27GP)	98 ± 2%	94 ± 6%
SL3261(pET3xa-env + pIP4)	100 ± 0%	96 ± 2%
SL3261(pET3xa-env + pIP5)	100 ± 0%	99 ± 1%

[a] P815 cells were infected with the different bacterial strains and cultured in the presence of gentamicin to kill extracellular bacteria. After 1 and 3 days the percentage intracellular bacteria still resistant to both ampicillin and kanamycin was determined. Represented is the mean plasmid stability ± SD of several independent experiments.

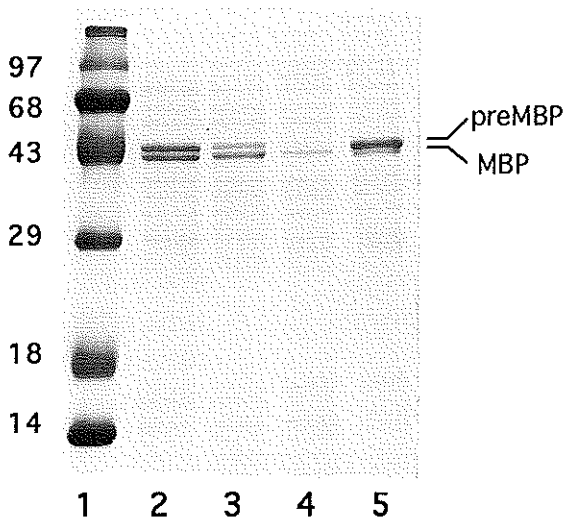

Fig. 6. Production of MBP by SL3261 strains containing the invertible or fixed promoter system. Bacteria were grown in the presence of appropriate antibiotics. Subsequently, equal amounts of bacteria were subjected to SDS-PAGE followed by fixation and staining of the gels. Lane 1, molecular weight markers; Lane 2, SL3261(pETMALp + pYZ27GP) grown at 37 °C; Lane 3, SL3261(pETMALp + pIP5) grown at at 37 °C; Lane 4, SL3261(pETMALp + pGP1-2) grown at 28 °C; Lane 5, SL3261(pETMALp + pGP1-2) grown at 28 °C and subsequently induced for 2 hours at 37 °C.

fixed P_L promoter to induce an immune response in mice was compared. Unfortunately, the gene-10-Env fusion protein expressed by SL3261 was poorly immunogenic in Balb/c mice (results not shown), making this antigen unsuitable for comparative studies. The B subunit of cholera toxin is less suitable for these studies too, as this protein is a strong mucosal antigen by itself. Like most proteins, the maltose binding protein (MBP) is not immunogenic by itself when administered orally (unpublished results), but immunisation by the oral route with *Salmonella* strains producing this antigen does induce MBP specific antibodies (32). Therefore, MBP was used as a model antigen in these experiments. Plasmid pETMALp (Fig. 1) contains the gene coding for the maltose binding protein (*malE*) under control of the T7 promoter. The strains SL5283 and SL3261 containing pETMALp in combination with pGP1-2 (which contains a fixed P_L promoter) were not able to grow at 37 °C, indicating that the amount of MBP produced was toxic at this temperature (not shown). SL3261 strains containing pETMALp in combination with pYZ27GP, pIP5 or the fixed promoter plasmid pGP1-2, produced large amount of MBP (Fig. 6). As

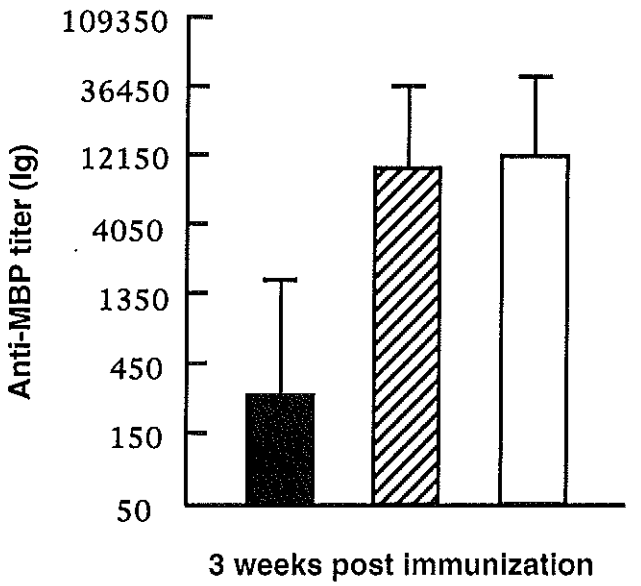

Fig. 7. Induction of MBP-specific antibodies by strains containing the two plasmid system with an invertible or fixed promoter. Mice were immunized orally with SL3261(pETMALp + pGP1-2) (filled bar); SL3261(pETMALp + pYZ27GP) (hatched bar) or SL3261(pETMALp + pIP5) (open bar), and antibody titers were determined 3 weeks post-infection. Each group contained 10 mice, with data presented as the mean titre + SD

expected, pIP5 revealed a lower production level of MBP than its higher copy number counterpart pYZ27GP. The production level obtained with SL3261(pETMALp + pYZ27GP) cultured at 37 °C was comparable with that of SL3261(pETMALp + pGP1-2) induced for 2 hours at 37 °C.

Three weeks after a single oral administration of the different MBP expressing strains, all mice had developed MPB specific antibodies. The average anti-MBP titers induced by oral immunisation with SL3261(pETMALp + pGP1-2) was nearly 40 times lower than the titers induced by the strains with the invertible promoter (Fig. 7).

DISCUSSION

Much effort has been put into the development of vectors for the stable production of heterologous antigens in *S.typhimurium* strains. Although chromosomal integration expression cassettes (12, 27), or linkage of an essential gene to the expression plasmid (10, 17) have proven to be very useful for the stable production of a number of antigens, these approaches will not allow stable production of antigens at levels that prevent cell growth. The use of promoters

that are induced *in vivo* is another approach that has been followed with some success. A more sophisticated kind of inducible promoter, delays toxic expression until the bacteria arrive in cells and tissues relevant for the immune system (7). This kind of promoters represent a significant improvement, but they too do not allow the production of toxic antigens during the complete route of infection nor will they allow further growth of the bacterial population once antigen production reaches toxic levels.

The essence of the phase variable expression system is, that it directs production of (toxic levels of) antigen in only a minor part of the bacterial population, allowing the non-producing bacteria to disseminate and segregate new producers. The two plasmid system described in this paper is an improvement of the previously described one plasmid system (31, 38, 39) in which the gene of interest is under direct control of an invertible P_L promoter. In the two plasmid system the invertible promoter controls the expression of T7 RNA polymerase gene, which in turn directs transcription of a gene under control of a T7 promoter, present on a second plasmid in the same bacterium. Therefore a single promoter inversion results in transcription of only one gene copy in the one plasmid system, but multiple gene copies in the two plasmid system. With the two plasmid system much higher levels of CtxB could be expressed compared to the one plasmid system (Fig. 2). This resulted in much stronger anti-CtxB IgG responses after oral and i.p. administration and higher specific serum and secretory IgA responses after oral administration (Fig. 3).

The two plasmid system can be used to produce antigens at toxic levels. This was demonstrated for the gene-10-Env fusion protein. When production of the Env fusion protein was dependent on a fixed, temperature inducible, P_L-promoter no colonies were recovered at the permissive temperature. However, when expression of the Env fusion protein was under control of the invertible P_L-promoter, a comparable number of colonies was recovered at permissive and non-permissive temperatures (Table 1).

The level of Env fusion protein (Fig. 5) and MBP (Fig. 6) production obtained with the two-plasmid system is probably a reflection of the percentage bacteria that is producing antigen due to a promoter inversion. Replacement of the p15A *ori* of the invertible promoter plasmids by the *ori* of the low copy number plasmid pSC101, resulted in invertible promoter plasmids that revealed a considerably lower antigen production level (Fig. 5 and 6). *In vivo* all invertible promoter vectors were essentially stable in combination with pET3xa-env, except for pIP1 which directs the highest production levels (Table 2). The extreme instability of the plasmid combination with pIP1 might be the result of

toxic antigen production in close to or more than 50% of the bacteria.

Plasmid stability *in vitro* can differ dramatically from *in vivo* stability. The different growth rates of *S.typhimurium in vitro* and *in vivo* may affect the plasmid stability in different ways. The longer generation time *in vivo* may result in an increased inversion frequency due to the longer time available for Gin to mediate the recombination event that results in promoter inversion. This will result in considerable plasmid loss when the inversion frequency approaches a level that prevents further expansion of the population. The restricted number of cell divisions *in vivo* will, on the other hand, limit the loss of plasmids. We have found that the plasmid stability after growth of *S.typhimurium* in cell lines correlates with *in vivo* stability (Table 2 and 3). Thus, an indication of plasmid stability *in vivo* may be obtained before animal experiments are initiated by determining the stability in cell lines.

Although satisfactory plasmid stability and expression levels of the Env fusion protein were obtained with a two plasmid system, we were not able to show the induction of Env-specific serum antibodies. A number of other studies also failed to demonstrated the induction of specific serum antibody responses against heterologous antigens produced by *S.typhimurium* vaccine strains, but showed the induction of specific helper T cells and class I restricted CTLs (1, 27, 36). Although not tested in the framework of this study, the phase variable expression system may have its merits for the generation of class I restricted CTL responses, as it allows abundant and stable production of antigens. Both these factors are of importance for the generation of class I restricted CTL responses (35, 37). Using MBP as a model antigen, we demonstrated that strains with the invertible promoter plasmids are able to induce a much stronger antibody response compared to strains with the fixed promoter plasmid pGP1-2, even though comparable production levels of MBP were obtained *in vitro*. Production of high levels of MBP was lethal for the bacteria and presumably strains with the invertible promoter, but not strains with a fixed promoter, are able to infect the GALT and the systemic lymphatics efficiently, thereby presenting much larger amounts of antigen to the immune system.

The phase variable expression system can be further improved by replacing the antibiotic resistance genes, by essential genes that have been deleted in the carrier strain (17). This will make it more acceptable for use in live bacterial vaccines and has the additional advantage that it may further stabilise the expression system.

ACKNOWLEDGEMENTS

We thank Geert van Amerongen, Herman Näring, Nico Schmidt and Ab van Arnhem, of the Animal Facilities Department for animal handling. The baculo derived recombinant HIV-1 IIIB gp120 glycoprotein was obtained from the MRC AIDS Directed Programme Reagent Project. The plasmids pGP1-2 and pT7-5 and the M13 phage mGP1-2 were kindly provided by Dr. S. Tabor.

This investigation was supported by a grant of the 'Raad voor Gezondheidsonderzoek', The Hague, The Netherlands (grant number 88-108/89027). Z.X. Yan was a scholarship recipient of Konrad-Adenauer-Stiftung, Bonn.

REFERENCES

1. **Aggarwal, A., S. Kumar, R. Jaffe, D. Hone, M. Gross, and J. Sadoff.** 1990. Oral *Salmonella*: Malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. *J. Exp. Med.* **172:**1083-1090.
2. **Bernardi A., and F. Bernardi.** 1984. Complete sequence of pSC101. *Nucleic Acid Res.***12:**9415-9426.
3. **Bullas, L.R. , and J.I. Ryu.** 1983. *Salmonella typhimurium* LT2 strains which are r-m+ for all three chromosomally located systems of DNA restriction and modification. *J. Bacteriol.* **156:**471-474.
4. **Chamberlin, M., J. McGrath, and L. Waskell.** 1970. New RNA polymerase from *Escherichia coli* infected with bacteriophage T7. *Nature* **228:**227-321.
5. **Chamberlin, M., and J. Ring.** 1973. Characterization of T7-specific ribonucleic acid polymerase. I. General properties of the enzymatic reaction and the template specificity of the enzyme. *J. Biol. Chem.* **248:**2235-2244.
6. **Charbit A., P. Martineau, J. Ronco, C. Leclerc, R. Lo-Man, V. Michel, D. O'Callaghan, and M. Hofnung.** 1993. Expression and immunogenicity of the V3 loop from the envelope of human immunodeficiency virus type 1 in an attenuated *aroA* strain of *Salmonella typhimurium* upon genetic coupling to two Escherichia coli carrier proteins. *Vaccine* **11:**1221-1228.
7. **Chatfield S.N., I.G. Charles, A.J. Makoff, M.D. Oxer, G. Dougan, D. Pickard, D. Slater and N.F. Fairweather.** 1992. Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. *Bio/Technology* **10:**888-892.
8. **Clements, J.D., F.L. Lyon, K.L. Lowe, A.L. Farrand, and S. El-**

Morshidy. 1986. Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. *Infect. Immun.* **53**:685-692.

9. **Curtiss III, R., S.M. Kelly, P.A. Gulig, and K. Nakayama.** 1989. Selective delivery of antigens by recombinant bacteria. *Curr. Top. Microbiol. Immunol.* **146**:35-49.
10. **Curtiss III, R., K. Nakayama, and S.M. Kelly.** 1989. Recombinant avirulent *Salmonella* vaccine strain with stable maintenance and high level expression of cloned genes *in vivo*. *Immunol. Invest.* **18**:583-596.
11. **Dröge, P., and F.M. Pohl.** 1991. The influence of an alternate template conformation on elongating phage T7 RNA polymerase. *Nucleic Acid Res.* **19**:5307-5312.
12. **Flynn, J.L., W.R. Weiss, K.A. Norris, H.S. Seifert, S. Kumar, and M. So.** 1990. Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Mol. Microbiol.* **4**:2111-2118.
13. **Gao, X.M., J.P. Tite, M. Lipscombe, S. Rowland-Jones, D.J.P. Ferguson, and A.J. McMichael.** 1992. Recombinant *Salmonella typhimurium* strains that invade nonphagocytic cells are resistant to recognition by antigen-specific cytotoxic T lymphocytes. *Infect. Immun.* **60**:3780-3789.
14. **Gonzalez C., D. Hone, F.R. Noriega, C.O. Tacket, J.R. Davis, G. Losonsky, J.P. Nataro, S. Hoffman, A. Malik, E. Nardin, M.B. Sztein, D.G.Heppner, T.R. Fouts, A. Isibasi, and M.M. Levine.** 1994. *Salmonella typhi* vaccine strain CVD908 expressing the circumsporozoite protein of *Plasmodium falciparum*: Strain construction and safety and immunogenicity in humans. *J. Inf. Diseases* **169**:927-931
15. **Hoiseth, S.K. and B.A.D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**:238-239.
16. **Kamp. D., Kahmann, R., Zipser D., Broker T.R. and Chow L.T.** (1978) Inversion of the G DNA segment of phage Mu controls phage infectivity. *Nature* **271**, 577-580.
17. **Nakayama, K., S.M. Kelly and R. Curtiss.** 1988. Construction of an *asd*$^+$ expression-cloning vector: Stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**:693-697.
18. **Newton, S.M.C., C.O. Jacob, and B.A.D. Stocker.** 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. *Science*

244:70-72.

19. **Pfeifer, J.D., M.J. Wick, R.L. Roberts, K. Findlay, S.J. Normark, and C.V. Harding.** 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361:**359-362.
20. **Plasterk, R.H.A., A. Brinkman, and P. van de Putte.** 1983. Site specific recombination by Gin of bacteriophage Mu: Inversion and deletions. *Virology* **127:**24-35.
21. **Plasterk, R.H.A., R. Kanaar, and P. van de Putte.** 1984. A genetic switch *in vitro*: DNA inversion by Gin protein of phage Mu. *Proc. Natl. Acad. Sci.* USA **81:**2689-2692.
22. **Poirier, T.P., M.A. Kehoe, and E.H. Beachey.** 1988. Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. *J. Exp. Med.* **168:**25-32.
23. **Rosenberg, A.H., B.N. Lade, D.S. Chui, S.W. Lin, J.J. Dunnn, and F.W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56:**125-135.

24.**Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. *A Laboratory Manual*, 2nd ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbor, N.Y.

25. **Sanderson K.E. and B.A.D. Stocker.** 1987 p. 1220-1228, *In* Neidhardt et al., (ed.), *E. coli* and S. typhimurium, Cellular and Molecular Biology, American Society for Microbiology, Washington, D.C.
26. **Schödel, F., S.M. Kelly, D.L. Peterson, D.R. Milich, and R. Curtiss III.** 1994. Hybrid hepatitis B virus core-pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. *Infect. Immun.* **62:**1669-1676.
27. **Strugnell, R.A., D. Maskell, N. Fairweather, D. Pickard, A. Cockayne, C. Penn, and G. Dougan.** 1990. Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium* vaccine strains. *Gene* **88:**57-63.
28. **Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.***189:**60-89.
29. **Tabor, S., and C.C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82:**1074-1078.
30. **Tabor, S.** 1990. Expression using the T7 RNA polymerase/promoter

system, p. 16.2.1-16.2.11 *In* F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed.) Current protocols in Molecular Biology. Greene Publishing and Wiley-Interscience, New York.

31. **Tijhaar, E.J., Z.X. Yan, J.A. Karlas, T.F. Meyer, M.J. Stukart, A.D.M.E. Osterhaus, and F.R. Mooi.** 1994. Construction and evaluation of an expression vector allowing the stable expression of foreign antigens in a *Salmonella typhimurium* vaccine strain. *Vaccine* **12:**1004-1011.
32. **Tijhaar, E.J., K.H.J. Siebelink, J. A. Karlas, M. C. Burger, F. R. Mooi and A.D.M.E. Osterhaus.** 1997. Induction of feline immunodeficiency virus specific antibodies in cats with an attenuated *Salmonella* strain expressing the Gag protein.Vaccine: In press
33. **Tite, J.P., X.M. Gao, C.M. Hughes-Jenkins, M. Lipscombe, D. O'Callaghan, and G. Dougan.** 1990. Antiviral immunity induced by recombinant nucleoprotein of influenza-A virus. III. Delivery of recombinant nucleoprotein to the immune system using attenuated *Salmonella typhimurium* as a live carrier. *Immunol.*, **70:**540-546.
34. **Tucker, W.T., C.A. Miller, and S.N. Cohen.** 1984. Structural and functional analysis of the *par* region of the pSC101 plasmid. Cell **38:**191-201.
35. **Turner, S.J., F.R. Carbone, and R.A. Strugnell.** 1993. *Salmonella typhimurium delta aroA delta aroD* mutants expressing a foreign recombinant protein induce specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes in mice. *Infect. Immun.* **61:**5374-5380.
36. **Valentine, P.J., K. Meyer, M.M. Rivera, C. Lipps, D. Pauza, R.T. Maziarz, M. M. So and F. Heffron.** 1996. Induction of SIV capsid-specific CTL and mucosal sIgA in mice immunized with a recombinant *S. typhimurium aroA* mutant. *Vaccine* **14**:138-146.
37. **Wick, M.J., J.D. Pfeifer, K.A. Findlay, C.V. Harding, and S.J. Normark.** 1993. Compartmentalization of defined epitopes expressed in *Escherichia coli* has only a minor influence on efficiency of phagocytic processing for presentation by class I and class II major histocompatibility complex molecules to T cells. *Infect. Immun.* **161:**4848-4856.
38. **Yan, Z.X.** 1992. Two-phase system for the development of live recombinant bacterial vaccines. Ph.D. thesis. Max Planck Institute, Tübingen, Germany.
39. **Yan, Z.X., F. Reuss, and T.F. Meyer.** 1990. Construction of an invertible DNA segment for improved antigen expression by a hybrid *Salmonella* vaccine strain. *Res. Microbiol.* **141:**1003-1004.
40. **Yang D.M., N. Fairweather, L.L. Button, W.R. McMaster, L.P. Kahl, and F.Y. Liew.** 1990. Oral *Salmonella typhimurium* (AroA-)

vaccine expressing a major Leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against Leishmaniasis. *J. Immunol.* **145:**2281-2285.

CHAPTER 4

Expression of genes encoding two major *Theileria annulata* merozoite surface antigens in *Escherichia coli* and a *Salmonella typhimurium aroA* vaccine strain

Gene 172:33-39 (1996)

Expression of genes encoding two major *Theileria annulata* merozoite surface antigens in *Escherichia coli* and a *Salmonella typhimurium aroA* vaccine strain

(Protozoan parasite; 30/32-kDa proteins; immunodominant; His_6-tag; T7 RNA polymerase/promoter; invertible promoter; immunodetection of gene products

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Received by P.F.G. Sims: 31 July 1995; Revised/Accepted: 16 November/21 November 1995; Received at publishers: 27 February 1996

SUMMARY

The genes, *Tams1-1* and *Tams1-2*, encoding the 30-and 32-kDa major merozoite surface antigens of *Theileria annulata* (*Ta*), have recently been cloned and characterized. Both genes encode a protein of 281 amino acids (aa) containing a putative hydrophobic N-terminal signal peptide. Another hydrophobic stretch is predicted at the C terminus which probably functions to anchor the protein in the membrane of the merozoite and piroplasm. Here, we report the successful expression of both *Tams1-1* and *Tams1-2* in *Escherichia coli* (*Ec*) using gene fragments lacking both hydrophobic domains. Attempts to produce high amounts of the entire recombinant (re-) protein, or a fragment containing the N terminus only, were unsuccessful. This is presumably due to the toxicity of these re-proteins. The internal part of both genes was also expressed in *Salmonella typhimurium* (*St*) *aroA* vaccine strain SL3261. We employed a dual-plasmid expression system based on an invertible promoter and selected the most stable *St* construct in vitro using liquid cultures and a macrophage-like cell line. The re-Tams1-1 protein produced in *Ec*, as well as in *St*, was recognized by monoclonal antibody (mAb) 5E1 specific to the 30-kDa protein. Both re-Tams1-1 and re-Tams1-2 were recognized by *Ta* immune calf serum.

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Abbreviations: *A*, absorbance (1 cm); aa, amino acid(s); Ab, antibody(-ies); Anb, antibiotic(s); Ap, ampicillin; *aroA*, gene encoding 3-enolpyruvylshikimate-5-phosphate synthetase; bp, base pair(s); *DHFR*, gene encoding dihydrofolate reductase; DMEM, Dulbecco's modified Eagle's medium; *Ec*, *Escherichia coli*; FBS, fetal bovine serum; Gin, invertase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; His_6-tag, six consecutive His residues; IPTG, isopropyl β-D-thiogalactopyranoside; ISCOM, immunostimulating complex; kb, kilobase(s) or 1000 bp; Km, kanamycin; *lacI*, gene encoding the *lac* repressor; LB, Luria-Bertani (medium); mAb, monoclonal Ab; Ni-NTA, nickel nitrilo-tri-acetic acid; nt, nucleotide(s); p, plasmid; p_L, leftward major promoter of bacteriophage λ; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/10 mM Na_2HPO_4/1.8 mM KH_2PO_4 pH 7.4); PCR, polymerase chain reaction; PolIk, Klenow (large) fragment of *Ec* DNA polymerase I; re-, recombinant; *St*, *Salmonella typhimurium*; SDS, sodium dodecyl sulfate; *Ta*, *Theileria annulata*; *Tams1-1* and *Tams1-2*, genes encoding Tams1-1 and Tams1-2; Tams1-1 and Tams1-2, 30- and 32-kDa major merozoite surface antigens of *Ta*, respectively; [], denotes plasmid-carrier state.

INTRODUCTION

Theileria annulata (*Ta*) is a tick-borne protozoan parasite, which causes tropical theileriosis in cattle. The disease is distributed from Southern Europe and Northern Africa, through the Middle East into Central Asia (Uilenberg, 1981). Tropical theileriosis is characterized by a lympho-proliferative and a lympho-destructive phase, usually accompanied by a marked anaemia. *Ta* enters the bovine host during tick feeding as sporozoites, which rapidly invade mononuclear leukocytes. Here, they mature into macroschizonts and induce proliferation of the host cell. Macroschizonts develop ultimately into merozoites, which are released from the leukocyte and invade erythrocytes to develop into piroplasms (for a review, see Tait and Hall, 1990).

Tropical theileriosis is currently controlled by chemotherapy and vaccination using attenuated macroschizont-infected lymphoid cell lines, and by tick control using acaricides. Although attenuated live vaccines are being used with success in endemic areas, their application has a number of drawbacks. Therefore, considerable effort is currently undertaken to identify and produce immunogenic antigens by re-DNA techniques. Such antigens, if shown protective, could provide a basis for a subunit vaccine against tropical theileriosis.

The merozoite stage is invasive and thus a potential target for a protective immune response. A recent study identified a molecule of 30 kDa on the surface of *Ta* (Ankara stock) merozoites (Glascodine et al., 1990). This molecule is a major polypeptide of merozoites and piroplasms and strongly recognized by immune sera from cattle infected with *Ta*. It has a variable molecular mass in different parasite lines of clonal origin (Dickson and Shiels, 1993).

Recently, the genes *Tams1-1* and *Tams1-2* encoding the 30- and 32-kDa major *Ta* merozoite surface antigens (Ankara stock), respectively, have been cloned and characterized by sequence analysis (Shiels et al., 1994; 1995). In order to assess the potential of these molecules for use in diagnosis and as components in a multi-unit re-vaccine, this study was carried out to express the *Tams1-1* and *Tams1-2* genes in *Ec* and the *St aroA* vaccine strain SL3261.

RESULTS AND DISCUSSION

(a) Cloning and expression of both *Tams1-1* and *Tams1-2* in *Ec*

The nt sequences encoding the 30- and 32-kDa major merozoite surface antigens translate into polypeptides of 281 aa each. They are 88% identical at the aa level. Both proteins exhibit a strong hydrophobic N terminus (aa 1 through 24) characteristic of a signal peptide and a strong hydrophobic C terminus (aa 261 through 281) characteristic as anchor peptide (Shiels et al., 1994; 1995). *Tams1-1* and *Tams1-2* DNA was cloned in frame with a stretch of six His residues (His_6-tag) at the N terminus of expression vector pQE10 (QIAGEN Inc., Chatsworth, CA, USA). PCR primers were chosen to facilitate the cloning of the genes and specific fragments thereof (Fig. 1). These fragments included: (*i*) the complete gene (primers A and D), (*ii*) the complete gene lacking the region coding for the hydrophobic N terminus (primers B and D), (*iii*) an internal gene fragment without the sequences coding for both hydrophobic domains (primers B and C), and (*iv*) the complete gene lacking the region coding for the hydrophobic C terminus (primers A and C).

Whole cell extracts of uninduced and induced *Ec* M15[pREP4][pETams1-1], -[pETams1-2], -[pETams1-1+] and [pETams1-2+] (Fig. 1) were analysed by SDS-PAGE, followed by Western blotting using mAb 5E1 specific to the 30-kDa protein and *Ta* immune calf serum (Fig. 2). Upon induction by IPTG, *Ec* M15[pREP4][pETams1-1] and -[pETams1-2] synthesized large amounts of re-protein (Fig. 2A, lane 6 and 8, respectively). These proteins could not be detected in uninduced cells (Fig. 2A, lanes 5 and 7, respectively), or in the vector-only control (Fig. 2A, lane 2). The presence of the hydrophobic C terminus resulted in a considerable reduction in the production of re-protein (Fig. 2A, lanes 10 and 12 arrow). These re-proteins exhibit a similar mobility when compared to the re-proteins lacking the hydrophobic domains (Fig. 2A, compare lanes 6 and 8 with lanes 10 and 12). This may be due to charge differences which cause this aberrant mobility when performing SDS-PAGE. Attempts to express gene fragments containing the region encoding the hydrophobic N terminus were unsuccessful, presumably due to toxicity of the encoded protein. The mAb 5E1 recognized both re-Tams1-1 but not the re-Tams1-2 proteins (Fig. 2B, lanes 6 and 10), whereas *Ta* immune calf serum reacted with all re-Tams1-1/Tams1-2 proteins (Fig. 2C, lanes 6, 8, 10 and 12). The observed reactivity of 5E1 with the Tams1-1 re-antigens was an unexpected result. This is because, previously, treatment of native antigen by mild periodate oxidation significantly reduced detection by the Ab (Dickson and Shiels, 1993), indicating that it recognizes an epitope conferred by modification of the polypeptide by carbohydrate (Woodward et al., 1985). The same was shown for the re-Tams1-1 proteins (C. d'O. and F.J., unpublished data). Possession of this epitope on the Tams1-1 re-polypeptides implies that either they are being modified in a prokaryotic expression system or that a secondary structure of the polypeptide chain, which is insensitive to SDS-PAGE, is altered by mild treatment.

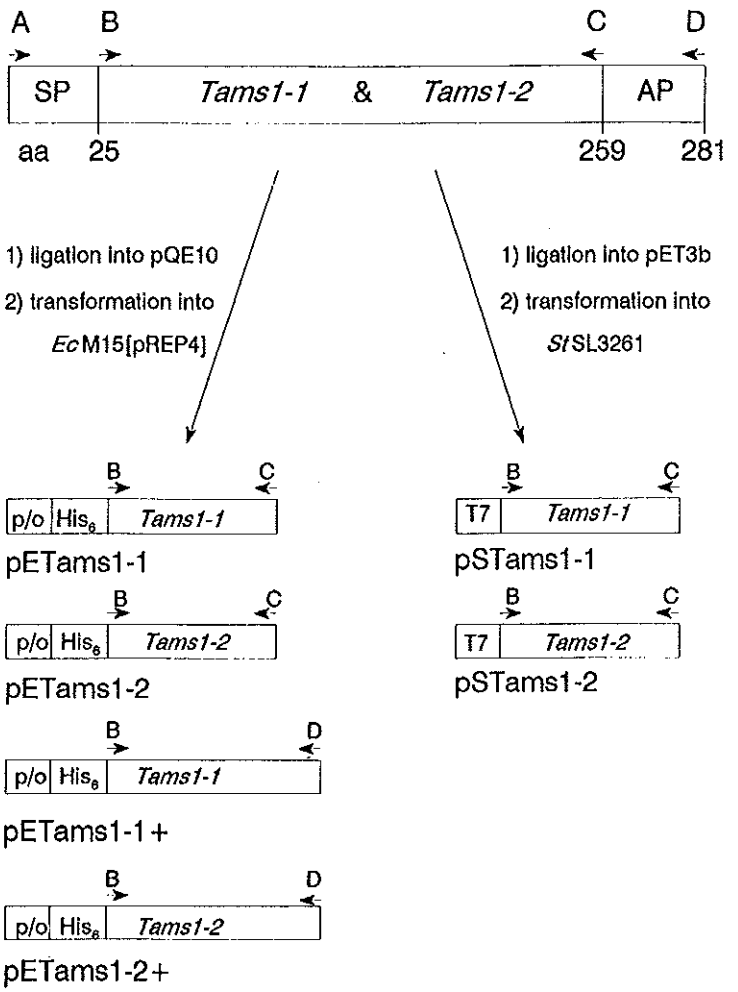

Fig. 1. Construction of expression cassettes for *Ec* and *St aroA*. PCR of *Tams1-1* and *Tams1-2* DNA using primers B (5'-AGGATGAAAAGAAAAAAGAAGGAAAAAAAAGACGTTG and C (5'-GTCGACAACTGGTTTGTAATC) or B and D (5'-GGGTTTTAAAGGAAGTAAAGG) resulted in 700- and 780-bp fragments, respectively, corresponding with aa 25 to 259 and aa 25 to 281, respectively.. Protruding ends were filled-in by PolIk and ligated into pQE10 vector digested with *Bam*HI and treated with PolIk. The resulting plasmids were verified by nt sequence analysis (Sanger et al., 1977) and used to transform *Ec* M15[pREP4] which resulted in M15[pREP4][pETams1-1], -[pETams1-2], [pETams1-1+] and -[pETams1-2+], respectively. In addition, the fragment generated with primers B and C was ligated into pET3b vector digested with *Bam*HI and treated with PolIk. Resulting plasmids pSTams1-1 and pSTams1-2 were verified by nt sequence analysis and used to transform *St aroA* SL3261 (Hoiseth and Stocker, 1981). SP, signal peptide; AP, anchor peptide; p/o, *Ec* phage T5 regulated promoter containing two *lac* operator sequences; His_6, six consecutive His residues; T7, *Ec* phage T7 regulated promoter.

Expression of the gene encoding mouse DHFR from plasmid pQE16 served as a control for expression. Neither the His_6-tag nor the re-DHFR protein reacted with mAb 5E1 (Fig. 2B, lane 4) or immune calf serum (Fig. 2C, lane 4). Pre-immune serum taken from the same animal did not react with the re-proteins (data not shown).

(b) Purification of re-Tams1-1 protein from *Ec*

His_6-tagged re-Tams1-1 protein was purified under native conditions according to the manufacturer's instructions (QIAGEN). Fig. 3 shows SDS-PAGE and Western analysis of the purified re-Tams1-1 and re-Tams1-2 proteins together with *Ta* piroplasm extract

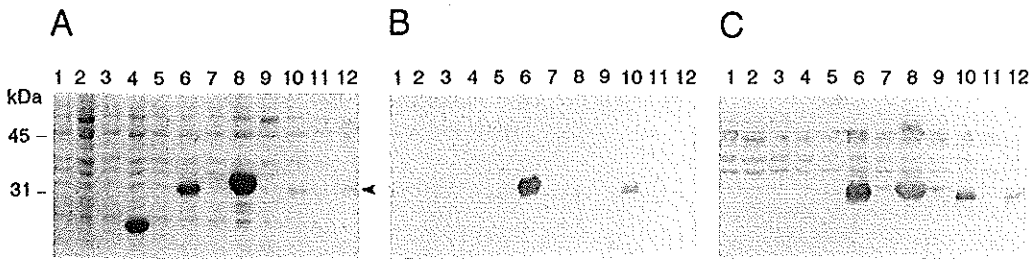

Fig. 2. Expression of *Tams1-1* and *Tams1-2* gene fragments by *Ec* transformants. (A) Gel stained with Coomassie brilliant blue. Lanes: 1 and 2, uninduced and induced *Ec* M15[pREP4][pQE10]; 3 and 4, uninduced and induced M15[pREP4][pQE16]; 5 and 6, uninduced and induced M15[pREP4][pETams1-1]; 7 and 8, uninduced and induced M15[pREP4][pETams1-2]; 9 and 10, uninduced and induced M15[pREP4][pETams1-1+]; 11 and 12, uninduced and induced M15[pREP4][pETams1-2+]. (B) Western blot using mAb 5E1. (C) Western blot using *Ta* immune calf serum. Methods: Single colonies of *Ec* transformants were grown for 16 h at 37°C in LB containing 100 µg Ap/ml and 25 µg Km/ml. A small aliquot was used to inoculate 10 ml of LB+Ap+Km medium and the bacteria were allowed to grow at 37°C while shaking. When the culture reached an $A_{600\,nm}$ of 0.7 an 1 ml aliquot was withdrawn as 'uninduced' sample, and subsequently 2 mM IPTG was added to induce expression. Induction was continued for 4 h, at which point a 250 µl aliquot was withdrawn as 'induced' sample. Cells were pelleted by centrifugation for 1 min at 10000 × *g* and resuspended in 50 µl of SDS-PAGE sample buffer (50 mM Tris·HCl pH 6.8/1% β-mercaptoethanol/2% SDS/0.1% bromophenol blue/10% glycerol). All samples were heated at 95°C for 10 min, centrifuged as described above and 10 µl aliquots of the supernatant was separated by 0.1% SDS-12% PAGE (Sambrook et al., 1989).

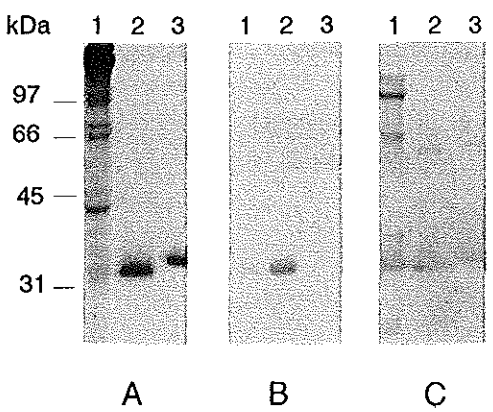

Fig. 3. Purification of re-Tams1-1 and re-Tams1-2 from *Ec* transformants. (A) Gel stained with Coomassie brilliant blue. Lanes: 1, piroplasm extract; 2, re-Tams1-1; 3, re-Tams1-2. (B) Western blot using mAb 5E1. (C) Western blot using *Ta* immune calf serum. Methods: Column-purified re-Tams1-1 and re-Tams1-2 (2 µg) were separated by SDS-PAGE (see legend to Fig. 2). As a control, purified piroplasms from infected bovine erythrocytes was used.

as a control. The mAb 5E1 recognized the 30-kDa protein in piroplasm extract (Fig. 3B, lane 1). The weak signal may be due to a low amount of available epitope for the mAb to react with. *Ta* immune calf serum detected the 30/32-kDa doublet as well as an array of other polypeptides (Fig. 3C, lane 1). Pre-immune serum taken from the same animal did not react with piroplasm extract (data not shown). Re-Tams1-1 protein is recognized by mAb 5E1 (Fig. 3B, lane 2) and both the re-Tams1-1 and re-Tams1-2 proteins were recognized by *Ta* immune calf serum (Fig. 3C, lanes 2 and 3, respectively). Plasmids pETams1-1 and pETams1-2 (Fig. 1) were verified by nt sequence analysis. They both encode a polypeptide of approximately 34 kDa. Therefore, the difference in mobility of the re-proteins seen on SDS-PAGE is presumably due to small differences in aa composition (Fig. 3A, compare lanes 2 and 3).

(c) Cloning and expression of both *Tam1-1* and *Tams1-2* in *St aroA*

In *Ec* the *Tams1-1* and *Tams1-2* gene sequences lacking the sequences that code for the N- and C terminus were well expressed. Therefore, these partial gene sequences were selected to be expressed in the *St aroA* vaccine strain SL3261 (Hoiseth and Stocker, 1981).

To obtain stable, high level expression we made use of a dual plasmid system based on an invertible promoter (Tijhaar et al., 1994; Tijhaar et al., submitted), as originally proposed by Podhajska et al. (1985). In this system the gene of interest is cloned downstream from the phage T7 promoter, so that expression becomes dependent on the presence of T7 RNA polymerase. The polymerase is encoded by a second plasmid present in the same bacterium. Transcription of the T7 RNA polymerase gene is controlled by a temperature inducible λ p_L promoter that inverts at random. This inversion is the result of recombination between two inverted repeats that border the λ p_L promoter by site-specific recombinase Gin. The T7 RNA polymerase gene is only expressed in one orientation of the promoter. The essence of the system is that the promoter inverts only in a minor part of the bacterial population. Therefore, expression of (toxic levels of) antigen will

be directed only in this part of the population, allowing the major non-producing part to disseminate and segregate new producers, thereby ensuring the continued production of re-antigen in the immunized host until SL3261 is cleared.

Both *Tams1-1* and *Tams1-2* gene fragments were cloned downstream from the phage T7 promoter in expression vector pET3b (Novagen, Madison, WI, USA) to yield plasmids pSTams1-1 and pSTams1-2, respectively (Fig. 1). Co-transformation of either *St* SL3261 [pSTams1-1] or -[pSTams1-2] cells with one of the four pIP plasmids, namely pIP2 (also called pYZ27bGP), pIP3 (also called pYZ27GP), pIP4 or pIP5 (Tijhaar et al., submitted), generated *St* SL3261[pSTams1-1][pIP] and SL3261[pSTams1-2][pIP], respectively. Plasmids pIP2 to pIP5 provide the T7 RNA polymerase gene under control of an invertible λ p_L promoter. They were constructed in such a way that the inversion frequency decreases from pIP2 to pIP5, resulting in a diminished percentage of the bacterial population producing re-protein. The percentage of the population producing re-protein may be of influence to the plasmid stability.

Whole cell extracts of the different SL3261 constructs grown at 37°C to induce p_L, were analysed by SDS-PAGE and Western blotting using mAb 5E1 and *Ta* immune calf serum (Fig. 4). As expected, the negative controls SL3261[pET3b], -[pSTams1-1], -[pSTams1-2] and -[pET3b][pIP5] did not show detectable expression of heterologous genes (Fig. 4A, B and C lanes 1 to 4, respectively). In contrast, SL3261[pSTams1-1] in combination with the different pIP constructs produced substantial amounts of re-Tams1-1 protein (Fig. 4A, lanes 5 to 8). Similar results were obtained for pSTams1-2 (Fig. 4A, lanes 9 to 12). The mAb 5E1 reacted with re-Tams1-1 protein only (Fig. 4B, lanes 5 to 8), whereas immune calf serum recognized both re-proteins (Fig. 4C, lanes 5 to 12). Plasmids pSTams1-1 and pSTams1-2 (Fig. 1) were verified by nt sequence analysis. Both translated products encode a polypeptide of approximately 31 kDa. The difference in mobility on SDS-PAGE between the re-Tams1-1 and re-Tams1-2 proteins was seen in different independent experiments and is probably due to small differences in aa composition (Fig. 4A, compare lanes 5 to 8 with lanes 9 to 12).

(d) Plasmid stability in *St aroA* SL3261

To determine the stability of the different plasmids in liquid cultures, the transformed *St* SL3261 strains were grown for 16 and 32 h at 37°C in the absence of Ap and Km. Aliquots from individual cultures were taken and subsequently grown on plates for 16 h at 37°C in the presence or absence of both antibiotics (Anb) (Table I). Two general observations could be made: (*i*) when comparing the re-Tams1-1 with the re-Tams1-2 producing bacteria after 32 h of growth in LB, the bacterial population producing re-Tams1-1 protein had a reduced ability to stably maintain both plasmids of the dual-plasmid expression system (Table I, column 3); (*ii*) when comparing the effect of the different pIP plasmids in both the re-Tams1-1 and re-Tams1-2 producing bacterial populations, the bacteria bearing pIP5 showed the highest degree of stable maintenance of both pIP and pSTams1-1 or pSTams1-2 (Table I, column 3). All plasmids were stably maintained when grown for 32 h at 37°C in the presence of Anb (data not shown).

More relevant is the in vivo stability of the different plasmid combinations. The in vivo plasmid stability has been shown to correlate well with the plasmid stability after growth of SL3261 strains in eukaryotic cell lines (Tijhaar et al., submitted). We, therefore, infected the macrophage cell line J774.16 (Ralph et al., 1975) with different SL3261 strains and subsequently determined the stability of the plasmids after a 48 h period of growth at 37°C (Table I). Again, two general observations could be made: (*i*) the bacterial population producing re-Tams1-2 protein maintained both their plasmids more stably than SL3261[pIP2][pSTams1-1] or -[pIP5][pSTams1-1] (Table I, column 4); (*ii*) when comparing both bacterial

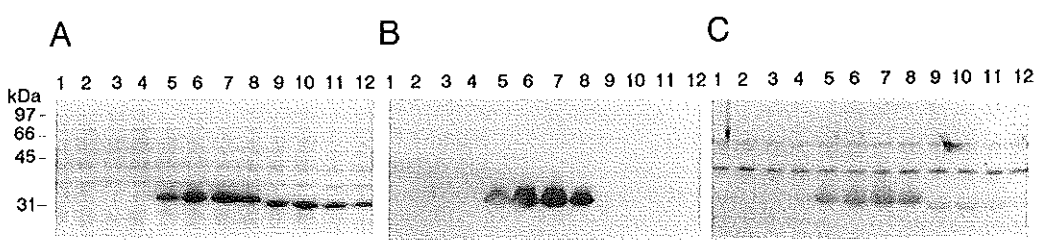

Fig. 4. Expression of *Tams1-1* and *Tams1-2* gene fragments by *St aroA* SL3261 transformants. (A) Gel stained with Coomassie brilliant blue. Lanes: 1, SL3261[pET3b]; 2, SL3261[pSTams1-1]; 3, SL3261[pSTams1-2]; 4, SL3261[pET3b][pIP5]; 5 to 8, SL3261[pSTams1-1][pIP2 to pIP5]; 9 to 12, SL3261[pSTams1-2][pIP2 to pIP5]. (B) Western blot using mAb 5E1. (C) Western blot using *Ta* immune calf serum. Methods: Single colonies were grown overnight at 37°C in 5 ml LB medium containing 100 μg Ap/ml and 25 μg Km/ml. Samples were taken for SDS-PAGE (see legend to Fig. 2).

TABLE I

Plasmid stability in *St aroA* SL3261

St aroA SL3261 [plasmids][a]	Plasmid stability		
	L broth[b]		J774.16 cells[c]
	16 h (%)	32 h (%)	48 h (%)
(1)	(2)	(3)	(4)
[pSTamsl-1][pIP2]	35	17	26
[pSTamsl-1][pIP3]	78	44	ND
[pSTamsl-1][pIP4]	56	33	ND
[pSTamsl-1][pIP5]	94	64	51
[pSTamsl-2][pIP2]	94	53	45
[pSTamsl-2][pIP3]	89	56	ND
[pSTamsl-2][pIP4]	92	95	ND
[pSTamsl-2][pIP5]	100	98	92

[a] Plasmids carried by this host are described in Fig. 1.

[b] Bacteria were grown in LB for 16 h at 37°C in the absence of both Ap and Km (16 h time point). Aliquots were taken for another 16 h of growth (32 h time point). After both time points, aliquots of appropriate dilutions were taken for growth on plates at 37°C in the presence (100 μg Ap/ml and 25 μg Km/ml) or absence of Anb. Colonies were counted and the plasmid stability of the different strains was defined as: [(number of resistant bacteria : total number of bacteria) × 100%].

[c] J774.16 cells, a continuous macrophage-like cell line derived from a reticulum cell sarcoma (Ralph et al., 1975), was grown in DMEM supplemented with 10% FBS/20 mM HEPES/2 mM L-glutamine/4.5 mg/ml D-glucose/1.25 μg fungizone/ml, further refered to as DMEM complete medium. Macrophages (5×10^5) were allowed to adhere to each well of a 6-well plate. An aliquot of an overnight SL3261 culture was serial diluted with DMEM and bacteria were added to each well at a ratio of approximately 1 bacterium per macrophage. The cells were incubated for 1 h at 37°C to permit phagocytosis. Free bacteria were removed by three washes with PBS and subsequently killed by incubation for 2 h at 37°C in DMEM complete medium supplemented with 150 μg gentamicin/ml. The medium was replaced by DMEM complete medium, supplemented with 20 μg gentamicin/ml. Wells were sampled at 48 h post infection by washing the cells 3 × with PBS and lysing with PBS/1% Triton-X100. Aliquots of appropriate dilutions were grown on plates for 16 h at 37°C in the presence and absence of Anb. The plasmid stability was expressed as described above. ND, not done.

populations producing re-Tams1-1 or re-Tams1-2, the bacteria bearing pIP5 showed the highest degree of stable maintenance of both pIP5 and pSTams1-1 or pSTams1-2 (Table I, column 4).

Clearly, the re-Tams1-1 protein has a negative effect on the stability of the plasmid combinations in *St* SL3261. This is probably due to a higher degree of toxicity of the re-Tams1-1 protein compared to re-Tams1-2. The bacteria bearing pIP5 maintained their plasmids more stably than bacteria bearing pIP2. This may be due to the fact that pIP5 has a different origin of replication that results in a lower plasmid number per bacterium and therefore a lower percentage of re-protein producing cells resulting in a lower selection pressure against the plasmids (Tijhaar et al., submitted).

(e) Conclusions

(*1*) Provided that they lack both the N- and C-terminal hydrophobic domains, the genes encoding the 30- and 32-kDa *Ta* major merozoite antigens can readily be expressed in *Ec* and *St* SL3261. When both *Tams1-1* and *Tams1-2* contained their signal sequences, no detectable expression could be obtained. The presence of hydrophobic N-terminal sequences is presumable highly toxic to the expression bacteria. Low-level expression is obtained from the constructs containing the hydrophobic C terminus.

The purified re-Tams1-1 and Tams1-2 proteins can now be incorporated into immunostimulating complexes (ISCOMs) (Morein et al., 1987) for immunization trials in cattle that are subsequently challenged with *Ta*. Successful immunization trials using ISCOMs have been reported for other protozoan parasites such as *Trypanosoma cruzi* (Araujo et al., 1991), *Toxoplasma gondii* (Lunden et al., 1993) and *Eimeria falciformis* (Kazanji et al., 1994).

(*2*) When infected macrophage-like cells (J774.16) were grown for 48 h at 37°C, *St* SL3261[pSTams1-1][pIP5] and SL3261[pSTams1-2][pIP5] retained their plasmids with the highest degree of efficiency. These are therefore the strains of choice for delivering both *Ta* merozoite re-proteins in future vaccination trials in cattle.

It has been shown that immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens induces humoral as well as cellular immune responses (Smith et al., 1984; for a review, see Cardenas and Clements, 1992; Coulson et al., 1994).

ACKNOWLEDGEMENTS

We thank Professor B.A.D. Stocker (Stanford University, USA) for kindly supplying *St* SL3261 and Dr. J.G. Kusters (Free University, Amsterdam, The Netherlands) for kindly supplying J774.16 cells. Drs. A.H.M. van Vliet, J.G. Kusters and A.W.C.A. Cornelissen are thanked for their interest throughout the work and for critical reading of the manuscript.

This work was supported by the European Union (Directorate General XII), under contract no. TS3*-CT91-0019 entitled 'The characterization of protective antigens and their genes from *Theileria annulata*'.

REFERENCES

Araujo, F.G. and Morein, B.: Immunization with *Trypanosoma cruzi* epimastigote antigens incorporated into ISCOMs protects against lethal challenge in mice. Infect. Immun. 59 (1991)2909–2914.

Cardenas, L. and Clements, J.D.: Oral immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens. Clin. Microbiol. Rev. 5 (1992) 328–342.

Coulson, N.M., Fulop, M. and Titball, R.W.: *Bacillus anthracis* protective antigen, expressed in *Salmonella typhimurium* SL3261, affords protection against anthrax spore challenge. Vaccine 12 (1994) 1395–1401.

Dickson, J. and Shiels, B.R.: Antigenic diversity of a major merozoite surface molecule in *Theileria annulata*. Mol. Biochem. Parasitol. 57 (1993) 55–64.

Glascodine, J., Tetly, L., Tait, A., Brown, C.G.D. and Shiels, B.R.: Developmental expression of a *Theileria annulata* merozoite surface antigen. Mol. Biochem. Parasitol. 40 (1990) 105–112.

Hoiseth S.K. and Stocker, B.A.D.: Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature 291 (1981) 238–239.

Kazanji, M., Laurent, F. and Péry, P.: Immune responses and protective effect in mice vaccinated orally with surface sporozoite protein of *Eimeria falciformis* in ISCOMs. Vaccine 9 (1994) 798–804.

Lunden, A., Lovgren, K., Uggla, A. and Araujo, F.G.: Immune responses and resistance to *Toxoplasma gondii* in mice immunized with antigens of the parasite incorporated into immunostimulating complexes. Infect. Immun. 61 (1993) 2639–2643.

Morein, B., Lovgren, K., Hoglund, S. and Sundquist, B.: The ISCOM: an immunostimulating complex. Immunol. Today 8 (1987) 333–338.

Podhajska, A.J., Hasan, N. and Szybalski, W.: Control of cloned gene expression by promoter inversion in vivo: construction of the heat-pulse-activated *att-nut*L-*p-att-N* module. Gene 40 (1985) 163–168.

Ralph, P., Prichard, J. and Cohn, M.: Reticulum cell sarcoma: an effector cell in antibody- dependent cell-mediated immunity. J. Immunol. 114 (1975) 898–905.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. CSHL Press, Cold Spring Harbor, NY, 1989.

Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.

Shiels, B.R., Smyth, A., Dickson, J., McKellar, S., Tetley, L., Fujisaki, K., Hutchinson, B. and Kinnaird, J.H.: A stoichiometric model of stage differentiation in the protozoan parasite *Theileria annulata*. Mol. Cell. Diff. 2 (1994) 101–125.

Shiels, B.R., d'Oliveira, C., McKellar, S., Ben-Miled, L., Kawazu, S. and Hide, G.: Selection of diversity at putative glycosylation sites in the immunodominant merozoite/piroplasm surface antigen of *Theileria* parasites. Mol. Biochem. Parasitol. 72 (1995) 149–162.

Smith, B.P., Reine-Guerra, M., Hoiseth, S.K., Stocker, B.A.D., Habasha, F., Johnson, E. and Merritt, F.: Aromatic-dependent *Salmonella typhimurium* as modified live vaccines for calves. Am. J. Vet. Res. 45 (1984) 59–66.

Tijhaar, E.J., Zheng-Xin, Y., Karlas, J.A., Meyer, T.F., Stukart, M.J., Osterhaus, A.D.M.E. and Mooi, F.R.: Construction and evaluation of an expression vector allowing the stable expression of foreign antigens in a *Salmonella typhimurium* vaccine strain. Vaccine 11 (1994) 1004–1011.

Tijhaar, E.J., Karlas, J.A., Zheng-Xin, Y., Meyer, T.F., Osterhaus, A.D.M.E. and Mooi, F.R.: High level and stable expression of heterologous antigens in *Salmonella typhimurium* vaccine strains using a two component plasmid system. Infect. Immun. (1996) in press.

Tait, A. and Hall, F.R.: *Theileria annulata*: control measures, diagnosis and the potential use of subunit vaccines. Rev. Sci. Tech. Off. Int. Epiz. 9 (1990) 387–403.

Uilenberg, G.: Theilerial species of domestic livestock. In: Irvin, A.D., Cunningham, M.P. and Young, A.S. (Eds.), Advances in the Control of Theileriosis. Martinus Nijhoff, The Hague, The Netherlands, 1981, pp. 4–37.

Woodward, M.P., Young Jr, W.W. and Bloodgood, R.A.: Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Meth. 78 (1985)143–153.

CHAPTER 5

Induction of feline immunodeficiency virus specific antibodies in cats with an attenuated *Salmonella* strain expressing the Gag protein

Vaccine15: 587-596 (1997)

Induction of feline immunodeficiency virus specific antibodies in cats with an attenuated *Salmonella* strain expressing the Gag protein.

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ABSTRACT

Salmonella typhimurium aroA strains (SL3261), expressing high levels of the Gag protein of feline immunodeficiency virus (FIV) fused with maltose binding protein (SL3261-MFG), were constructed using an invertible promoter system that allows the stable expression of heterologous antigens at levels toxic for bacteria. A SL3261 strain expressing the B subunit of cholera toxin by a similar system (SL3261-CtxB) served as a control in FIV-immunization experiments. Cats immunized once orally or intraperitoneally with SL3261-MFG or SL3261-CtxB all developed serum antibodies to SL3261 lipopolysaccharide and against maltose binding protein or the B subunit of cholera toxin, respectively. Two intraperitonial immunizations with SL3261-MFG also resulted in the development of Gag specific serum antibodies. Two oral immunizations with SL3261-MFG primed for a Gag specific response, which was demonstrated upon FIV challenge. All challenged cats became infected and no significant differences in viral loads were found between SL3261-MFG and SL3261-CtxB immunized cats.

INTRODUCTION

Feline immunodeficiency virus (FIV) infection of cats shows many similarities with human (HIV) and simian immunodeficiency virus (SIV) infections. It infects its natural host persistently, is T lymphotropic, causes a progressive loss of $CD4^+$ T cells, can infect macrophages and astrocytes, and eventually causes severe immunodeficiency or feline AIDS[1-6]. Because of these similarities, FIV infection of cats is considered a useful small-animal model for the evaluation of vaccine strategies relevant for controlling HIV-1 infection of humans.

In the search for effective lentivirus vaccines different strategies like inactivated complete virus[7], subunits[8-12], peptides[13], live attenuated virus[14-17], viral carriers[18-20], bacterial carriers[21, 22] and nucleic acid immunizations[23, 24] are being explored. In the FIV-cat system, vaccination of cats with paraformaldehyde fixed T cells (FL-4 cells) persistently infected with FIV and

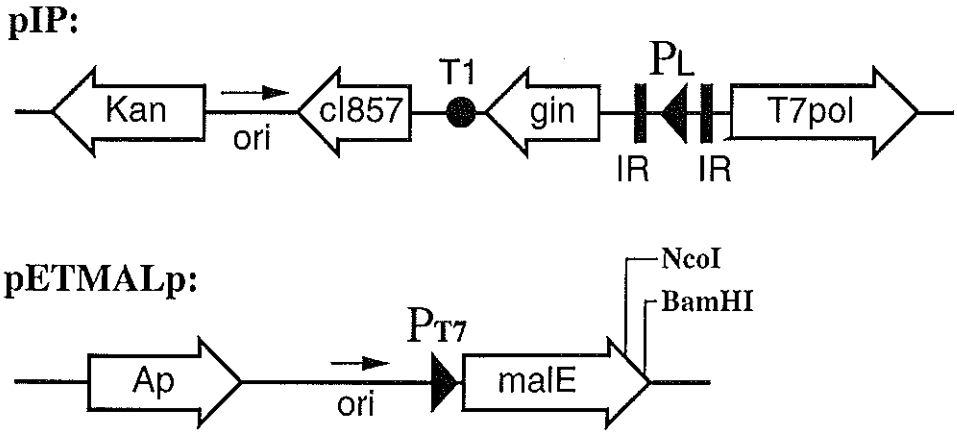

Fig. 1 The two plasmid invertible promoter system. P_L, leftward promoter of bacteriophage lambda; IR, inverted repeat; T1, rrnB T1 transcription terminator; gin, invertase gene; cI587, phage lambda temperature sensitive repressor protein gene; Kan, kanamycin resistance gene; Ap, ampicillin resistance gene; malE, gene coding for maltose binding protein; T7pol, T7 RNA polymerase gene; P_{T7}, *gene-10* promoter of bacteriophage T7. The invertible promoter of pIP controls transcription of the T7 RNA polymerase gene, which in turn directs transcription of a gene located under control of the P_{T7} promoter on a second plasmid (e.g. pETMALp, pETMALgag (not shown) or pT7ctxB (not shown)). In this study four different pIP-vectors (pIP2, -3, -4 and -5) were used. The vector pIP3 (=pYZ27GP) differs from pIP2 (=pYZ27bGP) by having an additional adenine in the untranslated leader sequence of the *gin* gene. The vectors pIP4 and pIP5 are derivatives from pIP2, respectively pIP3, in which the *p15A* origin of replication(*ori*) that results in an intermediate copy number, is replaced by the *ori* and partitioning (*par*) region of the low copy number plasmid pSC101. The *par* region ensures equal distribution of the plasmid over the daughter cells during cell division.

with paraformaldehyde inactivated FIV derived from the same cells, proved to be protective against homologous and to a lesser extent heterologous challenge[25, 26]. In the SIV-macaque system, vaccination with whole inactivated virus induced partial protection against challenge with PBMC from a SIVmac infected macaque[27]. Earlier reported protection against challenge with cell-free virus after immunizing with inactivated whole SIVmac was almost certainly mediated by the immune response to cellular proteins incorporated into both the immunogen and the challenge viruses[27, 28]. To date, the most effective protection of macaques against SIV infection has been obtained with live attenuated SIV[14, 29]. However, safety issues are still a major subject of debate[17].

Live recombinant carriers like pox-viruses, BCG and attenuated *Salmonella* strains are being evaluated as alternative approaches for lentivirus vaccine development. Attenuated *Salmonella* strains have been used successfully

to present heterologous bacterial[30, 31], viral[32-34] and protozoal[35-37] antigens to the immune system of mammals. Systemic humoral and cellular responses, including class I restricted cytotoxic T lymphocytes (CTL), and mucosal humoral responses have been shown to result from vaccination with recombinant *Salmonella* strains[35, 38-41].

The development of *Salmonella* bacteria as carriers for heterologous antigens has largely been hampered by problems with stability of expression or production levels caused by the toxicity of the antigens for the producing *Salmonella* strains. Especially expression of non-bacterial antigens may cause problems due to different codon usage or the presence of toxic (e.g. hydrophobic) sequences. Therefore we[42-45] developed a system that allows the stable expression of antigens at levels toxic for individual bacteria. The system is based on an invertible promoter that controls the expression of T7 RNA polymerase. A single promoter inversion results in expression of T7 RNA polymerase, which in turn directs expression from genes located under the control of a T7 promoter present on another vector in the same cell (Fig. 1). The key feature of the system is that the promoter controlling the T7 RNA polymerase expression inverts with a frequency that results in toxic levels of antigen expression in only a sub-population of the bacteria. Therefore the major part of the bacterial population will not be affected by expression of the heterologous antigen and will continuously segregate new antigen-producing bacteria.

Here we describe the use of this invertible promoter system to express high levels of FIV core protein as a fusion with the maltose binding protein (MBP) in the *S.typhimurium aroA* vaccine strain SL3261 and the use of this strain to induce specific immunity in cats.

MATERIAL AND METHODS

Bacterial strains, plasmids and media

Escherichia coli strain DH5α (BRL Life Technologies, Breda, The Netherlands) was used as a host for all plasmid constructions. Before introduction into the *S.typhimurium aroA* vaccination strain SL3261[46] plasmids were passaged through the restriction deficient, modification proficient *S. typhimurium* strain SL5283, a *galE503* derivative of LB5000[47, 48].

Strain BL21DEpLysS[49] is a lysogen of the *E.coli* strain BL21 that contains a copy of the T7 RNA polymerase gene in the chromosome under control of the IPTG inducible *lac*UV5 promoter[50] and the pLysS plasmid which

codes for T7 lysozyme; a natural inhibitor of T7 RNA polymerase. Strain BL21DEpLysS was used to express genes under the control of a T7 promoter. The T7 RNA lysozyme, encoded by pLysS, reduces the basal activity of T7 RNA polymerase in uninduced cells, thereby allowing the establishment of target genes whose basal expression would otherwise have been toxic to the cells. The vector pLysS confers resistance to chloramphenicol and is compatible with plasmids containing a ColE1 origin of replication[49]. All bacteria were grown aerobically at 37°C in Luria broth (LB), unless indicated otherwise. Ampicillin was used at 100 μg/ml in LB agar plates and 200 μg/ml in liquid media. Kanamycin and chloramphenicol were used at concentrations of 50 μg/ml and 35 μg/ml, respectively, in agar plates as well as in liquid media.

Construction of the plasmids pYZ27bGP (=pIP2), pYZ27GP (=pIP3), pIP4 and pIP5 has been described previously[43, 45]. The vectors pET3xa[49] and pMal-p were purchased from Invitrogen and New England Biolabs, respectively.

DNA manipulations

Isolation of plasmid DNA, preparation of DNA fragments and ligations were carried out using standard methods[51]. Plasmids were introduced into bacteria by electroporation as previously described[44].

Construction of plasmids used in this study

To increase the copy number of pET3xa[49] the *rop* gene, which negatively regulates plasmid copy numbers of ColE1 based plasmids, was removed by cleavage with *Bgl*II and *Pvu*II, followed by blunting of the ends with T4 DNA polymerase and religation of the plasmid. From the resulting plasmid, designated pET3xa1, a number of inconvenient restriction sites were removed by cleavage with *Eco*RI and *Eco*RV followed by blunting with T4 DNA polymerase and religation. The resulting plasmid was designated pET3xa2. The *malE* gene, which codes for precursor maltose binding protein (preMBP), was obtained by PCR using pMal-p (cat #800-61, New England Biolabs, Inc., Beverly, MA, USA) as template and the oligo nucleotides 5'-CGAGCATATGAAAATAAA AACAGGTGCACG-3' and 5'-GAATTCAGGCCTACCCTCGATGGATCC-3' as amplimers. The first amplimer was designed to introduce a *Nde*I site (underlined at the position of the ATG start codon of *malE*. The second amplimer overlapped partially with the multiple cloning site (MCS) of pMAL-p, so that the PCR product would contain the 5'-part of the MCS, which includes a unique *Kpn*I site. The PCR product was cleaved with *Nde*I and *Kpn*I and cloned into the corresponding sites of pET3xa2 to generate pETMALp (Fig. 1). In this plasmid

transcription of the *malE* gene is under the control of the T7 promoter.

The vector pETMALgag was constructed by replacing the small *Nco*I-*Bam*HI fragment of pETMALp (Fig. 1) by the *Nco*I-*Bam*HI fragment of pMAL-gag, which contained the 3'-end of the *malE* gene in frame with the complete coding part of the *gag* gene of the molecular FIV clone 19k1. In pETMALgag, transcription of the *malE-gag* fusion is under the control of the T7 promoter.

Western blot analysis of bacteria

For Western blot analysis SDS-PAGE was immediately followed by transfer of the proteins to nitro-cellulose. Blots were incubated for 30 minutes at room temperature with blocking buffer (BB) [0.5 % Tween-20 and 0.5% low fat milk powder in PBS]. Subsequently, the blots were incubated for one hour at room temperature with serum of an experimentally infected SPF cat (cat 89176), diluted 100 times in BB. After washing 3 times over a period of 30 minutes in PBS containing 0.5% Tween-20, the blot was incubated for 1 hour at room temperature with a biotinylated anti-cat IgG monoclonal (Sigma Immuno Chemicals, clone CT-21, cat.nr. B-226) diluted in BB. After washing, the blot was incubated for 30 minutes at room temperature with alkaline phosphatase conjugated ExtrAvidin (Sigma Immuno Chemicals, cat.nr. E2636) diluted in BB. Finally the blot was washed three times, soaked for 10 minutes in substrate buffer (0.1M Tris pH9.5, 0.1M NaCl, 0.05M $MgCl_2$) and developed in substrate buffer containing Nitro Blue Tetrazolium (NBT) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP).

Detection of antigen expression by electron microscopy.

To obtain an indication of the percentage of bacteria of strain SL3261-MFG4 (Table 1) that, due to a promoter inversion, expressed the MBP-Gag fusion protein, bacteria were grown in the presence of ampicillin and kanamycin until they reached the logarithmic phase. Subsequently, the bacteria were spun down (13.000 rpm) for 2 minutes, washed once with ice cold PBS and fixed with 4% paraformaldehyde in 0.1 M NaCacodylate (pH7.4). Then the bacteria were transferred to 2% gelatine, impregnated in 2.3 M sucrose and frozen in liquid nitrogen. Finally ultra thin cryosections were made. The cryosections were subjected to immunogold labelling using the Gag specific moAB 2-11[52] diluted 100x in PBS containing 0.5% BSA and 0.1% gelatine, and protein A conjugated to gold particles with a diameter of 10 nm (Aurion, Wageningen, The Netherlands, nr. 110.111) diluted 20x in the same buffer. The sections were stained and embedded into methyl cellulose according to the method of

Tokuyasu[53]. Subsequently, the sections were analysed by transmission electron microscopy (Phillips EM400). Bacteria were arbitrarily classified according to the amount of labelling. For this determination only complete longitudinal cross sections of bacteria were used.

Plasmid stability of bacterial strains during growth in thymocytes.
The assay for determination of plasmid stability of SL3261 strains after invasion of eukaryotic cells was essentially performed as described previously[45]. In short, thymocytes derived from a specified pathogen free (SPF) cat were stimulated for 3 days with Concanavalin A, washed and subsequently maintained in culture medium (RPMI 1640 [Gibco] supplemented with 10% (v/v) FCS, 2 mM L-glutamine, penicillin [100 IU/ml], streptomycin [100 µg/ml], L-glutamine [2mM] and IL2 [100 IU/ml, Cetus) at 37°C / 5% CO_2. About 10^8 bacteria from logarithmic phase cultures were added to 10^6 thymocytes in a total volume of 1 ml RPMI 1640 without any additives. After 2 hours incubation at 37°C the cells were washed and subsequently maintained at 37°C/5%CO_2 in 10 ml culture media containing gentamicin (50 µg/ml) to kill extracellular bacteria. After 24 hours the plasmid stability was determined by lysing the thymocytes and comparing the number of CFUs that could be recovered on plates without and with the appropriate antibiotics.

Immunization and FIV challenge of cats.

For immunization, bacteria from logarithmically growing cultures were harvested by centrifugation, washed, and resuspended in PBS. Subsequently, six months old specified pathogen free (SPF) cats, without detectable antibody levels against *Salmonella* LPS or CtxB, received 4 ml of $2.5x10^8$ cells/ml by the intraperitoneal (i.p.) route or 10 ml of $5x10^{10}$ cells/ml directly into the stomach to simulate oral administration. This "oral" immunization was performed with anaesthetized cats using a syringe connected to a thin tube that reached into the stomach. Just prior to oral administration of the bacteria, gastric juices were neutralized by administration of 10ml 10% $NaHCO_3$, using the same device.

Two cats were immunized with SL3261 expressing Gag, one cat by the oral route and the other by the i.p. route. The primary immunization (week 0) was performed with SL3261-MFG4, whereas the secondary immunization (week 9) was performed with equal amounts of strains SL3261-MFG2 and SL3261-MFG4 (Table 1). As a control, two cats were immunized with the CtxB expressing strain SL3261-CtxB (Table 1) at week 0 and 9. Again, one cat was

Table1 SL3261 strains used in this study.

Strain	Plasmids	Heterologous antigen
SL3261	no	no
SL3261(pETMALgag)	pETMALgag	no
SL3261-MFG2	pETMALgag + pIP2	MFG[a]
SL3261-MFG3	pETMALgag + pIP3	MFG[a]
SL3261-MFG4	pETMALgag + pIP4	MFG[a]
SL3261-MFG5	pETMALgag + pIP5	MFG[a]
SL3261-CtxB	pT7ctxB + pIP2	CtxB[b]

[a] MFG: fusion protein of Maltose binding protein and FIV-Gag.
[b] CtxB: B subunit of cholera toxin.

immunized by the oral route and the other by the i.p. route. Eighteen weeks after the primary immunization all cats were challenged intramuscularly with 30 ID50 of the molecular FIV clone 19k1[54]. Serum samples were taken at 1, 2, 3, 4, 5, 9, 10, 13 and 15 weeks after the primary immunization, immediately prior to FIV challenge and 4, 7, 10, 14, 19, 24, 28, 33 and 67 days post challenge.

Detection of FIV infection.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood and cultured in limiting dilutions as previously described[54] to enumerate percentages of FIV infected cells. The presence of FIV antigen in culture supernatants was detected with a FIV antigen capture ELISA as previously described[55].

ELISAs

LPS-ELISA. The detection of *S. typhimurium* LPS specific antibodies was essentially performed as previously described[44]. The incubation step with HRP conjugated anti-mouse antibody was replaced by an incubation for 90 minutes at 37°C with a biotinylated anti-cat IgG monoclonal antibody (Sigma Immuno Chemicals, clone CT-21, cat.nr. B-226) diluted 1:1000, followed by incubation for one hour at 37°C with a streptavidin-biotin-HRP complex (cat.nr. RPN1051, Amersham) also diluted 1:1000. The titer was defined as the highest dilution of test samples at which the O.D.450 was higher than the mean O.D.450 + 3 times S.D. of pre-immune sera.

CtxB-ELISA. The ELISA for the detection of CtxB specific antibodies

differed from the LPS ELISA in the coating step, which was performed in PBS containing 5.0 µg/ml CtxB (List Biological Labs, Campbell, CA, USA) and the dilutions of the biotinylated anti-cat IgG monoclonal antibody (1:2000) and the streptavidin HRP-conjugate (1:2500). In addition the ELISA-buffer in which the cat sera were diluted, was supplemented with 1% SL3261 sonificate prepared from a 50 times concentrated o/n bacterial culture to reduce background reactions. The titer was defined as the highest dilution of test samples at which the O.D.450 was at least three times higher than that of the corresponding serum dilution of the control cat immunized in a similar way with SL3261 expressing MBP-Gag instead of CtxB.

MBP-ELISA. The ELISA for the detection of MBP specific antibodies was performed identical to that for CtxB except that for the coating step 3.0 µg/ml MBP was used. The MBP was isolated from strain DH5α(pMALc) by affinity chromatography with maltose-Sepharose using a commercially available kit (Protein Fusion and Purification System, cat #800, New England Biolabs, Inc., Beverly, MA, USA). The titer was defined as the highest dilution of test samples at which the O.D.450 was at least two times higher than that of the corresponding serum dilution of the control cat immunized in a similar way with SL3261 expressing CtxB instead of MBP-Gag.

Gag-ELISA. Antibody titers against p17 and p24 core proteins were determined using a commercially available ELISA (FIV-p24/p17 antibody test kit, cat. nr. F1002-AB01, European Veterinary Laboratory BV., Woerden, The Netherlands).

RESULTS

Expression of FIV-Gag

For use in the two plasmid invertible promoter system (Fig. 1), the *malE* gene was placed under the control of the T7 promoter. The resulting plasmid, designated pETMALp (Fig. 1), was transferred to the *E.coli* strain BL21DEpLysS which contains on its chromosome the T7 RNA polymerase gene under control of the IPTG inducible *lac*UV5 promoter.

IPTG-induction of the T7 RNA polymerase gene in BL21DEpLysS-(pETMALp) resulted in the expression of the *malE* gene (Fig. 2). A prominent band with a Mw corresponding to preMBP, as well as a weaker band with a Mw corresponding to MBP from which the signal sequence had been cleaved-off, were visible on a Coomassie Blue stained SDS-PAGE gel.

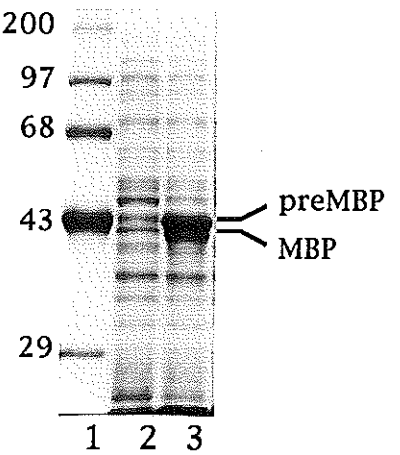

Fig. 2 Expression of MBP by BL21DEpLysS(pETMALp). Five hours after the addition of 1 mM IPTG to logarithmically growing bacteria, cells were harvested and total bacterial lysates were subjected to SDS-PAGE followed by staining of the gel. Lanes: 1. molecular weight markers; 2. BL21DEpLysS; 3. BL21DEpLysS-(pETMALp). Numbers on the left refer to the molecular sizes of the marker proteins in kDa.

To obtain high expression levels of the *gag* gene of the molecular clone FIV 19k1, it was cloned in frame with the *malE* gene of pETMALp and the resulting plasmid was designated pETMALgag. The plasmid pIP2 (=pYZ27bGP) (Fig. 1) contains the T7 RNA polymerase gene under control of an invertible P_L-promoter. *S.typhimurium* strain SL3261, harbouring pETMALgag in combination with pIP2, designated SL3261-MFG2, expressed high amounts of the MBP-Gag fusion protein (Fig. 3A). The presence of the Gag-moiety of this fusion protein was confirmed by Western blotting which shows the full-length MBP-Gag band and a number of break-down products (Fig. 3B).

Inversion frequency and plasmid stability

Previously[45] it had been demonstrated that pIP3, which differs from pIP2 by having an extra nucleotide in the untranslated leader sequence of *gin*, reveals slightly lower expression levels of genes under the control of a T7-promoter than pIP2. The vectors pIP4 and pIP5 are lower copy number derivatives of pIP2 and pIP3, respectively, which reveal significantly lower expression levels than the parental plasmids. The pIP-vectors were analyzed by SDS-PAGE (Fig. 4A) and Western blotting (Fig. 4B). Three individual clones were tested for each

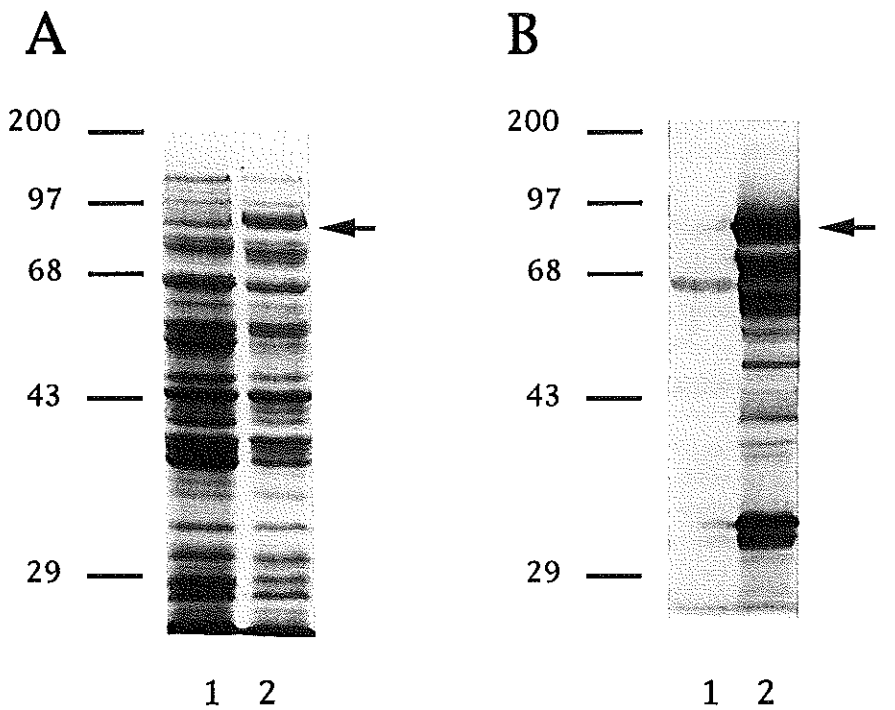

Fig. 3 Expression of MBP-Gag by SL3261 containing the two-plasmid invertible promoter system. Cells were grown at 37°C, lysed and subjected to SDS-PAGE. Lanes: 1. SL3261; 2. SL3261-MFG2. (A) Coomassie-blue stained SDS-PAGE gel; (B) Western blot using serum of an experimentally FIV-infected SPF cat. The arrow indicates the position of the full-length MBP-Gag fusion protein.

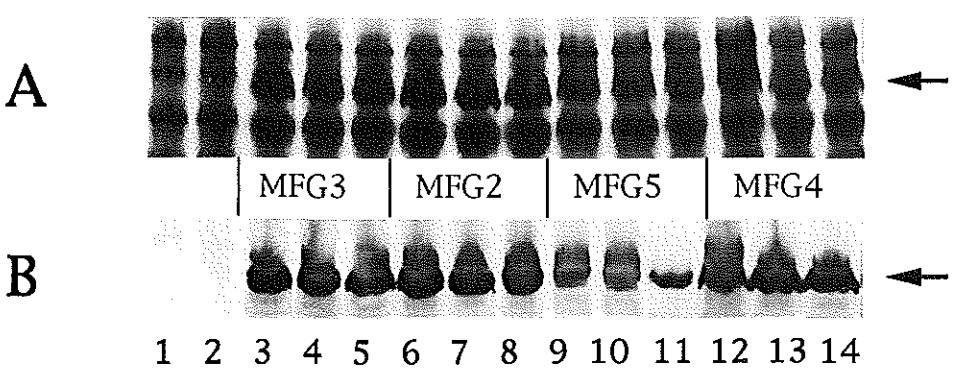

Fig. 4 Expression of MBP-Gag by different SL3261-MFG strains. Total cell lysates of bacteria grown at 37°C are shown. For each strain three individual clones were analysed. Lanes: 1. SL3261; 2. SL3261(pETMALgag); 3-5. SL3261-MFG3; 6-8. SL3261-MFG2; 9-11. SL3261-MFG5; 12-14. SL3261MFG4; (A) Coomassie-blue stained SDS-PAGE gel; (B) Western blot using serum of an experimentally FIV-infected SPF-cat. The arrow indicates the position of the MBP-Gag fusion protein. Only the relevant part of the SDS-PAGE and WB is shown.

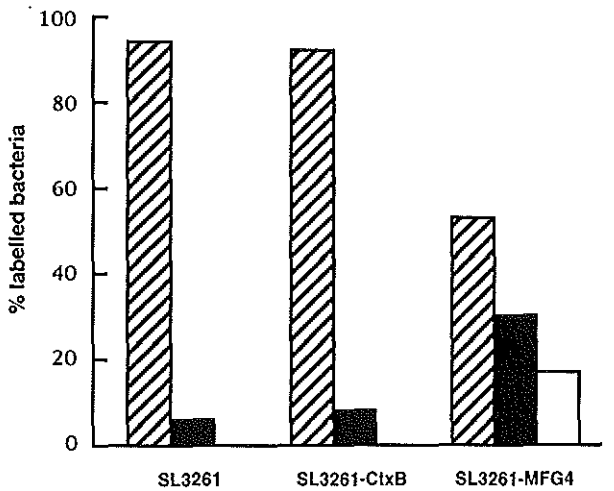

Fig. 5 Percentage immunogold labelled bacteria using a Gag specific monoclonal antibody. Logarithmically growing bacteria were fixed, immunogold labelled and analysed by transmission electron microscopy. Bacteria were classified according to the amount of goldparticles/cell: 0 (striped); 1-3 (black); more than 3 (dotted).

of the plasmid combinations. The highest MBP-Gag production was obtained for SL3261-MFG2, followed by SL3261-MFG3 and SL3261-MFG4, which reveal comparable expression levels, and finally SL3261-MFG5.

The plasmid stability of SL3261 strains after growth in eukaryotic cell lines gives an indication of the *in vivo* plasmid stability[45]. Therefore the plasmid stability of the SL3261-MFG strains was determined in cat thymocytes cultured *in vitro* (Table 3). In this invasion assay pETMALgag alone was stably maintained in SL3261, but the combinations of pETMALgag with pYZ27bGP (=pIP2), and pYZ27GP (=pIP3) were not. The majority of the bacteria harbouring one of these combinations lost one or both plasmids within 24 hours. Furthermore, the bacteria that still contained both plasmids, appeared to be mutants that did not express the fusion protein. In contrast, the combinations of pETMALgag with pIP4 or pIP5 were relatively stable and all the tested bacteria still expressed the MBP-Gag fusion protein.

Of the two stably expressing strains the highest expression levels were obtained with SL3261-MFG4. To obtain an indication of the percentage of the cells, that expressed the MBP-Gag fusion protein in this strain, bacteria from

Table 2. Immunization schedule of cats with SL3261 strains expressing heterologous antigens.

Cat (no)	Strain	Route	Immunizations (week)	Challenge[a] (week)
1	SL3261-CtxB	oral	0 and 9	18
2	SL3261-CtxB	i.p.	0 and 9	18
3	SL3261-MFG	oral	0 and 9	18
4	SL3261-MFG	i.p.	0 and 9	18

[a]Challenge was performed i.m. with 30 cat ID50 of the molecular clone FIV 19k1.

logarithmic growth cultures were fixed, immunogold labelled on ultra-thin cryosections and analysed by means of transmission electron microscopy. Bacteria were arbitrary classified according to the amount of labelling (Fig. 5). Of the cells of the negative control strains SL3261 and SL3261-CtxB, 94% and 92% were not labelled, respectively. The remaining 6% and 8% respectively, were labelled with only one to three gold particles. Of strain SL3261-MFG4 53% of the cells were not labelled, 30% contained one to three gold particles and 17% contained more than 3 particles, with an average of 10.5 particles/bacterium. Taking the 7% background labelling of the control strains into consideration, the percentage cells from strain SL3261-MFG4 expressing MBP-Gag is approximately 40% (=30-7+17) of the total population.

Immunogenicity of SL3261-MFG and SL3261-CtxB in the cat

Two cats were immunized with SL3261-MFG4 and two cats with strain SL3261-CtxB, which previously induced high titers of CtxB specific antibodies in mice[45]. Within each group one of the cats was immunized orally and the other one intraperitoneally (i.p.) (Table 2). All animals developed antibodies to *S. typhimurium* LPS within one week after the first immunization (Fig 6A). After administration of SL3261-CtxB, high anti-CtxB serum titers were induced (Fig 6B). After the primary immunization with SL3261-MFG4, considerable anti-MBP IgG titers were induced in the intraperitonially immunized cat (Fig 6C). The orally immunized cat developed a weak specific IgG response after the first immunization, but the second immunization at week 9 resulted in a considerably increased response.

After the primary immunization with SL3261-MFG4, no antibodies specific for the Gag-moiety of the MBP-Gag fusion protein could be detected.

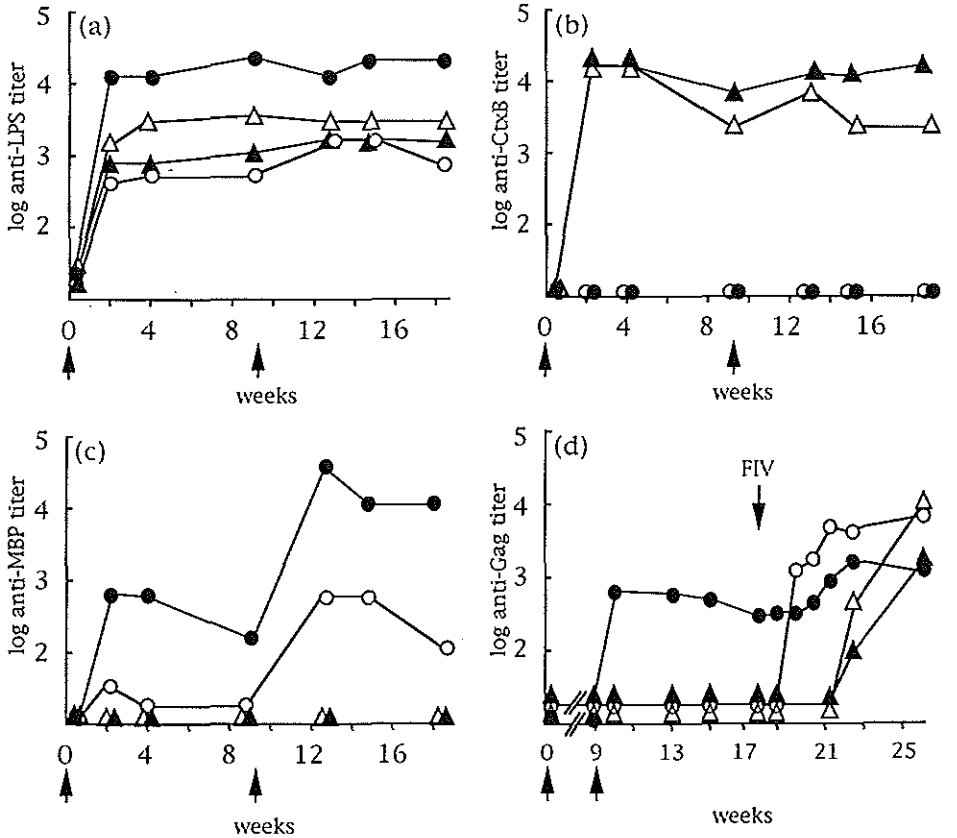

Fig. 6 The IgG antibody titers in cat sera immunized with recombinant *S.typhimurium* vaccine strains and challenged with FIV. Cats were immunized orally (open symbols) or intraperitonially (closed symbols) at week 0 and 9 with SL3261-MFG (circles) or SL3261-CtxB (triangles). Eight weeks after the booster immunization the cats were challenged intramuscularly with a dose of 30 cat ID_{50} FIV. (A) LPS-specific antibody titer; (B) CtxB-specific antibody titer; (C) MBP-specific antibody titer; (D) core proteins p17/p24-specific antibody titer.

This strain was selected for the primary immunization, because the plasmids in this strain were relatively stable in the invasion assay (see Table 3). The second immunization was performed with equal amounts of SL3261-MFG4 and SL3261-MFG2. Although the plasmid combination of SL3261-MFG2 was not stably maintained in the invasion assay, it was incorporated in the second immunization because it expressed higher levels of MBP-Gag. The idea was that SL3261-MFG2 would present a higher amount of MBP-Gag early in infection, while SL3261-MFG4 would give a lower but more stable expression. One week after the second immunization Gag-specific antibodies were detected in the serum of the cat immunized by the i.p. route (Fig. 6D). No Gag-specific antibodies were detected in the serum of the orally immunized cat.

To determine whether immunization with SL3261-MFG had induced

Table 3. Stability of recombinant SL3261 strains in cat thymocytes cultured *in vitro*.

Strain	Stability	
	plasmids[a]	expression[b]
SL3261(pETMALgag)	99.5 ± 0.5%	
SL3261-MFG2	17 ± 2%	0/2
SL3261-MFG3	7 ± 2%	0/2
SL3261-MFG4	71 ± 21%	4/4
SL3261-MFG5	79 ± 19%	3/3

[a] Cat thymocytes were infected with the different bacterial strains and cultured for 24 hours in the presence of gentamicin to kill extracellular bacteria. The plasmid stability was determined by lysing the thymocytes and comparing the number of CFU on LB-agar plates with and without all the appropriate antibiotics.
[b] Number of MBP-Gag expressing colonies / total number of tested colonies harbouring both plasmids.

Table 4. Detection of FIV in cat PBMCs after challenge.

Immunization: strain/route	presence of FIV at day (p.c.):						
	4	7	10	14	19	24	60
SL3261-MFG/oral	-	-	-	+	+	+	+
SL3261-MFG/i.p.	-	-	-	+	+	+	+
SL3261-CtxB/oral	-	-	-	-	+	+	+
SL3261-CtxB/i.p.	-	-	-	-	+	+	+

PBMC were stimululated with 10 µg/ml ConA for three days, washed and maintained in culture medium containing 100 IU/ml r-IL-2. The presence of FIV-antigen was detected using an antigen capture ELISA.

protective cellular immune responses, all cats were challenged intramuscularly with 30 ID50 of the homologous molecular clone FIV 19k1, eighteen weeks after the first immunization. Between 14 to 19 days post challenge (p.c.) all the animals had developed a persistent viraemia (Table 4). No significant differences in viral load were observed between the cats immunized with SL3261-MFG or SL3261-CtxB (Table 5). Both cats immunized with the SL3261-MFG strains developed an anamnestic response to Gag as antibody titers to Gag started to rise

Table 5. Viral load after FIV challenge.

Immunization: antigen/route	Infected cells per 10^6 PBMC (days p.c.):			
	14	19	33	67
SL3261-MFG/oral	NT	12.5	58	75
SL3261-MFG/i.p.	<12.5	NT	133	375
SL3261-CtxB/oral	<12.5	NT	79	150
SL3261-CtxB/i.p.	<12.5	NT	79	112

two weeks earlier in these cats than in the cats immunized with SL3261-CtxB (Fig. 6d).

DISCUSSION

In the present paper we have shown that the *S.typhimurium* strain SL3261 can be used efficiently as a vector to present heterologous antigens to the feline immune system. Cats immunized with SL3261-MFG developed a Gag specific immune response, but were not protected from intramuscular challenge with the homologous molecular clone FIV 19k1.

The development of candidate vaccines based on recombinant *Salmonella* as live carriers, has largely been hampered by the inability to stably express heterologous antigens at levels that are toxic for the producing bacteria. However, we previously[45] described a two plasmid invertible promoter system which may solve this problem. The system is based on the pIP-vectors which contain the T7 RNA polymerase gene under control of a promoter that inverts at random. When the promoter inverts to the ON position, T7 RNA polymerase is expressed which in turn directs expression of the gene of interest that has been positioned under the control of a T7 promoter on a second plasmid. The system can be stably maintained when a pIP vector is used that results in toxic antigen expression in only a minor part of the bacterial population.

In the present study we used the invertible promoter vectors pIP2 (=pYZ27bGP), pIP3 (=pYZ27GP), pIP4 and pIP5 to express the Gag protein of FIV as a fusion product with MBP. *S.typhimurium aroA* strains SL3261-MFG (Table 1) expressed high levels of the MBP-Gag fusion protein. The highest expression levels were obtained with SL3261-MFG2 followed by SL3261-MFG3 and SL3261-MFG4, which revealed similar expression levels, and finally SL3261-MFG5. Of the different SL3261-MFG strains, only SL3261-MFG4 and

SL3261-MFG5 stably maintained their plasmids after invasion of a cat thymocyte cell line (Table 3). Although SL3261-MFG3 and SL3261-MFG4 expressed similar amounts of the MBP-Gag fusion protein, indicating the presence of comparable percentages of antigen expressing bacteria, a striking difference in plasmid stability between both strains was observed. The higher plasmid stability of SL3261-MFG4 compared to SL3261-MFG3, is most likely the result of the partitioning (*par*) region present on pIP4 which is absent on pIP3. This *par* region ensures equal distribution of the plasmid copies over the daughter cells during cell division, what greatly enhances plasmid stability[56].

Immunogold labelling of SL3261-MFG4 demonstrated that about 40% of the bacteria expressed MBP-Gag (Fig. 5). This does not mean that all these expressing bacteria actually contain a pIP plasmid with the P_L promoter in the ON position. It may be speculated that a number of these cells have been derived from cells that divided after a promoter inversion occurred, but before antigen expression reached a level that prevented further cell divisions.

The *S.typhimurium* vaccination strain SL3261 appeared to be immunogenic in cats. After i.p. as well as after oral immunization, high specific antibody titers to *S.typhimurium* LPS were induced. Moreover, SL3261 can also be used as a vector to present heterologous antigens to the immune system of cats, as high serum antibody titers were induced to CtxB and MBP. The responses to FIV Gag were considerably weaker. A second immunization was necessary to induce detectable Gag specific serum antibodies upon i.p. immunization. After two oral immunizations no Gag-specific antibodies were detected, but the immune system had clearly been primed for Gag protein recognition, as after FIV challenge Gag-specific antibodies could be detected two weeks earlier than in the control cats. It is unlikely that this is the result of enhancement of FIV infection, as we observed with other FIV vaccine candidates[57], since the FIV loads of this cat at 33 and 67 days post challenge were even lower than those of the control cats. It should be stressed that the specific antibody responses were mainly determined as a marker to demonstrate that the heterologous antigens expressed by SL3261 are presented to the immune system of cats, since it cannot be expected that antibodies against the internal Gag proteins would neutralize FIV. In general *S. typhimurium* preferentially induces cellular immune responses, including cytotoxic T cell responses[35-38, 40] which may contribute to protective immunity[58]. No FIV specific T cell responses were determined in the framework of these experiments. However, if indeed Gag specific T helper and CTL responses were induced by these immunizations, they were apparently not sufficient to protect the cats against the homologous

challenge with 30 CID50 FIV.

We are currently investigating the immune responses and priming effects in cats, immunized with SL3261 strains expressing the Gag and envelope proteins of FIV in order to generate a more complete immune response to FIV proteins that will hopefully result in protection.

ACKNOWLEDGEMENTS

We thank Geert van Amerongen, Herman Näring, Nico Schmidt and Ab van Arnhem, of the Animal Facilities Department for animal handling and R. van Herwijnen (EVL) for performing the Gag specific ELISA.
This investigation was supported by a grant of the 'Raad voor Gezondheidsonderzoek', The Hague, The Netherlands (grant number 88-108/89027).

REFERENCES

1 Brunner, D., and Pedersen, N.C. Infection of peritoneal macrophages *in vitro* and *in vivo* with feline immunodeficiency virus. *J. Virol.* 1989, **63**, 5483-5488

2 Ackley, C.D., Yamamoto, J.K. Levy, N., Pedersen, N.C. & Cooper, M. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *J. Virol.* 1990, **64**, 5652-5655

3 Dow, S.W., Poss, M.L. and Hoover, E.A. Feline immunodeficiency virus: a neurotropic lentivirus. *AIDS* 1990, **3**, 658-668

4 Torten, M., Franchini, M., Barlough, J.E., George, J.W., Mozes, E., Lutz, H. and Pedersen, N.C. Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus. *J. Virol.* 1991, **65**, 2225-2230

5 Hoffmann-Fezer, G., Thum, J., Ackley, C., Herbold, M., Mysliwietz, J., Thefeld, S., Hartmann, K., Kraft, W. Decline in CD4+ cell numbers in cats with naturally acquired feline immunodeficiency virus infection. *J. Virol.* 1992, **66**, 1484-1488

6 Siebelink, K.H.J., Chu, I., Rimmelzwaan, G.F., Weijer, K., Van Herwijnen, R., Knell, P., Egberink, H.F., Bosch, M.L. and Osterhaus, A.D.M.E. Feline

immunodeficiency virus (FIV) infection in the cat as a model for HIV infection in man: FIV induced impairment of immune function. *AIDS Res. Hum. Retrov.* 1990, **6**, 1373-1378

7 The European Concerted Action on 'Macaques models for AIDS reseach. Protection of macaques against simian immunodeficiency virus infection with inactivated vaccines: comparison of adjuvants, doses and challenge viruses. *Vaccine* 1995, **13**, 295-300

8 Mannhalter, J.W., Fischer, M.B., Wolf, H.M., Küpcü, Z., Barrett, N., Dorner, F., Eder, G. and Eibl, M.M. Immunization of chimpanzees with recombinant gp160, but not infection with human immunodeficiency virus type I, induces envelope-specific Th1 memory cells. *J. Inf. Dis.* 1995, **171**, 437-440

9 Bruck, C., Thiriart, C., Fabry, L., Francotte, M., Pala, P., van Opstal, O., Culp, J., Rosenberg, M., de Wilde, M., Heidt, P. and Heeney, J. HIV-1 envelope-elicited neutralizing antibody titres correlate with protection and virus load in chimpanzees. *Vaccine* 1994, **12**,1141-1148

10 Vaslin, B., Le Grand, R., Vogt, G., Benveniste, O., Gras, G., Roques, P., Stoeckel, P., Salk, P. L., Salk, J. and Dormont, D. Induction of humoral and cellular immunity to simian immunodeficiency virus: what are the requirements for protection? *Vaccine* 1994, **12**,1132-1140

11 Hulskotte, E.G.J., Geretti, A.M., Siebelink, K.H.J., van Amerongen, G., Cranage, M.P., Rud, E.W., Norley, S.G., de Vries, P. and Osterhaus, A.D.M.E. Vaccine-induced virus neutralizing antibodies and cytotoxic T cells do not protect macaques from experimental infection with simian immunodeficiency virus SIVmac32H (J5). *J. Virol.* 1995, **69**, 6289-6296

12 Hu, S.-L, Abrams, K., Barber, G.N., Moran, P., Zarlin, J.M., Langlois, A.J., Kuller, L., Morton, W.R. and Benveniste, R.E. Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. *Science* 1992, **255**, 456-459

13 Golding B., Inman, J., Highet, P., Blackburn, R., Manischewitz, J., Blyveis, N., Angus, R. D. and Golding, H. *Brucella abortus* conjugated with

a gp120 or V3 loop peptide derived from human immunodeficiency virus (HIV) type 1 induces neutralizing anti-HIV antibodies, and the V3-*B. abortus* conjugate is effective even after CD4+ T-cell depletion. *J. Virol.* 1995, **69**, 3299-3307

14 Daniel, M.D., Kirchhoff, F., Czafak, S.C., Sehgal, P.K. and Desrosiers, R.C. Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. *Science* 1992, **258**,1938-1941

15 Putkonen, P., Walther, L., Zhang,Y.-J., Li, S.-L., Nilsson, C., Albert, J., Biberfeld, P., Thorstensson, R. and Biberfeld, G. Long-term protection against SIV-induced disease in macaques vaccinated with a live attenuated HIV-2 vaccine. *Nature Medicine* 1995, **1**, 914-918

16 Clements, J.E., Montelaro, R.C., Zink, M.C., Amedee, A.M., Miller, S. Trichel, A.M., Jagerski, B., Hauer, D., Martin, L.N., Bohm, R. P. and Murphey-Corb, M. Cross-protective immune responses induced in rhesus macaques by immunization with attenuated macrophage-tropic simian immunodeficiency virus. *J. Virol.* 1995, **96**, 2737-2744

17 Dittmer, U., Nißlein, T., Bodemer,W., Petry, H., Sauermann, U., Stahl-Hennig, C. and Hunsmann, G. Cellular immune response of rhesus monkeys infected with a partially attenuated *nef* deletion mutant of simian immunodeficiency virus. *Virol.* 1995, **212**, 392-397

18 Gallimore, A., Cranage, M., Cook, N., Almond, N., Bootman, J., Rud, E., Silvera, P., Dennis, M., Corcoran, T., Stott, J., McMichael, A. and Gotch, F. Early suppression of SIV replication by CD8+ *nef*-specific cytotoxic T cells in vaccinated macaques. *Nature Medicine* 1995, **1**, 1167-1173

19 Muster, T., Ferko, B., Klima, A., Purtscher, M., Trkola, A., Schulz, P., Grassauer, A., Engelhardt, O.G., García-Sástre, A., Palese, P. and Katinger, H. Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. *J. Virol.* 1995, **69**, 6678-6686

20 Mossman, S.P., Bex, F., Berglund, P., Arthos, J., O'Niel, S.P., Riley, D.,

Maul, D.H., Bruck, C., Momin, P., Burny, A., Fultz, P.N., Mullins, J.I., Liljeström, P. and Hoover, E.A. Protection against lethal simian immunodeficiency virus SIVsmmPBj14 disease by a recombinant Semliki Forest virus gp160 vaccine and by a gp120 subunit vaccine. *J. Virol.* 1996, **70**, 1953-1960

21 Fouts, T.R., Lewis, G.K. and Hone, D.M. Construction and charaterization of a *Salmonella typhi*-based human immunodeficiency virus type 1 vector vaccine. *Vaccine* 1995, **13**, 561-569

22 Winter, N., Lagranderie, M., Gangloff, S., Leclerc, C., Gheorghiu, M. and Gicquel, B. Recombinant BCG strains expressing the SIVmac251 nef gene induce proliferative and CTL responses against nef synthetic peptides in mice. *Vaccine* 1995, **13**, 471-478

23 Fuller, D.H. and Haynes, J.R. A qualitative progression in HIV type-1 glycoprotein-120 specific cytotoxic cellular and humoral responses in mice receiving a DNA-based glycoproteine-120 vacine. *AIDS Res. Hum. Retrov.* 1994, **10**, 1433-1441.

24 Wang B., Boyer, J.D., Gilbert, L., Dang, K.C., Ugen, K.E., Srikantan, V., Ayyvoo, V., Agadjanyan, M.G., Williams,W.V., McCallus, D., Carrano, R., Coney, L., Weiner, D.B. DNA-based immunization against HIV-1: induction of multiple *in vivo* immune responses. *AIDS Res. Hum. Retrov.*1995, **11**, p.S 136

25 Yamamoto, J.K., Okuda, T., Ackley, C.D., Louie, H., Pembroke, E., Zochlinski, H., Munn, R.J. and Gardner, M.B. Experimental vaccine protection against feline immunodeficency virus. *AIDS Res. Hum. Retrov.* 1991, **7**, 911-922

26 Yamamoto, J.K., Hohdatsu, T., Olmsted, R.A., Pu, R., Louie, H., Zochlinski, H.A., Acevedo, V., Johnson, H.M., Soulds, G.A. and Gardner, M.B. Experimental vaccine protection against homologous and heterologous strains of feline immunodeficency virus. *J. Virol.* 1993, **67**, 601-605

27 De Vries, P., Heeney, J.L., Boes, J., Dings, M.E.M., Hulskotte, E.G.J., Dubbes, R., Koornstra, W., ten Haaft, P., Akerblom, L., Eriksson, S.,

Morein, B., Norley, S. and Osterhaus, A.D.M.E. Protection of rhesus macaques from SIV infection by immunization with different experimental SIV vaccines. *Vaccine* 1994, **12**,1443-1452

28 Stott, E.J. Anti-cell antibody in macaques. Nature 1991, **353**, 393.

29 Lohman, B.L., McChesney, M.B., Miller, C.J., McGowan, E., Joye, S.M., Van Rompay, K.K., Reay, E., Antipa, L., Pedersen, N.C. and Marthas, M.L. A partially attenuated Simian Immumodeficiency Virus induces host immunity that correlates with resistance to pathogenic virus challenge. *J Virol.* 1994. **68**, 7021-7029.

30 Clements, J.D., Lyon, F.L., Lowe, K.L., Farrand, A.L., and El-Morshidy, S. Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. *Infect. Immun.* 1986, **53**, 685-692.

31 Poirier, T.P., Kehoe, M.A. and Beachey, E.H. Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. *J. Exp. Med.* 1988,**168,** 25-32.

32 Charbit A., Martineau, P., Ronco, J., Leclerc, C., Lo-Man, R., Michel, V., O'Callaghan, D. and Hofnung, M. Expression and immunogenicity of the V3 loop from the envelope of human immunodeficiency virus type 1 in an attenuated *aroA* strain of *Salmonella typhimurium* upon genetic coupling to two Escherichia coli carrier proteins. *Vaccine* 1993, **11**,1221-1228

33 Schödel, F., Kelly, S.M., Darrell, L., Milich, D.R. and Curtiss III, R. Hybrid hepatitis B virus core-pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. *Infect. Immun.* 1994, **62**,1669-1676

34 Tite, J.P., Gao, X.M., Hughes-Jenkins, C.M., Lipscombe, M., O'Callaghan, D. and Dougan, G. Antiviral immunity induced by recombinant nucleoprotein of influenza-A virus. III. Delivery of recombinant nucleoprotein to the immune system using attenuated *Salmonella typhimurium* as a live carrier. *Immunol.*, 1990, **70**, 540-546

35 Aggarwal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M. and Sadoff, J. Oral *Salmonella*: Malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. *J. Exp. Med.* 1990, **172**,1083-1090.

36 Sadoff, J.C., Ballou, W.R., Baron, L.S., Majarian, W.R., Brey, R.N., Hockmeyer, W.T., Young, J.F., Cryz, S.J., Ou, J., Lowell, G.H. and Chulay, J.D. Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science* 1988, **240**, 336-338.

37 Yang, D.M., Fairweather, N., Button, L.L., McMaster, W.R., Kahl, L.P. and Liew, F.Y. Oral *Salmonella typhimurium* (AroA-) vaccine expressing a major Leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against Leishmaniasis. *J. Immunol.* 1990, **145**, 2281-2285

38 Flynn, J.L., Weiss, W.R., Norris, K.A., Seifert, H.S., Kumar, S. and So, M. Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Mol. Microbiol.* 1990, **4**, 2111-2118.

39 Gao, X.M., Tite, J.P., Lipscombe, M., Rowland-Jones, S., Ferguson, D.J.P. and McMichael, A.J. Recombinant *Salmonella typhimurium* strains that invade nonphagocytic cells are resistant to recognition by antigen-specific cytotoxic T lymphocytes. *Infect. Immun.* 1992, **60**, 3780-3789

40 Turner, S.J., Carbone, F.R. and Strugnell, R.A. *Salmonella typhimurium delta aroA delta aroD* mutants expressing a foreign recombinant protein induce specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes in mice. *Infect. Immun.* 1993, **61**, 5374-5380

41 Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J. and Harding, C.V. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 1993, **361**, 359-362

42 Yan, Z.X., Reuss, F. and Meyer, T.F. Construction of an invertible DNA segment for improved antigen expression by a hybrid *Salmonella* vaccine strain. *Res. Microbiol.* 1990, **141**, 1003-1004

43 Yan, Z.X. 1992. Ph.D. thesis. Max Planck Institute, Tübingen.

44 Tijhaar, E.J., Yan, Z.X., Karlas, J.A., Meyer, T.F., Stukart, M.J., Osterhaus, A.D.M.E. and Mooi, F.R. Construction and evaluation of an expression vector allowing the stable expression of foreign antigens in a *Salmonella typhimurium* vaccine strain. *Vaccine,* 1994, **12**, 1004-1011

45 Tijhaar, E.J., Karlas, J.A., Yan, Z.X., Meyer, T.F., Osterhaus, A.D.M.E. and Mooi, F.R. Induction of enhanced immune responses by an expression system that allows continuous production of toxic antigens in a *Salmonella typhimurium* vaccine strains. *Submitted for publication.*

46 Hoiseth, S.K. and Stocker, B.A.D. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 1981, **291**, 238-239

47 Bullas, L.R. and Ryu, J.I. *Salmonella typhimurium* LT2 strains which are r-m+ for all three chromosomally located systems of DNA restriction and modification. *J. Bacteriol.* 1983, **156**, 471-474

48 Sanderson and Stocker, p. 1220-1228, in "*E. coli* and *S. typhimurium*, Cellular and Molecular Biology", Neidhardt et al., edit. 1987. Amer. Soc. Microbiol., Washington, D.C.

49 Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 1990, **189**, 60-89

50 Studier, F.W. and Moffatt, B.A. Use of T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 1986, **189**, 113-130

51 Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989, Molecular cloning. *A Laboratory Manual*, 2nd ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbor, N.Y.

52 Rimmelzwaan, G.F., Siebelink, K.H.J., Broos, H., Drost, G.A., Weijer, K.,

van Herwijnen, R. and Osterhaus, A.D.M.E. Gag and Env specific serum antibodies in cats after natural and experimental infection with feline immunodeficiency virus. *Vet. Microbiol.* 1994, **39**,153-165

53 Tokuyasu, K.T. Application of cryoultramicrotomy to immuno-cytochemistry. *J. Microscopy*, 1986, **143**, 139-149

54 Siebelink, K.H.J., Chu, I., Rimmelzwaan, G.F., Weijer, K., Osterhaus, A.D.M.E. and Bosch, M.L. Isolation and partial characterization of infectious molecular clones of feline immunodeficiency virus obtained directly from bone marrow DNA of a naturally infected cat. *J. Virol.* 1992, **66**,1091-1097

55 Siebelink, C.H.J., Windrich, R.W., Chu, I., Groen, J., Weijer, K., UytdeHaag, F.G.C.M. and Osterhaus, A.D.M.E. An enzyme-linked immunosorbent assay (ELISA) for the detection of feline immunodeficiency virus (FIV) antigen in cell culture and FIV specific antibodies in feline serum. *Dev. Biol. Stand.* 1990, **72**, 189-196.

56 Manen, D., Goebel, T. and Caro, L. 1990. The *par* region of pSC101 affects plasmid copy number as well as stability. *Mol. Microbiol.* 1990, **4**, 1839-1846.

57 Siebelink, K.H.J., Tijhaar, E., Huisman, R.C., Huisman, W., De Ronde, A., Darby, I.H., Francis, M.J., Rimmelzwaan, G.F., and Osterhaus, A.D.M.E. Enhancement of feline immunodeficiency virus infectivity after immunization with envelope glycoprotein subunit vaccines. *J. Virol.* 1995, **69**, 3704-3711

58 Flynn, J.N., Beatty, J.A., Cannon, C.A., Stephens, E.B., Hosie, M.J., Neil, J.C., and Jarret, O. Involvement of gag- and env-specific cytotoxic T lymphocytes in protective immunity to feline immunodeficiency virus. *AIDS Res. Hum. Retroviruses* 1995, **11**, 1107-1113

CHAPTER 6

Salmonella typhimurium aroA recombinants and immune stimulating complexes as vaccine candidates for feline immunodeficiency virus

Journal of General Virology: In press (1997)

Salmonella typhimurium aroA recombinants and immune stimulating complexes as vaccine candidates for feline immunodeficiency virus

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ABSTRACT

Two experimental feline immunodeficiency virus (FIV) vaccines were tested, either alone or in combination, in four groups of cats (A-D). One vaccine (SL3261-FIV) was composed of live attenuated Salmonella typhimurium aroA *(SL3261) strains expressing the capsid (Gag) and part of the envelope (Env) proteins of FIV. The other was composed of FIV-Gag and -Env proteins incorporated into immune stimulating complexes (iscom-FIV). Cats of group A were immunized four times with SL3261-FIV and of group B twice with SL3261-FIV followed by two immunizations with iscom-FIV. Cats of group C were immunized twice with SL3261 expressing the B subunit of Cholera toxin (SL3261-CtxB), followed by two immunizations with iscom-FIV. Cats of group D, which served as negative controls, received SL3261-CtxB twice followed by two immunizations with iscom in which the Gag and Env proteins of simian immunodeficiency virus (SIV) had been incorporated (iscom-SIV). Two weeks after the last immunization all cats were challenged. At this time cats immunized with iscom-FIV (groups B and C) had shown strong plasma antibody responses to Gag and Env, while these responses had been weak or undetectable in the cats immunized four times with SL3261-FIV. Seven weeks after FIV-challenge, Env-specific antibody responses had increased considerably in cats of all the groups except the one immunized four times with SL3261-FIV. The mean viral loads in the cats of this group, proved to be lower than those of the other groups at all time points, indicating partial protection.*

INTRODUCTION

Feline immunodeficiency virus (FIV) infection causes a disease in cats similar to AIDS in humans. Like human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), it is a T-lymphotropic lentivirus that persistently infects its natural host and causes loss of $CD4^+$ T cells, finally resulting in a severe immunodeficiency (Brunner & Pedersen, 1989; Ackley *et al.*, 1990; Dow

et al., 1990; Torten *et al.*, 1991; Hoffmann-Fezer *et al.*, 1992). These similarities make FIV infection of cats a useful animal model for the evaluation of HIV vaccination strategies.

Many of the vaccine strategies that have been explored to induce protective immunity against lentiviruses have been without or with limited success. Inactivated whole virus vaccines have been effective in the FIV system. Cats vaccinated with paraformaldehyde fixed T cells (FL-4 cells) persistently infected with FIV, and cats vaccinated with paraformaldehyde inactivated FIV derived from the same cells proved to be protected against homologous and to a lesser extent against heterologous FIV challenge infection (Yamamoto *et al.*, 1991, 1993; Hosie *et al.*, 1995). Similar approaches with vaccine preparations derived from other cells were unsuccessful (Hosie *et al.*, 1992; Verschoor *et al.*, 1995). The most impressive protection against SIV infection in macaques has been obtained by vaccination with live attenuated virus (Daniel *et al.*, 1992; Clements *et al.*, 1995), but safety concerns (Dittmer *et al.*, 1995) may limit the use of this approach.

Live carriers like canary pox, BCG and attenuated *Salmonella* strains are being extensively evaluated as alternative lentivirus vaccine candidates. Attenuated *Salmonella* strains have been used successfully as live carriers to elicit mucosal and systemic humoral responses as well as cellular responses, including class I restricted cytotoxic T cells (CTL), against a number of bacterial (Wick *et al.*, 1993), viral (Tite *et al.*, 1990; Charbit *et al.*, 1993; Schödel *et al.*, 1994; Valentine *et al.*, 1996) and protozoal (Aggarwal *et al.*, 1990; Flynn *et al.*, 1990; Yang *et al.*, 1990; Gonzalez *et al.*, 1994) antigens. Induction of mucosal immunity may be of particular relevance to HIV-1 vaccine development, since the most common route of infection is via mucosal surfaces of the genital tract.

Although attenuated *Salmonella* strains may present antigens to the immune system, the development of vaccines using *Salmonella* strains as live carriers has been hampered by low and/or unstable expression of heterologous antigens. To solve this problem we (Yan *et al.*, 1990; Yan, 1992; Tijhaar *et al.*, 1994; E.J. Tijhaar, J.A. Karlas, Z.X. Yan, T.F. Meyer, A.D.M.E. Osterhaus & F.R. Mooi, unpublished results) developed an expression system that allows abundant and stable expression of heterologous antigens by a bacterial population, even if antigen expression is toxic for individual bacteria. The system is based on an invertible promoter that randomly inverts. After switching to the "ON" position, it directs the expression of T7 RNA polymerase. The polymerase in turn directs expression of genes located under the control of a T7 promoter present on another vector in the same cell. The bacterial population

consists therefore of two populations, one of which expresses the gene of interest due to the promoter inversion, while the major subpopulation does not produce the antigen. Not hindered by the expression of the heterologous antigen, the non-producing population may disseminate within the host and segregate new antigen producers allowing a wide spread expression of the antigen in the body.

Previously we have used this invertible promoter system to express the *gag* gene of FIV in *Salmonella typhimurium* (Tijhaar *et al.*, 1997). Cats immunized intraperitoneally (i.p.) with these *Salmonella typhimurium* strains developed a clear antibody response to the Gag protein. Oral administration did not induce Gag-specific antibodies, but did prime the immune system, as became evident by the considerably faster and stronger response to Gag after challenge with FIV as compared to the control cats. However, none of the thus immunized cats proved to be protected. In the present study we have extended the immunization regimen by co-administering *Salmonella typhimurium* strains that express part of the *env* gene and subsequent boosting with recombinant Gag and Env proteins incorporated into immune stimulating complexes (iscom). Here we demonstrate that immunization with FIV-iscom, either alone or in combination with *Salmonella typhimurium* strains expressing FIV-Env and -Gag, resulted in the induction of higher specific plasma antibody titers. However, this did not correlate with reduced viral loads upon challenge. In contrast, repeated immunization with the FIV-Env and -Gag expressing *Salmonella typhimurium* strains alone did result in lower mean cell associated FIV loads upon challenge.

METHODS

Bacterial strains and media

The bacterial strains and their growth conditions have been described previously (Tijhaar *et al.* 1997).

Construction of plasmids

Construction of the invertible promoter plasmids pIP2 (=pYZ27bGP) and pIP4, and the T7 RNA polymerase controlled expression vectors pETMALp and pETMALgag, has been described previously (Yan, 1992; Tijhaar *et al.*, 1997; E.J. Tijhaar, J.A. Karlas, Z.X. Yan, T.F. Meyer, A.D.M.E. Osterhaus & F.R. Mooi, unpublished results).

Vector pETMALc was created by deletion of codons 9 to 26 of the *malE* gene of pETMALp, which code for the stretch of hydrophobic amino acids in the signal sequence of pre-maltose binding protein (MBP) that is essential for transport to the periplasm. This mutation was introduced because the toxicity of

cytoplasmatically localized MBP-fusion proteins, as coded for by pETMALc, is usually less and the expression higher than of MBP-fusion proteins that are directed to the periplasm. The mutation was performed using the "Transformer Site-Directed Mutagenesis Kit" (catalog number K 1600-1; Clontech Laboratories Inc., Palo Alto, CA, USA). The oligo 5'-AAAACAGGTGCACGC GAAGAAGGTAAACTGG-3' was used for introducing the deletion, while oligo 5'-ACCACGATGCCCGCAGCAATGGC-3' was used as the selection oligo. This oligo introduced a silent mutation in the ß-lactamase gene of pETMALp that destroys the *Pst*I-site which allowed selection against unmutated plasmids by linearisation with *Pst*I prior to transformation.

Western blot analysis of bacteria

Western blots were performed as previously described (Tijhaar *et al.*, 1997).

Plasmid stability of bacterial strains during growth in thymocytes

Thymocytes derived from a specified pathogen free (SPF) cat were stimulated for 3 days with 5 µg/ml concanavalin A (ConA) and subsequently maintained in culture medium (RPMI 1640 (GIBCO-BRL) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamin (2 mM), and ß-mercaptoethanol (2×10^{-5} M)) containing 100 IU/ml IL2 (Cetus). Prior to infection with the SL3261 strains, thymocytes were washed twice in RPMI 1640 without any additives. Bacteria from logarithmic growth cultures were harvested by centrifugation and washed twice with RPMI 1640. Approximately 10^8 bacteria were added to 10^6 thymocytes in a total volume of 1 ml RPMI 1640. Subsequently, cells were incubated for 2 hours at 37°C, during which period they were resuspended every 20 minutes. Finally the cells were washed twice in RPMI and suspended in 10 ml culture media supplemented with gentamicin (50 µg/ml) to kill extracellular bacteria and IL2 (100 IU/ml). After 24 hours at 37°C, cells from 1 ml of culture were harvested, washed two times in PBS and lysed PBS containing 1% Triton x-100 to release intracellular bacteria. The bacteria were plated on media without antibiotics and colonies were allowed to form at 28°C. Subsequently, at least 100 colonies of each sample were tested for resistance on plates containing ampicillin and kanamycin. The plasmid stability was expressed as the percentage bacteria that was still resistant to both antibiotics.

Preparation of iscom

FIV envelope glycoprotein iscom were prepared as described previously

(Rimmelzwaan *et al.*, 1994) from lectin purified protein derived from BHK cells infected with the recombinant vaccinia virus vGR657x15. This recombinant vaccinia virus expressed a form of the envelope protein from which the cleavage site between the surface (SU) and transmembrane (TM) part of the protein had been deleted to facilitate incorporation in iscom.

For the preparation of FIV Gag iscom, the complete coding sequence of the *gag* gene of molecular FIV clone 19k1 (Siebelink *et al.*, 1992) was cloned in frame with the bacterial expression vector pMALc (New England Biolabs Inc., Beverly, MA, USA) using PCR. *E.coli* containing the resulting plasmid, expressed a MBP-Gag fusion protein after IPTG induction, which was affinity purified on an amylose column according to the procedure recommended by the manufacturer. The MBP-Gag fusion protein was coupled to palmatic acid (Reid, 1992) and then incorporated into iscom, via this hydrophobic anchor, by a procedure similar to that described for the preparation of the FIV envelope iscom.

Iscom containing vaccinia derived SIV envelope glycoprotein and iscom containing SIV Gag derived from an MBP-Gag fusion protein, which were both a kind gift from E. Hulskotte, have been described elsewhere (Hulskotte *et al.*, 1995).

Table 1. Immunization schedule

Group	Immunization at week: 0 and 4	Immunization at week: 10 and 13	Challenge (i.m.) week 15
A	SL3261-FIV*	SL3261-FIV	30 CID50 FIV 19k1
B	SL3261-FIV	iscom-FIV§	30 CID50 FIV 19k1
C	SL3261-CtxB	iscom-FIV	30 CID50 FIV 19k1
D	SL3261-CtxB	iscom-SIV¶	30 CID50 FIV 19k1

Each cat received a total of 10^{11} bacteria by the oral route and 10^{9} by the i.p. route per immunization with SL3261. The iscom-immunized cats received 10 µg of each protein by the subcutaneous route. *SL3261-FIV: equal amounts of SL3261 strains expressing the *gag* gene and strains expressing part of the *env* gene of FIV (19k1). §iscom-FIV: FIV Env and Gag incorporated in iscom. ¶iscom-SIV: SIV Env and Gag incorporated in iscom.

Immunization of cats and FIV challenge

Immunizations were performed as shown in Table 1. Two Env-expressing strains (i.e. SL3261(pETMALenv + pIP2) and SL3261(pETMALenv + pIP4)) and two Gag-expressing strains (i.e. SL3261(pETMALgag + pIP2) and SL3261(pETMALgag + pIP4)) were used. Although only the plasmid combination of pETMALgag with pIP4 was stably maintained in the invasion assay (Tijhaar *et al.*, 1997), SL3261(pETMALgag + pIP2) was included in the vaccine formulation because of the higher expression levels obtained by this strain. The idea was that the combination with pIP2 would present a higher amount of antigen early in infection, while the combination with pIP4 would result in a lower but more stable expression. Equal amounts of the four different strains were used. For convenience this combination of Gag and Env expressing strains is in short referred to as SL3261-FIV. As a control the CtxB-expressing strain SL3261(pT7ctxB + pIP2) (Tijhaar *et al.*, 1997), in short referred to as SL3261-CtxB, was used. Per immunization each cat received a total of $5x10^{11}$ bacteria by the oral route and 10^9 bacteria by the i.p. route. Preparation and administration of the bacteria was performed as described previously (Tijhaar *et al.*, 1997). The iscom-immunized cats received 10 µg of each protein by the subcutaneous route.

All cats were challenged intramuscularly with thirty 50% cat infectious doses (CID50) of the homologous molecular FIV clone 19k1 (Siebelink *et al.*, 1992) two weeks after the last immunization. Plasma samples were taken immediately before the primary immunization and after 2, 4, 6, 9.5, 10.5, 11, 13, 15, 17, 17.7, 18.5, 20, 22, 26 and 32 weeks.

Detection of FIV infection

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood and cultured as previously described (Siebelink *et al.*, 1992). The presence of FIV antigen in the culture supernatant was detected using an FIV antigen capture ELISA (Siebelink *et al.*, 1990).

Semi-quantitative PCR of proviral DNA

A nested PCR end-point titration was performed on chromosomal DNA prepared from PBMC taken 16 weeks post challenge (pc). Ten microlitre reaction product of the first PCR reaction was used as template in the second (nested) PCR. As a negative control chromosomal DNA isolated in parallel from uninfected cells was used. The primer combinations used in the first PCR reaction were GGCCCTCCACAGGCATATCC (corresponding to nucleotides

1014-1033) with GCATTTTATATCCTGGTGAGCC (nucleotides 1670-1692), and for the nested PCR reaction GGCAAGAGAAGGACTAGGAGG (nucleotides 1106 - 1126) with GCACAGCTCGAGGAGACTTAGC (nucleotides 1440-1461). Both PCRs were "touch-down"-PCRs starting with two cycles at an annealing temperature of 70°C, followed by two cycles at 68, 66, 64, 62 and finally 25 cycles at 60°C. PCR samples were analyzed by electrophoresis on an 1.5% agarose gel containing ethidiumbromide. The end-point was defined as the lowest concentration template DNA that resulted in a positive PCR with all higher concentrations being positive as well.

Cell associated virus load: infectious center test

The cell associated virus load in FIV challenged cats was determined as described previously (Siebelink *et al.*, 1995). In short, serial dilutions of PBMC samples from infected cats were cocultivated with ConA and IL-2 stimulated PBMC from an uninfected SPF cat. After three weeks the culture supernatants were tested for the presence of FIV antigen by ELISA (Siebelink *et al.*, 1990). The cell-associated virus load was based on those dilutions that resulted in wells positive and negative for FIV antigen, assuming that one infected PBMC gave rise to antigen production after three weeks of cocultivation.

ELISA

The ELISAs to determine specific antibody responses against LPS from *Salmonella typhimurium* and against the MBP, were performed as described previously (Tijhaar *et al.*, 1997).

Antibodies against the Gag proteins p24 and p17 were detected with a commercially available test kit, using recombinant p24 and p17 proteins (catalog number F1002-AB01; European Veterinary Laboratory BV, Woerden, The Netherlands).

An ELISA based on the detection of antibodies to the bacterial fusion proteins SU1 and SU3 (see Fig. 1) was performed as described elsewhere (De Ronde *et al.*, 1994). Plasma samples were used in a 1:100 dilution for this ELISA.

RESULTS

Construction and characterization of pETMALc and pETMALenv

The vector pETMALp (Table 2) contains the *malE* gene under control of a T7 promoter. When present in bacteria that produce T7 RNA polymerase, large amounts of the MBP are expressed from this *malE* gene (Tijhaar *et al.*, 1997).

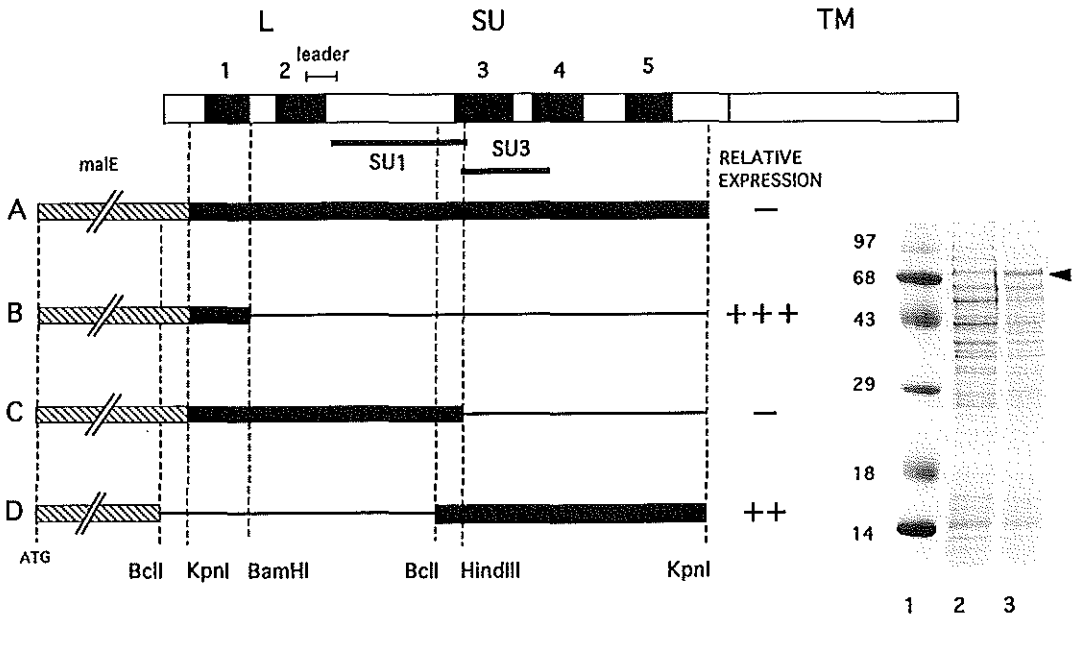


Fig. 1 Representation of different *MalE-env* fusions and their relative expression levels in SL3261(pIP4). The top bar represents the *env* gene of FIV encoding the envelope protein of FIV, including the leader (L), surface (SU) and transmembrane (TM) proteins. The hypervariable regions are represented as black boxes. The number of pluses are an arbitrary representation of the relative expression levels of the different *malE-env* fusions as determined by SDS-PAGE and Western blotting. The photo shows a stained gel of bacterial lysates subjected to SDS-PAGE. Lanes: 1. molecular mass markers (in kDa); 2. SL3261; 3. SL3261(pETMALenv (=construct D) + pIP4). The arrow head indicated the position of the MBP-Env fusion protein. The bacterial fusion products SU1 and SU3 used in the Env-ELISA are indicated as black bars.

The pIP-vectors (Table 2), which contain the T7 RNA polymerase gene under control of an invertible promoter, allowed the continuous production of an MBP-Gag protein by a bacterial population (Tijhaar *et al.*, 1997). The MBP-Gag protein was encoded by an in-frame fusion between the *malE* gene of pETMALp and the *gag* gene of FIV. Attempts to express parts of the FIV *env* gene using pETMALp were unsuccessful. In an attempt to reduce the toxicity of MBP-fusion proteins, the region of *malE* coding for the hydrophobic part of the signal sequence was deleted, so that the protein would remain localized within the cytoplasm. Expression of MBP by bacteria containing pIP4 (Table 2) in combination with the mutated plasmid, designated pETMALc, was at least as abundant as that from the unmutated plasmid pETMALp (Fig. 2). However, expression of the *Kpn*I-*Kpn*I *env*-fragment of the molecular clone FIV 19k1

Table 2. Main features of plasmids and bacterial strains used in this study

Plasmid or strain	Main features	Reference
plasmid		
pETMALp	*malE* (MBP), T7 promoter, Amp®	Tijhaar *et al.*, 1997
pETMALc	Δ signal seq. *malE*, T7 promoter, Amp®	this study
pETMALgag	*malE-gag* (MBP-Gag), T7 promoter, Amp®	Tijhaar *et al.*, 1997
pETMALenv	*malE-env* (MBP-Env), T7 promoter, Amp®	Tijhaar *et al.*, 1997
pT7ctxB	*ctxB* (CtxB), T7 promoter, Amp®	Tijhaar *et al.*, 1997
pIP2/pIP4*	T7 RNA polymerase gene, invertible promoter, Kan®	Tijhaar *et al.*, 1997
strain		
SL3261	*Salmonella typhimurium aroA* mutant	Hoiseth *et al.* 1981
SL3261-CtxB	SL3261 (pT7-CtxB + pIP2)	Tijhaar *et al.*, 1997
SL3261-FIV	*equal amounts of:*	
	SL3261 (pETMALgag + pIP2)	Tijhaar *et al.*, 1997
	SL3261 (pETMALgag + pIP4)	Tijhaar *et al.*, 1997
	SL3261 (pETMALenv + pIP2)	this study
	SL3261 (pETMALenv + pIP4)	this study

*Plasmid pIP4 is a lower copy number derivative of pIP2 (previously also designated pYZ27bGP), which directs lower expression levels of genes positioned under control of a T7 promoter than pIP2 (Tijhaar *et al.*, 1997).

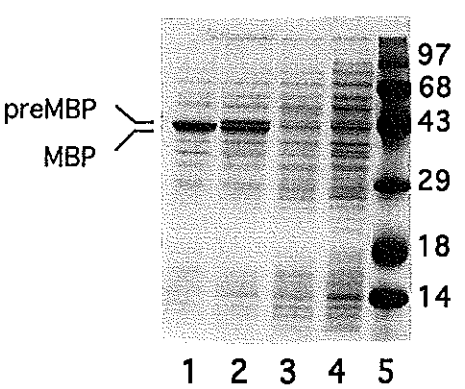


Fig. 2 Expression of MBP from *malE* with and without a functional signal sequence, using the two-plasmid invertible promoter system. Total bacterial lysates grown till late log phase at 37°C were subjected to SDS-PAGE followed by staining of the gel. Lanes: 1. SL3261(pETMALc + pIP4); 2. SL3261(pETMALp + pIP4); 3. SL3261(pETMALp); 4. SL3261; 5. molecular mass markers (in kDa).

(Siebelink *et al.*, 1992) in frame with the *malE* gene of pETMALc (Fig. 1: A) remained problematic. To determine which parts of the envelope gene were predominantly responsible for this lack of expression, different gene truncations were made (Fig. 1: B, C and D). The *malE* fusion with the *Kpn*I-*Bam*HI *env*-fragment was well expressed (Fig. 1: B). Introduction of a frameshift by filling in the *Hin*dIII-site (Fig. 1: C) resulted in a translational stop-codon within 7 codons of this site. No expression could be observed from this truncated *malE-env* fusion. The *Bcl*I-*Kpn*I fragment (Fig. 1: D) that codes for the variable regions V-3, V-4 and V-5 in which several neutralizing B cell epitopes are localized, was well expressed (Fig. 1). Therefore this plasmid, designated pETMALenv, was selected for immunization experiments.

Before the immunization experiments were started, the plasmid stability of SL3261 strains after invasion of cat thymocytes was determined. Plasmid pETMALenv proved to be stable in combination with pIP2 as well as with pIP4 (Table 3).

Plasma antibody responses after immunization

The day following the first *Salmonella* immunization (Table 1), two out of ten cats appeared not to be feeling well, as they were much less active than usually. Two to three days later these cats seemed to have recovered completely. No obvious side effects were observed following the *Salmonella* booster immunizations. All the cats developed plasma antibodies against *Salmonella typhimurium LPS*, within two weeks after the first immunization (Fig. 3). Considerable differences were observed between individual cats. Two weeks after the second immunization titers ranged from 800 to 50,000. The cats immunized with the MBP-Env and -Gag expressing SL3261 strains (groups A and B) developed antibodies specific for the MBP moiety of the fusion protein within two weeks after the first immunization. Two weeks after the second immunization the plasma titers were around 10,000 for most of the cats of groups A and B. Despite of the strong antibody responses to the MBP-moiety, no clear responses were detected against the Env or Gag moieties of the MBP fusion proteins. After the third immunization with SL3261-FIV, Gag-specific responses were detected in two of the three cats of group A (Fig. 3). After the first immunization with iscom-FIV (groups B and C), anti-Gag titers rose faster in the group that had previously been immunized with SL3261-FIV (group B), as compared to the group that had received SL3261-CtxB (group C) (Fig. 3) indicating a priming effect of the Gag-expressing SL3261 strains. The second iscom-FIV immunization of groups B and C at week 13, resulted in a

Table 3. Plasmid stability after invasion of cat thymocytes

Strain	Plasmid stability
SL3261(pETMALenv + pIP2)	93 ± 7%
SL3261(pETMALenv + pIP4)	92 ± 3%

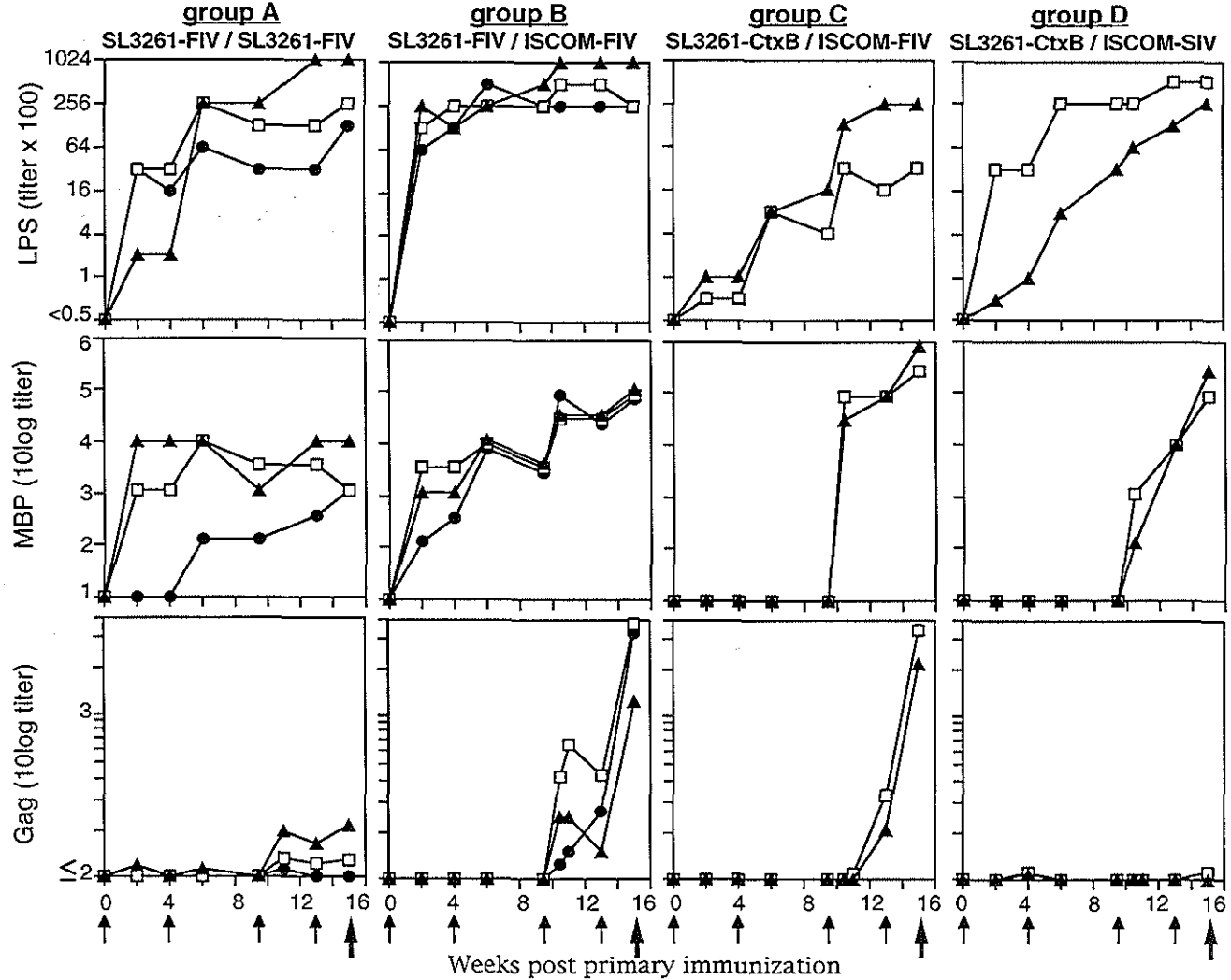
*Cat thymocytes were infected with the different bacterial strains and cultured in the presence of gentamycin to kill extracellular bacteria. After two days the percentage intracellular bacteria still resistant to both ampicillin and kanamycin was determined. Represented is the mean plasmid stability ± SD of three independent experiments.

considerable increase in the anti-Gag response (Fig. 3). The titers in the plasma samples of cats that had not been primed with SL3261-FIV (group C) were similar to those that had (group B). Even after a fourth immunization with SL3261-FIV, the Gag-specific antibody titer remained weak in the cats of group A. At the day of challenge only the plasma samples of the cats vaccinated twice with iscom-FIV (groups B and C) showed clear reactivity with the bacterial derived FIV envelope fusion protein SU3 (Fig. 4: 0 weeks post challenge). No significant antibody response to SU3 was detected after a single iscom-FIV immunization (results not shown). Cats of groups C and D showed an antibody response to MBP upon immunization with the respective iscom preparations, which contained MBP either as part of the Gag fusion protein (group C) or as a contaminant (group D) (Fig. 3).

Plasma antibody responses after FIV challenge

Two weeks after the last immunization all cats were challenged with homologous molecularly cloned FIV (FIV 19k1). Gag-specific antibody titers in the plasma samples from cats vaccinated with iscom-FIV (groups B and C) rose within three weeks pc. Cats of the control group (group D) and the group vaccinated four times with SL3261-FIV (group A) started to develop Gag-specific antibodies 5 weeks pc.

Within 2.5 weeks pc the antibody responses to bacterial derived FIV Env fusion proteins, started to increase in the cats immunized with iscom-FIV (groups B and C) (Fig. 4). At this time, both cats immunized with SL3261-CtxB/iscom-FIV (group C) and one out of three cats immunized with SL3261-FIV/iscom-FIV (group B), had also developed responses to SU1. At 5 weeks pc the plasma samples of all the cats of groups B and C displayed plasma antibodies specific for SU1 and SU3. Both cats of the negative control group (D) developed


group A
SL3261-FIV / SL3261-FIV
group B
SL3261-FIV / ISCOM-FIV
group C
SL3261-CtxB / ISCOM-FIV
group D
SL3261-CtxB / ISCOM-SIV
LPS (titer x 100)
1024
256
64
16
4
1
<0.5
MBP (10log titer)
6
5
4
3
2
1
Gag (10log titer)
3
≤2
0
4
8
12
16
Weeks post primary immunization

antibodies specific for SU3 between 2 and 7 weeks pc and one cat also developed antibodies to SU1. In contrast, the cats only immunized with SL3261-FIV (group A) did not develop detectable antibody responses to SU1 during the whole 16 week period of the experiment. Only one cat (no. 1) of this group developed antibodies to SU3, but later than the control cats (group D).

Development of viraemia after FIV challenge

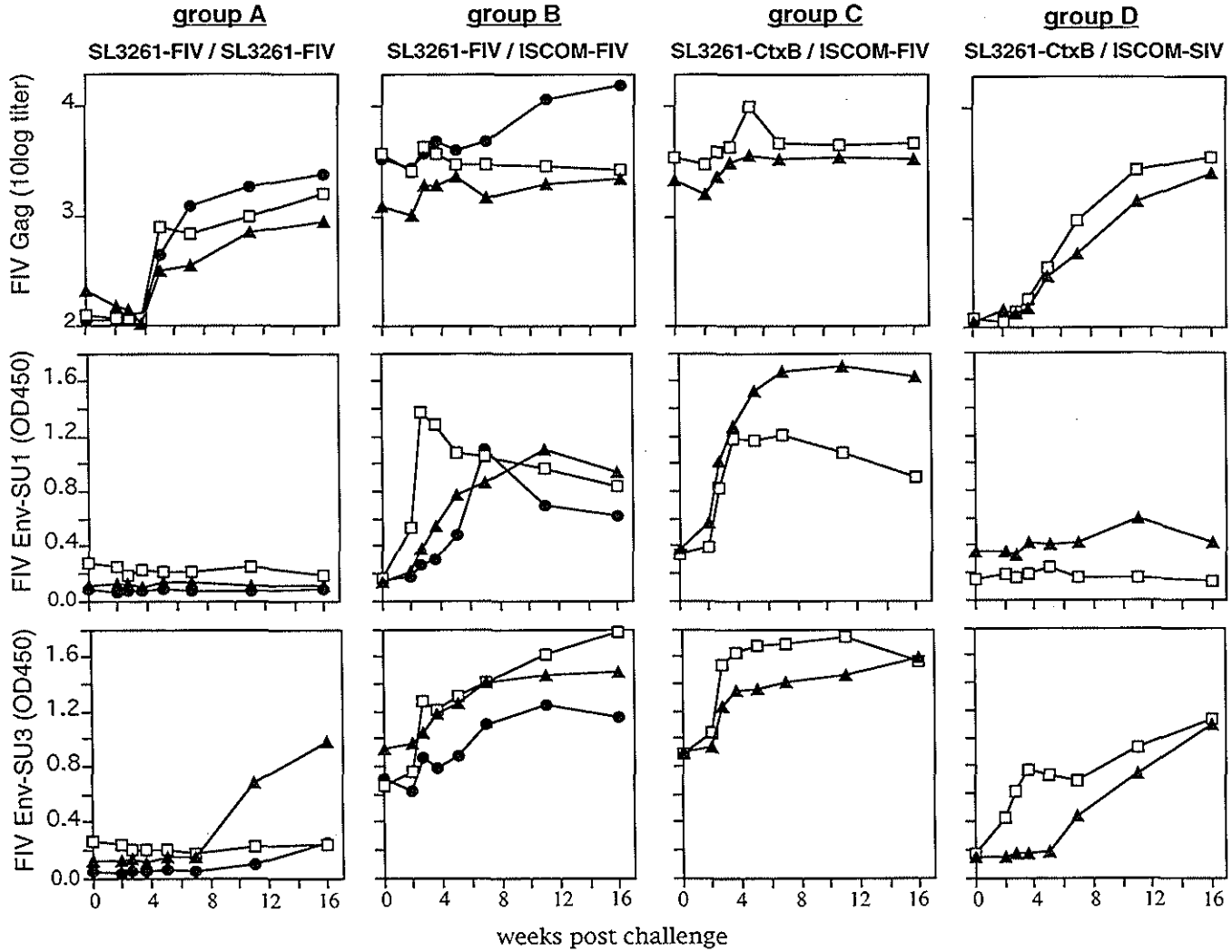
At 19 days pc FIV infected PBMC were detected in both cats of the control group (group D), in two out of three cats of group B and in one of the two cats of group C (Fig. 5). At this time no infected PBMC were detected in the cats immunized four times with SL3261-FIV (group A). At 25 days pc, FIV infected PBMC were detected in all cats, except for one cat of group A. At 110 days pc, FIV infected PBMC were demonstrated in all cats of groups B, C and D, but in only one cat of group A. The average cell-associated virus loads at this time point were 8, 217, 38 and 31 infected cells per 10^6 PBMC for groups A, B, C and D, respectively. PCR on chromosomal DNA isolated from PBMC taken at this time point demonstrated the presence of proviral FIV DNA in all cats (Fig. 6). The overall lowest levels of proviral DNA were observed in the cats immunized four times with SL3261-FIV (group A).

DISCUSSION

In the present study we have shown that cats immunized four times with FIV-Gag and -Env expressing *Salmonella typhimurium* strains, developed lower mean cell associated viral loads upon homologous FIV challenge than the other groups of cats studied. Immunization with FIV-iscom, either alone or in combination with the recombinant *Salmonella typhimurium*, did not result in a decrease of cell associated FIV load upon challenge. Due to the relatively small group sizes, the differences observed between the four groups can only provide an indication of a protective effect of vaccination in the first group of cats.

The Gag and Env proteins were expressed as fusions with MBP in the attenuated *Salmonella typhimurium aroA* strain SL3261. The expression levels of the different *env* gene truncations indicated that a region located between the *Bam*HI- and *Bcl*I-site of *env* (Fig. 1) had a prohibitive effect on the expression

Fig. 3 Development of antibody responses in cats after vaccination. Cats within the respective groups are represented by the same symbols in all graphs (filled triangles: cat no. 1; open square: cat no. 2; filled circle: cat no. 3). The small arrows indicate the days of immunization and the large arrow indicates the day of challenge.


group A
SL3261-FIV / SL3261-FIV
group B
SL3261-FIV / ISCOM-FIV
group C
SL3261-CtxB / ISCOM-FIV
group D
SL3261-CtxB / ISCOM-SIV
FIV Gag (10log titer)
FIV Env-SU1 (OD450)
FIV Env-SU3 (OD450)
weeks post challenge

by SL3261. It is likely that the region coding for the highly hydrophobic putative leader of the SU protein is mainly responsible for this effect. Antibodies to MBP, Gag and Env were induced more efficiently when these proteins were incorporated into iscom than when presented by SL3261 (Fig. 3 and Fig. 4). In fact, three immunizations with SL3261-FIV were required to induce detectable titers of Gag-specific antibodies and even four immunizations failed to induce Env-specific antibodies. However, the MBP fusion proteins expressed by the SL3261 strains were presented to the immune system of these animals, since MBP-specific antibodies were detected within two weeks after the first immunization in five out of six cats immunized with SL3261-FIV (groups A and B; Fig. 3). Although after the second immunization still no Gag-specific antibodies could be detected, the immune system had clearly been primed for this protein by SL3261-FIV, as specific antibodies developed faster after the first iscom-FIV immunization in group B (SL3261-FIV/iscom-FIV) than in group C (SL3261-CtxB/iscom-FIV). Priming with SL3261-FIV apparently had no beneficial effect on the level of the MBP-, Gag- or Env (SU1/SU3)-specific plasma antibody responses (compare groups B and C; Fig. 3 and Fig. 4).

None of the evaluated immunization strategies protected the cats from FIV infection upon homologous challenge, but the reduced mean cell-associated virus loads (Fig. 5), the reduced mean proviral DNA loads (Fig. 6) and the much weaker induction of antibodies against SU1/SU3 after challenge in the cats immunized four times with SL3261-FIV, as compared to cats of the other groups (Fig. 4), suggested that this immunization regimen, did have a protective effect. It is unlikely that the observed reduced FIV loads were the result of induced antibody responses, as no significant Env-specific antibodies and only weak Gag-specific antibody titers were present at the time of challenge. Furthermore, no reduced virus load was observed in the iscom-FIV immunized cats, which had much higher Gag- and Env-specific plasma antibody titers. This is not surprising in the view of our recent observations, which showed that enhancement of FIV infection may occur after immunization with Env-iscom (Siebelink *et al.*, 1995), which showed that the enhancement of FIV infection may occur after immunization with Env-iscom. It seems therefore more likely that, if indeed the cats of group A were partially protected, a cellular rather than a humoral

Fig. 4 Development of antibody responses in cats after challenge. Represented are the OD_{450} values obtained with plasma samples diluted 1:100. The same cats within a group are represented by the same symbols in all graphs (filled triangles: cat no. 1; open square: cat no. 2; filled circle: cat no. 3).

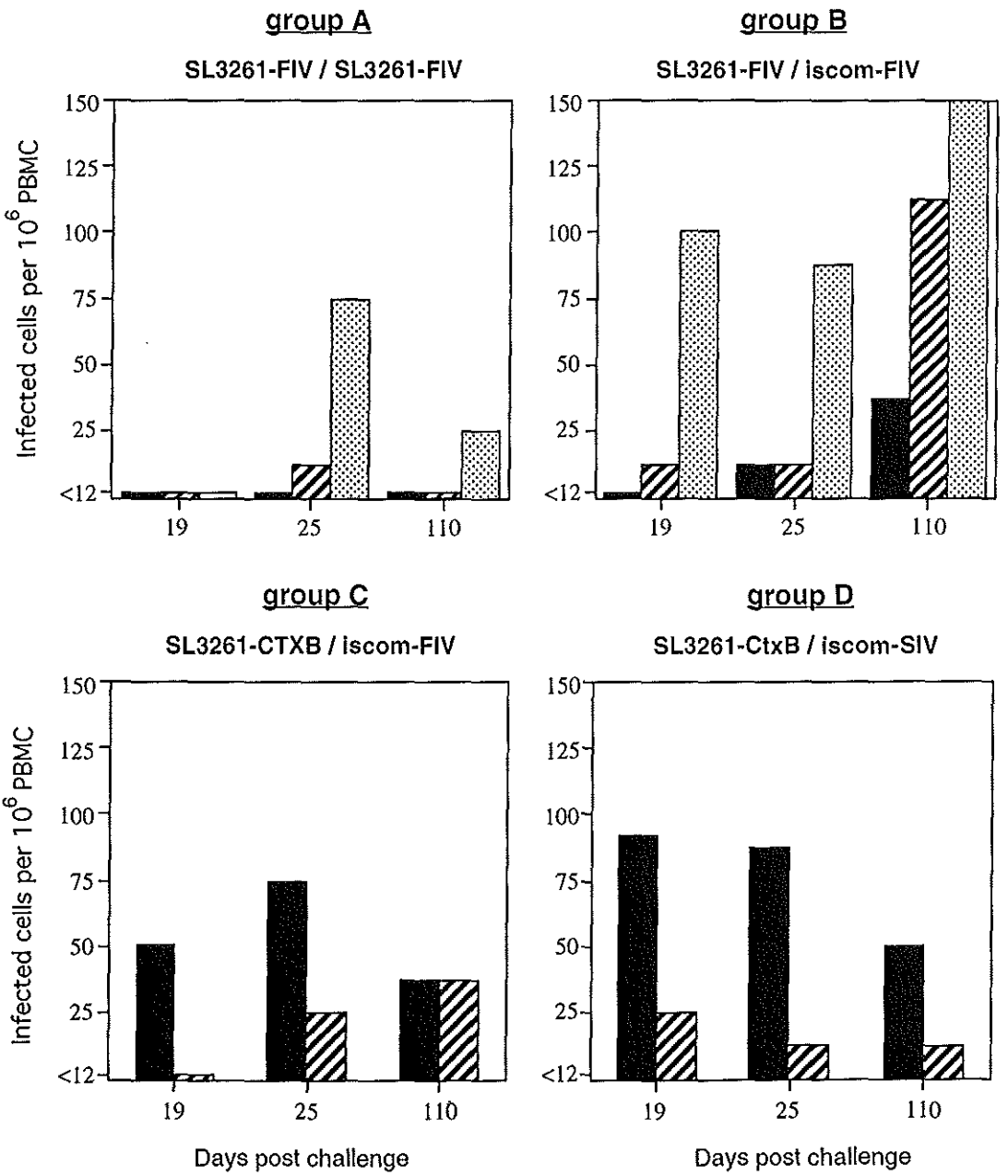


Fig. 5 Viral load in cats after challenge expressed as the number of FIV infected cells per 10^6 PBMC of individual cats at different time points (filled bar: cat no. 1; striped bar : cat no. 2; spotted bar: cat no. 3).

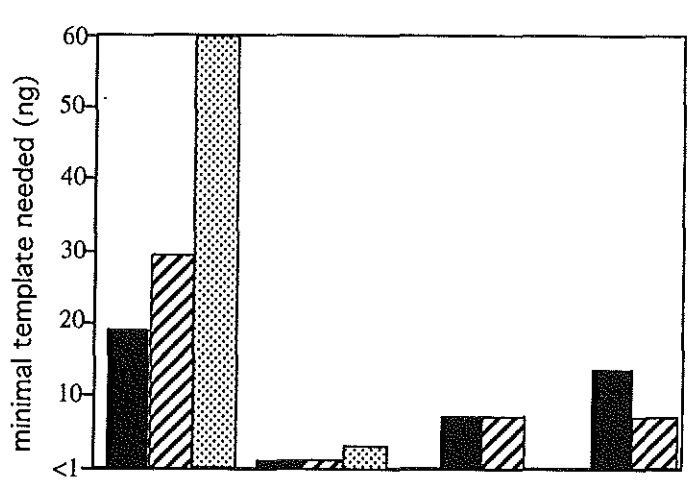


Fig. 6 Viral load in cats after challenge. The viral load is expressed as the minimal amount of chromosomal DNA, isolated from PBMC 110 days after challenge, required as template to obtain a positive FIV-specific PCR (filled bar: cat no. 1; striped bar : cat no. 2; spotted bar: cat no. 3).

immune response was at the basis of this protection. In our previous study cats immunized with a *Salmonella* strain only expressing the FIV *gag* gene had a comparable virus load after challenge as control cats (Tijhaar *et al.*, 1997). The reduced virus load in cats immunized with *Salmonella* strains expressing the *gag* and *env* genes observed in this study, indicates that the *env*-expressing *Salmonella* strains are mainly responsible for the observed reduced virus load. Interestingly, Flynn *et al.* (1996) found that vaccine induced protection against FIV infection correlated with Env- but not Gag-specific CTL responses. Alternatively, the reduced virus load observed in this study is not (only) due to the *env*-expressing *Salmonella* strains, but might be due to the increased number of immunizations, the different route of administration (oral or i.p. in the previous study vs oral and i.p. in this study) or a combination of all.

In conclusion, this study provided an indication of a protective effect of immunization with the *Salmonella typhimurium* recombinants expressing the FIV-Gag and -Env protein. However, further studies are required to evaluate the full potential of this approach for lentivirus vaccine development.

ACKNOWLEDGEMENTS

We thank Geert van Amerongen and Nico Schmidt for biotechnical assistance, Ellen Hulskotte for the SIV Gag- and Env-iscom and Conny Kruyssen for

assistance in preparing the manuscript. This investigation was supported by a grant of the 'Raad voor Gezondheidsonderzoek', The Hague, The Netherlands (grant number 88-108/89027).

REFERENCES

Ackley, C. D., Yamamoto, J. K., Levy, N., Pedersen, N. C. & Cooper, M.D. (1990). Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *Journal of Virology* **64**, 5652-5655.

Aggarwal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M. & Sadoff, J. (1990). Oral *Salmonella*: Malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. *Journal of Experimental Medicine* **172**, 1083-1090.

Brunner, D. & Pedersen, N. C. (1989). Infection of peritoneal macrophages *in vitro* and *in vivo* with feline immunodeficiency virus. *Jounal of Virology* **63**, 5483-5488.

Charbit A., Martineau, P., Ronco, J., Leclerc, C., Lo-Man, R., Michel, V., O'Callaghan, D. & Hofnung, M. (1993). Expression and immunogenicity of the V3 loop from the envelope of human immunodeficiency virus type 1 in an attenuated *aroA* strain of *Salmonella typhimurium* upon genetic coupling to two Escherichia coli carrier proteins. *Vaccine* **11**, 1221-1228.

Clements, J. E., Montelaro, R. C., Zink, M. C., Amedee, A. M., Miller, S., Trichel, A. M., Jagerski, B., Hauer, D., Martin, L. N., Bohm, R. P. & Murphey-Corb, M. (1995). Cross-protective immune responses induced in rhesus macaques by immunisation with attenuated macrophage-tropic simian immunodeficiency virus. *Journal of Virology* **69**, 2737-2744.

Daniel, M. D., Kirchhoff, F., Czafak, S. C., Sehgal, P. K. & Desrosiers, R. C. (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. *Science* **258**, 1938-1941.

De Ronde, A., Stam, J. G., Boers, P., Langedijk, H., Meloen, R.,

Hesselink, W., Keldermans, L. C. E. J. M., Van Vliet, A., Verschoor, E. J., Horzinek, M. C. & Egberink, H. F. (1994). Antibody response in cats to the envelope proteins of feline immunodeficiency virus: identification of an immunodominant neutralization domain. *Virology* **198**, 257-264.

Dittmer, U., Niblein, T., Bodemer, W., Petry, H., Sauermann, U., Stahl-Hennig, C. & Hunsmann, G. (1995). Cellular immune responses of rhesus monkeys infected with a partially attenuated nef deletion mutant of the Simian immunodeficiency virus. *Virology* **212**, 392-397.

Dow, S. W., Poss, M. L. & Hoover, E. A. (1990). Feline immunodeficiency virus: A neurotropic lentivirus. *AIDS* **3**, 658-668.

Flynn, J. L., Weiss, W. R., Norris, K. A., Seifert, H. S., Kumar, S. & So, M. (1990). Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Molecular Microbiology* **4**, 2111-2118.

Flynn, J. N., Keating, P., Hosie, M. J., Mackett, M., Stephens, E. B., Beatty, J. A., Neil, J. C. & Jarrett, O. (1996). Env-specific CTL predominate in cats protected from feline immunodeficiency virus infection by vaccination. *Journal of Immunology* **157**, 3658-3665.

Gonzalez C., Hone, D., Noriega, F. R., Tacket, C. O., Davis, J. R., Losonsky, G., Nataro, J. P., Hoffman, S., Malik, A., Nardin, E., Sztein, M. B., Heppner, D. G., Fouts, T. R., Isibasi, A. & Levine, M. M. (1994). *Salmonella typhi* vaccine strain CVD908 expressing the circumsporozoite protein of *Plasmodium falciparum*: Strain construction and safety and immunogenicity in humans. *Journal of Infectious Diseases* **169**, 927-931.

Hoffman-Fezer, G., Thum, J., Ackley, C., Herbold, M., Mysliwietz, J., Thefeld, S., Hartmann, K. & Kraft, W. (1992). Decline in CD4+ cell numbers in cats with naturally acquired feline immunodeficiency virus infection. *Journal of Virology* **66**, 1484-1488.

Hoiseth, S.K. and Stocker, B.A.D. (1991). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*

291, 238-239.

Hosie, M. J., Osborne, R., Reid, G., Neil, J. C. & Jarrett, O. (1992). Enhancement after feline immunodeficiency virus vaccination. *Veterinary Immunology and Immunopathology* **35**, 191-197.

Hosie, M. J., Osborne, R., Yamamoto, J. K., Neil, J. C. & Jarrett, O. (1995). Protection against homologous but not heterologous challenge induced by inactivated feline immunodeficiency virus vaccines. *Journal of Virology* **69**, 1253-1255.

Hulskotte, E. G. J., Geretti, A. M., Siebelink, K. H. J., Van Amerongen, G., Cranage, M. P., Rud, E. W., Norley, S. G., De Vries, P. & Osterhaus, A.D.M.E. (1995). Vaccine-induced virus neutralizing antibodies and cytotoxic T cells do not protect macaques from experimental infection with simian immunodeficiency virus SIVmac32H (J5). *Journal of Virology* **69**, 6289-6296.

Reid, G. (1992). Soluble proteins incorporated into iscoms after covalent attachment of fatty acids. *Vaccine* **10**, 597-602.

Rimmelzwaan, G. F., Siebelink, K. H. J., Huisman, R. C., Moss, B., Francis, M. J. & Osterhaus, A. D. M. E. (1994). Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation in immune-stimulating complexes. *Journal of General Virology* **75**, 2097-2102.

Schödel, F., Kelly, S. M., Darrell, L., Milich, D. R. & Curtiss III, R. (1994). Hybrid hepatitis B virus core-pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. *Infection and Immunity* **62**, 1669-1676.

Siebelink, C. H. J., Windrich, R. W., Chu, I., Groen, J., Weijer, K., UytdeHaag, F. G. C. M. & Osterhaus, A. D. M. E. (1990). An enzyme-linked immunosorbent assay (ELISA) for the detection of feline immunodeficiency virus (FIV) antigen in cell culture and FIV-specific antibodies in feline serum. *Developments in Biological Standardizations* **72**, 189-196.

Siebelink, K. H. J., Chu, I. H., Rimmelzwaan, G. F., Weijer, K., Osterhaus, A. D. M. E. & Bosch, M. (1992). Isolation and partial characterization of infectious molecular clones of feline immunodeficiency virus obtained directly from bone marrow DNA of a naturally infected cat. *Journal of Virology* **66**, 1091-1097.

Siebelink, K. H. J., Tijhaar, E., Huisman, R. C., Huisman, W., De Ronde, A., Darby, I. H., Rimmelzwaan, G. F. & Osterhaus, A. D. M. E. (1995). Enhancement of feline immunodeficiency virus infectivity after immunization with envelope glycoprotein subunit vaccines. *Journal of Virology* **69**, 3704-3711.

Tijhaar, E. J., Yan, Z. X., Karlas, J. A. Meyer, T. F., Stukart, M. J., Osterhaus, A. D. M. E. & Mooi, F. R. (1994). Construction and evaluation of an expression vector allowing the stable expression of foreign antigens in a *Salmonella typhimurium* vaccine strain. *Vaccine* **12**, 1004-1011.

Tijhaar, E. J., Siebelink, K. H. J., Karlas, J. A., Burger, M. C., Mooi, F. R. & Osterhaus A. D. M. E. (1997). Induction of feline immunodeficiency virus-specific antibodies in cats with an attenuated *Salmonella* strain expressing the Gag protein. *Vaccine*, in press.

Tite, J. P., Gao, X. M., Hughes-Jenkins, C. M., Lipscombe, M., O'Callaghan, D. & Dougan, G. (1990). Antiviral immunity induced by recombinant nucleoprotein of influenza-A virus. III. Delivery of recombinant nucleoprotein to the immune system using attenuated *Salmonella typhimurium* as a live carrier. *Immunology* **70**, 540-546.

Torten, M., Franchini, M., Barlough, J. E., George, J. W., Mozes, E., Lutz, H. & Pedersen, N. C. (1991). Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus. *Journal of Virology* **65**, 2225-2230.

Valentine, P. J., Meyer, K., Rivera, M. M., Lipps, C., Pauza, D., Maziarz, R. T., So, M. & Heffron, F. (1996). Induction of SIV capsid-specific CTL and mucosal sIgA in mice immunized with a recombinant *S. typhimurium aroA* mutant. *Vaccine* **14**, 138-146.

Verschoor, E. J., Van Vliet, A. L. W., Egberink, H. F., Hesselink, W., Van Alphen, W. E., Joosten, I., Boog, C. J. P., Horzinek, M. C. & De Ronde, A. (1995). Vaccination against feline immunodeficiency virus using fixed infected cells. *Veterinary Immunology and Immunopathology* **46**, 139-149.

Wick, M. J., Pfeifer, J. D., Findlay, K. A., Harding, C. V. & Normark S. J. (1993). Compartmentalization of defined epitopes expressed in Escherichia coli has only a minor influence on efficiency of phagocytic processing for presentation by class I and class II major histocompatibility complex molecules to T cells. *Infection and Immunity* **161**, 4848-4856.

Yamamoto, J. K., Okuda, T., Ackley, C. D., Louie, H., Pembroke, E., Zochlinski, H. A., Munn, R. J. & Gardner, M. B. (1991). Experimental vaccine protection against feline immunodeficiency virus. *AIDS Research and Human Retroviruses* **7**, 911-922.

Yamamoto, J. K., Hohdatsu, T., Olmsted, R. A., Pu, R., Louie, H., Zochlinski, H. A., Acevedo, V., Johnson, H. M., Soulds, G. A. & Gardner, M. B. (1993). Experimental vaccine protection against homologous and heterologous strains of feline immunodeficiency virus. *Journal of Virology* **67**, 601-605.

Yan, Z. X. (1992). Two-phase system for the development of live recombinant bacterial vaccines. Ph.D. thesis. Max Planck Institute, Tübingen, Germany.

Yan, Z. X., Reuss F. & Meyer, T. F. (1990). Construction of an invertible DNA segment for improved antigen expression by a hybrid *Salmonella* vaccine strain. *Research in Microbiology* **141**, 1003-1004.

Yang D. M., Fairweather, N., Button, L. L., McMaster, W. R., Kahl, L. P. & Liew F. Y. (1990). Oral *Salmonella typhimurium* (AroA-) vaccine expressing a major Leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against Leishmaniasis. *Journal of Immunology* **145**, 2281-2285.

CHAPTER 7

Enhancement of feline immunodeficiency virus infectivity after immunization with envelope glycoprotein vaccines

Journal of Virology 69: 3704-3711 (1995)

Enhancement of Feline Immunodeficiency Virus Infection after Immunization with Envelope Glycoprotein Subunit Vaccines

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Received 4 January 1995/Accepted 9 March 1995

Cats were immunized three times with different recombinant feline immunodeficiency virus (FIV) candidate vaccines. Recombinant vaccinia virus (rVV)-expressed envelope glycoprotein with (vGR657) or without (vGR657x15) the cleavage site and an FIV envelope bacterial fusion protein (β-Galactosidase–Env) were incorporated into immune-stimulating complexes or adjuvanted with Quil A. Although all immunized cats developed antibodies against the envelope protein, only the cats vaccinated with the rVV-expressed envelope glycoproteins developed antibodies which neutralized FIV infection of Crandell feline kidney cells. These antibodies failed to neutralize infection of thymocytes with a molecularly cloned homologous FIV. After the third immunization the cats were challenged with homologous FIV. Two weeks after challenge the cell-associated viral load proved to be significantly higher in the cats immunized with vGR657 and vGR657x15 than in the other cats. The cats immunized with vGR657 and vGR657x15 also developed antibodies against the Gag proteins more rapidly than the cats immunized with β-Galactosidase–Env or the control cats. This suggested that immunization with rVV-expressed glycoprotein of FIV results in enhanced infectivity of FIV. It was shown that the observed enhancement could be transferred to naive cats with plasma collected at the day of challenge.

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus that causes feline AIDS, which is similar to AIDS in humans (1, 3, 9, 12, 20, 29, 39, 41, 45, 51). The similarities between FIV and human immunodeficiency virus (HIV) on the one hand and between the pathogeneses of the syndromes they cause on the other hand have led to the use of FIV infection of cats as an animal model to evaluate the potential of preventive and therapeutic measures for HIV infection in humans. Since FIV infection is widespread among cats all over the world, the development of preventive and therapeutic measures for feline AIDS is also of major veterinary importance.

Different vaccination strategies for lentivirus infections have been evaluated with varying degrees of success. Most attempts to develop candidate vaccines against lentivirus infections were without success (for a review, see reference 22). Nevertheless, in some experiments chimpanzees were successfully vaccinated against HIV type 1 (HIV-1) (4) and macaques were successfully vaccinated against HIV-2 or simian immunodeficiency virus (SIV) infections with candidate whole inactivated virus, live attenuated virus, recombinant virus, subunit virus vaccines, or combinations of these candidate vaccines (6, 7, 17, 21, 28, 30, 32, 38). The mechanisms by which protective immunity was established in these systems are not well understood at present, although there are indications for a major role of both virus-neutralizing (VN) antibodies and cell-mediated immunity (10, 24, 31). However, contradictory data have also been obtained in some of these studies (2, 5, 16, 18, 36). In the macaque SIV model it was shown that at least part of the protective immunity induced could be attributed not to virus-specific but rather to cell-specific antibodies (23, 46). In the SIV system it was recently shown that vaccine-induced protection against infection with SIV-infected cells correlated with the presence of a certain major histocompatibility complex class I genotype of the monkeys, indicating the involvement of major histocompatibility complex class I-restricted cytotoxic T-lymphocyte responses (11).

Several approaches to develop a preventive vaccine against FIV infection have also proven to be unsuccessful (16, 48). However, Yamamoto et al. reported the induction of protective immunity in cats against homologous and to a lesser extent also to heterologous FIV challenge, by vaccination with inactivated whole virus or FIV-infected cells (49, 50). This protective effect could be transferred to naive cats with plasma from vaccinated animals, indicating that antibodies may be at the basis of this protective immunity (13). It was shown that serum antibodies against FIV envelope glycoproteins, with different VN domains, correlated more with protective immunity than did antibodies to other viral proteins.

FIV vaccines based on recombinant envelope proteins would have clear advantages over inactivated or attenuated virus vaccines. However, so far vaccination strategies using FIV purified envelope glycoproteins or fractions of these proteins as immunogens have failed (16, 26). Here we report the results of a series of vaccination experiments in cats with different envelope proteins of FIV, expressed by recombinant vaccinia viruses (rVV) or as a bacterial fusion protein and presented in the context of different adjuvant systems. The most striking finding of these studies is that vaccines containing intact envelope glycoprotein induced enhancement of infectivity rather than protective immunity against homologous FIV infection. The observed enhancement could be transferred to naive cats with the plasma of the vaccinated cats.

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MATERIALS AND METHODS

Cells and challenge virus. Peripheral blood mononuclear cells (PBMC) and thymocytes were derived from an 8-week-old specific-pathogen-free (SPF) cat (42). These cells were stimulated with concanavalin A (5 μg/ml) in culture medium (RPMI 1640 [GIBCO, Gaithersburg, Md.], penicillin [100 IU/ml], streptomycin [100 μg/ml], L-glutamine [2 mM], 2-mercaptoethanol [2×10^{-5} M], interleukin-2 [100 IU/ml]) and 10% fetal calf serum. After 3 days the cells were washed and cultured further in culture medium. An FIV-susceptible clone of Crandell feline kidney (CrFK) cells, named CrFK 1D10, was kindly provided by N. Pedersen (51). The FIV AM19 strain was isolated from PBMC of a cat naturally infected with FIV (42). Concanavalin A- and interleukin-2-stimulated cells were infected with FIV AM19. When FIV antigen was detected in culture supernatant by enzyme-linked immunosorbent assay (ELISA) (40), it was filtered through a 220-nm-pore-size filter, aliquoted, and stored at −135°C. This FIV stock was titrated in vivo. Groups of four SPF cats were inoculated intramuscularly with 0.5 ml of 1:100, 1:400, 1:1,600, 1:6,400, or 1:25,600 dilutions of the FIV stock. All cats receiving 1:100 and 1:400 dilutions became infected by 4 to 8 weeks postinfection as shown by seroconversion and virus isolation. Three of the four cats inoculated with 1:1,600-diluted FIV stock and one of the four cats inoculated with 1:6,400-diluted stock became virus isolation positive and seropositive. One 50% cat infectious dose (CID_{50}) was therefore estimated to be 0.5 ml of a 1:3,200 dilution of the FIV stock.

FIV AM6c was isolated from PBMC of a cat naturally infected with FIV and adapted to replicate in CrFK 1D10 cells as previously described (43). *env* gene sequence homology of FIV AM6c and FIV19k1 is 94.8%. CrFK 1D10 cells were infected with FIV AM6c, and after 6 days the culture medium was refreshed. After another 4 days of culture, when FIV antigen was detected in the culture supernatant, it was filtered through a 220-nm-pore-size filter and stored in aliquots at −135°C. This FIV stock was titrated in CrFK 1D10 cells and the highest dilution (20 $TCID_{50}$s) which consistently resulted in detectable antigen production within 8 days was used in the VN assay (see below).

Preparation of candidate FIV vaccines. The envelope glycoproteins of FIV AM19 were expressed by an rVV in BHK cells either in their native form (vGR657) or after deletion of the cleavage site between the surface (SU) and the transmembrane (TM) proteins (vGR657x15) to facilitate incorporation into immune-stimulating complexes (iscoms) (33). After lectin purification these proteins were incorporated into iscoms, which resulted in two iscom preparations—vGR657 and vGR657x15 iscoms—as previously described (33). An 1,870-bp fragment of the envelope gene was excised from pBluescript, containing the whole envelope gene, by using the restriction enzymes *Bam*HI and *Bgl*II (nucleotide positions 350 and 2220, respectively) and subcloned into *Bam*HI-digested pEX vector (Stratagene, La Jolla, Calif.). This vector allows inducible expression of proteins as β-galactosidase (β-Gal) fusion protein. This protein was partially purified as inclusion bodies, solubilized, and mixed with Quil A as an adjuvant.

SIV envelope glycoprotein iscoms were prepared by E. Hulskotte using a method similar to that used for the cleavage site-deleted FIV envelope glycoprotein iscoms (19).

Vaccination and challenge infection of cats. Six groups of six SPF cats each were vaccinated three times subcutaneously according to the following schedule: group 1, vGR657 iscoms; group 2, vGR657x15 iscoms; group 3, vGR657x15 plus Quil A; group 4, β-Gal–FIV Env plus Quil A; group 5, SIV Env iscoms; and group 6, phosphate-buffered saline. The cats were vaccinated with 10 μg of protein at weeks 0, 4, and 10. Two weeks later the cats were challenged by the intramuscular route with 20 CID_{50}s of FIV AM19. PBMC and plasma samples were collected every 2 weeks postchallenge (p.c.) during an 8-week period.

Serological assays. Antibodies against the Gag proteins p24 and p17 were detected with a commercially available test kit, using recombinant p24 and p17 proteins (catalog number FI002-AB01; European Veterinary Laboratory B.V., Woerden, The Netherlands). Antibody titers were expressed as the optical density at 450 nm (OD_{450}) value of the serum multiplied by the dilution divided by three times the OD_{450} value of the negative control serum.

Antibodies against the envelope protein were detected by ELISA using synthetic peptides and bacterial fusion proteins, representing different regions of the envelope protein as shown in Fig. 1. The synthetic peptides were purchased from European Veterinary Laboratory B.V. (catalog numbers: SU peptide, EVS-000-PE-003; TM peptide, EVS-000-PE-004). The first synthetic peptide contains the immunodominant VN epitope within variable region 3 spanning amino acid residues 396 to 412 of the surface protein of the Petaluma strain (SU peptide) (25). The second peptide contains a B-cell epitope between amino acid positions 695 and 706 of the transmembrane protein of the same FIV strain (TM peptide). Antibody titers were calculated as described for the Gag ELISA.

An ELISA based on the detection of antibodies to a series of bacterial fusion proteins as shown in Fig. 1 was performed as described elsewhere (8).

VN assays. VN serum antibodies were determined in two different VN assays. The feline lymphocyte VN assay was based on inhibition of infection of thymocytes with molecularly cloned FIV 19k1 as previously described (44). The CrFK VN assay was based on the inhibition of infection of CrFK 1D10 cells with FIV AM6c, which is adapted to replicate in these cells. For this assay CrFK 1D10 cells (3.5×10^3) were seeded into a 96-well plate in 100 μl of Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. The next day the cells were washed and incubated for 1 h at 37°C with Polybrene (8 μg/ml) in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. A mixture of equal volumes of the diluted (see above) virus stock of FIV AM6c and twofold serial dilutions of heat-inactivated serum was incubated for 1 h at 37°C. Then the CrFK cells were washed and incubated with the virus-serum mixture at 37°C. After 24 h the CrFK cells were washed twice and propagated in Dulbecco modified Eagle medium supplemented with 2% fetal calf serum. After 8 days the culture supernatant was tested for the presence of FIV antigen by ELISA (40). The neutralizing antibody titer was expressed as the highest dilution of the serum still preventing FIV antigen production in the culture supernatant.

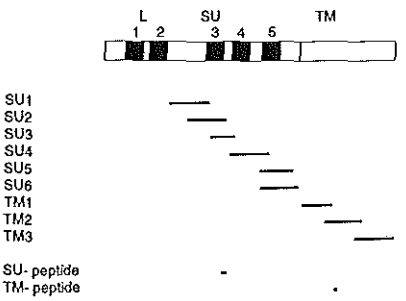


FIG. 1. Surface (SU1 to SU6) and transmembrane (TM1 to TM3) bacterial fusion products and SU and TM peptides used in the ELISA to detect antibodies against the envelope protein. The top bar represents the envelope protein of FIV, including the leader (L), surface (SU), and transmembrane (TM) proteins. The black boxes represent hypervariable regions in the envelope protein. The different bacterial fusion products and the peptides are indicated as bars.

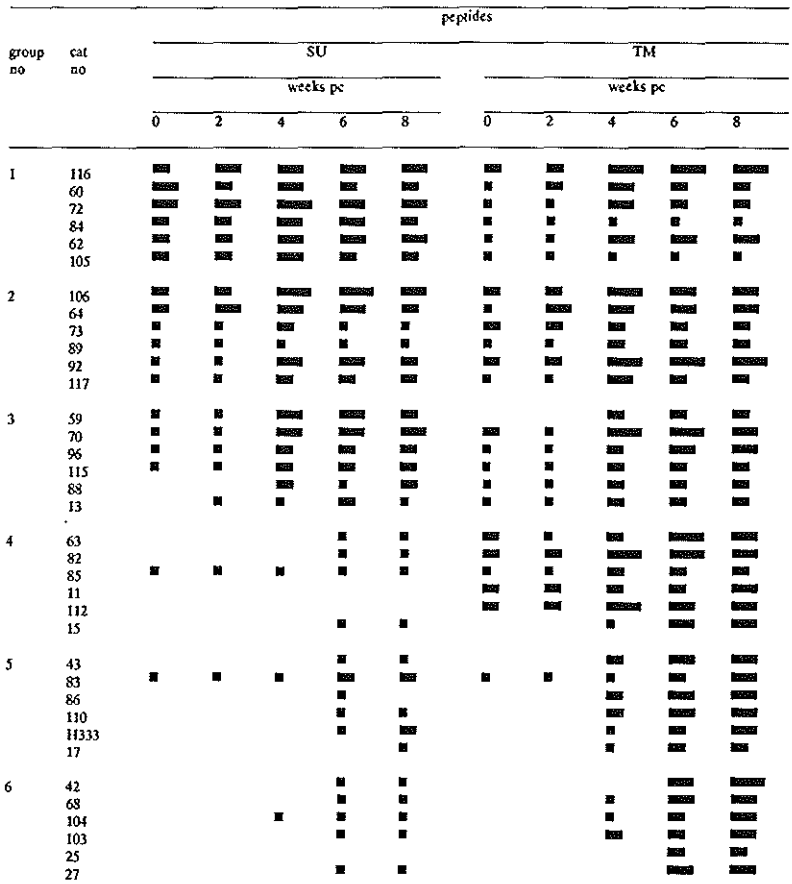
Cell-associated virus load: infectious center test. Serially diluted PBMC samples (1×10^4, 3×10^3, and 1×10^3 cells) were prepared from cats before and after challenge as previously described (42). These cells were cocultivated with 10^5 concanavalin A- and interleukin-2-stimulated PBMC from an SPF cat in eight duplicate wells. After 3 weeks the culture supernatants were tested for the presence of FIV antigen by ELISA. The number of infected cells in the PBMC in vivo was calculated from the in vitro culture by assuming that one infected cell gave rise to antigen production after cocultivation with concanavalin A- and interleukin-2-stimulated PBMC from an SPF cat, when one or more cultures tested in eight duplicate wells were negative for FIV antigen production.

Plasma transfer. A plasma pool was prepared by mixing equal volumes of the plasma samples derived from all the vaccinated cats from groups 1 and 2 at the day of challenge (plasma pool A). A second pool (plasma pool B) was prepared similarly by mixing plasma samples from all the cats of group 6 at the day of challenge. Two groups of four SPF cats, 10 weeks old, weighing between 800 and 1,250 g were used in the transfer experiment. Cats of group A and group B were injected intravenously with 7 ml of plasma pool A and plasma pool B, respectively. Six hours later the cats were challenged by the intramuscular route with 20 CID_{50}s of FIV AM19 as described above. PBMC and plasma samples of the cats were collected at weekly intervals and tested for viremia by virus isolation and for the development of plasma antibodies against the Gag protein.

RESULTS

Development of FIV-specific plasma antibodies upon vaccination. All plasma samples collected at the day of challenge were tested in the SU and TM peptide ELISA (Table 1, week p.c. 0). All the cats vaccinated with the vGR657 and vGR657x15 iscoms (groups 1 and 2) had developed plasma antibody titers to these peptides, ranging from 200 to 25,000, whereas all the cats vaccinated with Quil A-adjuvanted vGR657x15 (group 3) had developed antibody titers to at least one of the two peptides ranging from 50 to 3,000. Five of six cats vaccinated with the Quil A-adjuvanted bacterial FIV envelope fusion protein (group 4) had also developed serum antibody titers to the TM peptide, ranging from 50 to 3,000, whereas only one animal had developed a titer of 300 to the SU peptide. One cat of the two control groups, groups 5 and 6, showed an apparently nonspecific plasma antibody titer to the SU and TM peptides, which already existed before the start of the immunization procedure (not shown). Subsequently the

TABLE 1. Antibody response against SU and TM peptides in vaccinated cats at different times p.c.[a]



[a] SU peptide ELISA results: no symbol, titer < 200; ■, titer between 200 and 1,000; ■, titer between 1,000 and 5,000; ■, titer between 5,000 and 25,000; ■, titer > 25,000. TM peptide ELISA results: no symbol, titer < 50; ■, titer between 50 and 300; ■, titer between 300 and 3,000; ■, titer between 3,000 and 10,000; ■, titer > 10,000.

same plasma samples were tested in ELISA for antibody titers against the respective bacterial FIV envelope SU and TM fusion proteins (Table 2). Vaccination with the vGR657- and vGR657x15 iscoms (groups 1 and 2) resulted in high plasma antibody responses to all the SU and TM regions tested with the exception of the SU5 and TM1 regions, against which lower or no antibody levels were detected. Antibody levels induced with the native glycoprotein iscoms (group 1) and the cleavage site-deleted envelope iscoms (group 2) are comparable. The overall OD_{450} values induced with the cleavage site-deleted FIV envelope glycoprotein adjuvanted with Quil A (group 3) were lower than those found in the cats of groups 1 and 2. Vaccination with the β-Gal–FIV envelope fusion protein adjuvanted with Quil A failed to induce antibodies against the SU1, SU2, SU4, and SU5 regions. No significant levels of antibody to these fusion proteins were found in the cats of the two control groups (groups 5 and 6).

All the plasma samples collected at the day of challenge were also tested in the two VN antibody assays. None of the samples exhibited VN activity in the feline lymphocyte VN assay (not shown). However, VN antibodies could be demonstrated in cats of groups 1, 2, and 3 in the CrFK VN assay (Fig. 2). The VN titers in cats vaccinated with vGR657 iscoms (group 1) ranged from 160 to 640, with a mean titer of 506. Those of vGR657x15 iscom-vaccinated cats (group 2) ranged from 40 to 640 with a mean of 206, which is not significantly different from the values found for group 1. VN plasma antibody titers of cats vaccinated with the Quil A-adjuvanted vGR657x15 protein varied from 10 to 40, with a mean titer of 28, a value which is significantly lower than that found for

TABLE 2. Levels of antibody against envelope fragments in vaccinated cats at the day of challenge[a]

group no	cat no	Envelope regions								
		SU1	SU2	SU3	SU4	SU5	SU6	TM1	TM2	TM3
1	116	■	■	■	■		■		■	■
	60	■	■	■	■	■	■		■	■
	72	■	■	■	■	■	■		■	■
	84	■	■	■	■	■	■		■	■
	62	■	■	■	■	■	■		■	■
	105	■	■	■	■	■	■		■	■
2	106	■	■	■	■	■	■	■	■	■
	64	■	■	■	■	■	■		■	■
	73	■	■	■	■	■	■	■	■	■
	89	■	■	■	■		■		■	■
	92	■	■	■	■	■	■	■	■	■
	117	■	■	■	■		■		■	■
3	59	■	■	■	■		■	■	■	
	70	■	■	■	■		■	■	■	
	96				■		■	■	■	■
	115				■		■		■	
	88				■		■	■	■	■
	13				■				■	
4	63			■			■	■	■	■
	82			■			■	■	■	■
	85			■				■		■
	11			■			■	■	■	■
	112			■			■	■	■	■
	15			■			■	■	■	
5	43									
	83									
	86									
	110									
	H333									
	17									
6	42									
	68									
	104									
	103									
	25									
	27									

[a] No symbol, $OD_{450} < 0.4$; ■, OD_{450} between 0.4 and 0.8; ■, OD_{450} between 0.8 and 1.2; ■, $OD_{450} > 1.2$.

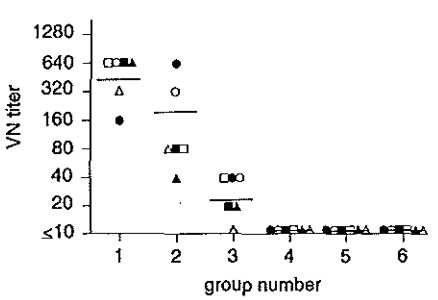


FIG. 2. Plasma VN antibody titers of the individual cats of the six different vaccination groups as measured in the CrFK VN assay at the day of challenge. The titers in the individual cats are indicated with different symbols. The mean titer per group is indicated by a bar.

groups 1 and 2 (Student t test; $P < 0.05$). In cats vaccinated with the β-Gal–FIV envelope fusion protein (group 4) and the cats of the control groups 5 and 6, no VN antibody response could be demonstrated at the day of challenge.

As expected, none of the cats had developed plasma antibodies to the FIV Gag protein at the day of challenge (Fig. 3).

Kinetics of FIV-specific plasma antibodies after FIV challenge. Plasma antibody titers against the SU and TM peptides of most of the cats immunized with the recombinant FIV envelope proteins (groups 1 to 4) increased 3- to 10-fold within 4 weeks after challenge infection. Within 8 weeks all the cats, including the control animals in groups 5 and 6, had developed anti-SU and anti-TM peptide plasma antibodies (Table 1). At 8 weeks p.c. the SU peptide-specific antibody titers in the cats vaccinated with rVV-expressed FIV envelope glycoprotein were significantly higher than those of the nonvaccinated cats (Table 1; $P < 0.05$).

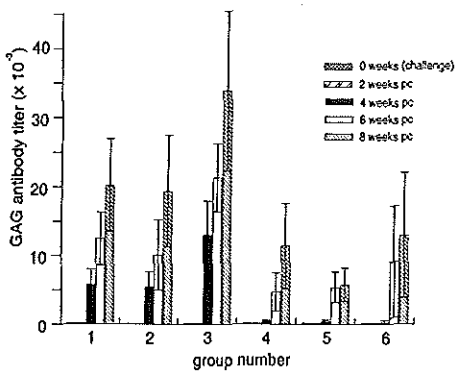


FIG. 3. Kinetics of the plasma antibody titer development against the Gag protein, in cats from the different vaccination groups. Mean anti-Gag titers at different times after challenge are presented.

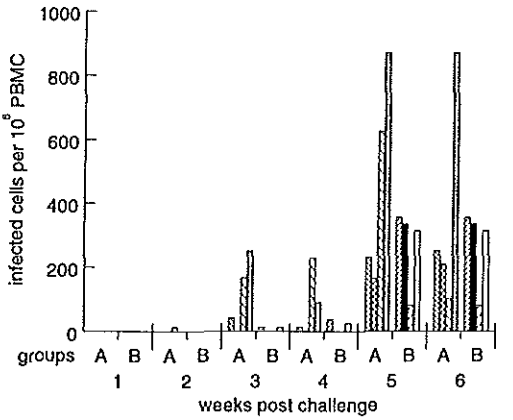


FIG. 5. Kinetics of numbers of FIV-infected cells per 10^6 PBMC in individual cats (indicated by different bars) transferred with plasma pool A (groups 1 and 2) or plasma pool B (group 6), measured over 6 weeks p.c.

Plasma antibodies to the FIV Gag protein could be detected 4 weeks after challenge in all the cats vaccinated with rVV-expressed FIV envelope glycoproteins (groups 1, 2, and 3) (Fig. 3). In the cats vaccinated with the β-Gal–FIV envelope fusion protein (group 4) and in the cats of the two control groups, groups 5 and 6, it took about 2 weeks longer before FIV Gag-specific antibodies were detected. Not only was the induction of FIV Gag-specific antibodies in cats of groups 1 to 3 faster, but also the 8-week p.c. plasma titers tended to be higher in these animals (Fig. 3).

Cell-associated virus load. Two weeks after infection FIV-infected PBMC were demonstrated in all the cats of groups 1 and 2 and in four of six cats of group 3 (Fig. 4). The numbers of FIV-infected PBMC varied from 50 to 210, 30 to 1,000, and 0 to 106 per 10^6 PBMC, respectively. In the other three groups no infected PBMC were demonstrated at this time. Two weeks later, FIV-infected PBMC could be demonstrated in all the cats of all groups. Although a lower average FIV load was observed in cats of group 4 compared with that in the other groups, the differences observed between the groups proved not to be significant.

Plasma transfer experiment. Pools of plasma collected at the day of challenge from the cats of groups 1 and 2 (pool A) and from group 6 (pool B) were prepared, and VN titers were determined. The titers, measured in the CrFK VN assay, were 320 and <10, respectively. Two groups of four SPF kittens were inoculated intravenously with 7 ml of plasma pool A or plasma pool B. No plasma VN antibodies could be detected 6 h after the transfer. FIV could be demonstrated in PBMC of one of four cats of group A (20 infected cells per 10^6 PBMC) but not in PBMC of cats of group B 2 weeks after challenge with 20 CID_{50}s of FIV AM19 (Fig. 5). Three weeks after challenge three of four cats of group A and two of four cats of group B exhibited cell-associated viremia, with significantly higher numbers of FIV-infected cells in the cats of group A ($P < 0.05$). The mean numbers of infected PBMC in the cats from groups A and B at 3 weeks p.c. were 115 and 6 per 10^6 PBMC, respectively (Fig. 5). At 4 weeks p.c. the mean numbers of FIV-infected cells were 111 and 16 per 10^6 PBMC, respectively. Five and six weeks after challenge FIV could be reisolated from PBMC from all cats of both groups with no clear differences in cell-associated virus load.

Four weeks after challenge, plasma antibodies to the Gag

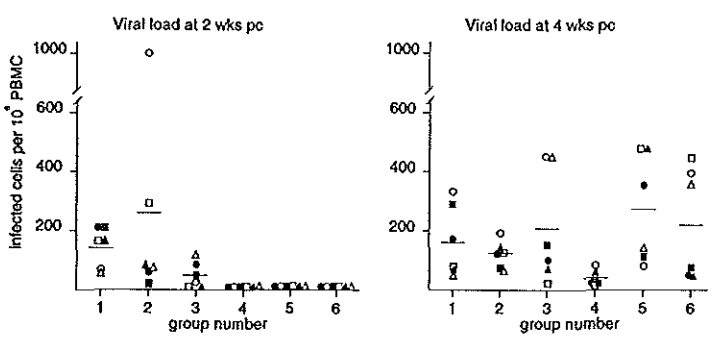


FIG. 4. Numbers of FIV-infected cells per 10^6 PBMC in the individual cats (indicated by different symbols) of the different vaccination groups at 2 and 4 weeks p.c. The mean numbers of infected PBMC per group are indicated by a bar.

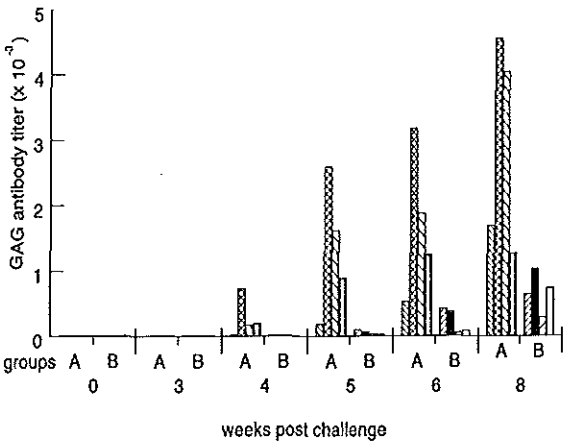


FIG. 6. Kinetics of plasma antibody titer development against the Gag protein in individual cats (indicated by different bars) transferred with plasma of pool A (groups 1 and 2) or plasma of pool B (group 6).

protein could be detected in three of four cats of group A (with ELISA titers ranging from 170 to 800) and in none of the cats of group B (Fig. 6). At 5 weeks p.c. anti-FIV Gag plasma antibodies were demonstrated in all cats of group A (titers from 180 to 2,600) and in only two of four cats from group B (titers from 70 to 100). In the plasma samples collected from all the cats of both groups at 6 and 8 weeks p.c., antibodies against the Gag protein were demonstrated. The titers were significantly higher ($P < 0.05$) in the cats of group A than those in the cats of group B from 4 weeks p.c. onward.

DISCUSSION

The present paper describes the evaluation of the potential of different candidate FIV vaccines in a vaccination challenge experiment in SPF cats. The most striking finding was that immunization with rVV-expressed FIV glycoproteins, resulting in VN plasma antibodies, led to enhanced FIV infection upon challenge in these cats (groups 1, 2, and 3). This was demonstrated by the more rapid development of PBMC-associated viremia and Gag-specific plasma antibodies. The enhancement effect could be transferred to naive cats with plasma collected from cats immunized with these candidate vaccines. In contrast, cats immunized with an FIV envelope bacterial fusion protein (group 4) did not develop VN plasma antibodies and developed PBMC-associated viremia and Gag-specific plasma antibodies with the same kinetics as the two control groups.

Comparison of the antibody levels induced in the first three groups of cats at the day of challenge indicated that the immunogenicity of vGR657 and vGR657x15 incorporated into iscoms was higher than that of vGR657x15 presented with Quil A. Both VN plasma antibodies and plasma antibodies to the different SU regions proved to be higher in the first two groups (Fig. 2; Table 2). Plasma antibody levels against most of the SU regions were lower or absent in group 4 at the day of challenge (Table 2). It is not clear why the presence of VN plasma antibodies at the day of challenge correlated not with protective immunity but rather with enhanced susceptibility to FIV infection. It should be noted, however, that the VN activity was demonstrable only in the CrFK VN assay and not in the feline lymphocyte assay. The latter assay should probably be considered more relevant in terms of protective immunity against in vivo FIV infection since, in contrast to feline kidney cells, feline lymphocytes are natural targets for FIV infection. In the transfer experiment it was shown that the enhancement phenomenon could be transferred to naive cats with the plasma of cats of groups 1 and 2. Although not formally proven, this indicated that the enhancement was mediated by FIV envelope-specific antibodies. The mechanism of enhancement proved to be operational at relatively high dilutions: after plasma transfer, no VN antibody activity could be demonstrated in the plasma of the kittens which subsequently showed enhanced FIV infectivity. It has also been shown for HIV-1 that antibody-dependent enhancement (ADE) could still be demonstrated at high dilutions (up to 1:65,000), whereas VN antibody activity can rarely be demonstrated at dilutions higher than 1:1,000 (35). As no reliable systems are available at present to quantify or even detect FIV-enhancing antibodies in vitro, it should be realized that all FIV-neutralizing antibodies found, per definition, should be considered to be the net result of neutralization and enhancement of FIV infectivity measured in vitro.

To date different mechanisms of ADE have been described for lentivirus infections. Complement- and Fc receptor-mediated ADEs have been shown to play a role in HIV-1, HIV-2, and SIV infections (14, 15, 27, 34, 47). Recently, another mechanism of ADE was described, in which antibodies neutralized or enhanced HIV-1, dependent on the phenotype of the virus involved (37). From our data it cannot be concluded which mechanism was involved in the observed enhancement of FIV infectivity. Indications for enhanced infectivity after FIV vaccination have been observed before (16). In the experiments of Yamamoto et al. (50) the presence of VN antibodies, demonstrated in an FeT1 cell (feline lymphoid cell line) VN assay, correlated with protective immunity rather than with enhancement of infectivity. This immunity could be transferred to naive animals with plasma of immunized animals (13). The main difference between their vaccination approach and ours is that in our experiments recombinant envelope proteins were used whereas Yamamoto et al. used inactivated whole virus or virus-

infected cells as immunogens. Since transfer experiments showed that probably plasma antibodies are involved in the observed mechanisms of enhancement and protection in both series of experiments, it may be speculated that the differences in configuration in which the FIV envelope glycoproteins were presented in both vaccines led to VN antibodies with different affinities. This may have had direct consequences for their in vivo effects.

Furthermore, the cell substrates used for the production of challenge viruses may have contributed to the observed differences in outcome of the vaccination experiments. The challenge virus used in our experiments was propagated in primary feline lymphocytes, whereas the challenge viruses in the experiments of Yamamoto et al. were propagated in a feline T-cell line (50). Like in the HIV-1 system, T-cell line-adapted FIV may be neutralized more efficiently than virus isolates from primary lymphocyte cultures, which may result in neutralization of the virus in the presence of enhancing antibodies. Furthermore, it may be speculated that, as was recently demonstrated for HIV-1 isolates from one individual (37), T-cell line-adapted FIV is less susceptible to ADE.

Taken together, it should be stressed that the mechanisms leading to the observed phenomenon of FIV enhancement upon vaccination and passive transfer are not fully understood at present. For the development of an effective FIV vaccine, the elucidation of the underlying mechanisms may be crucial. This may also lead to a more rational strategy for the development of HIV-1 vaccines.

ACKNOWLEDGMENTS

We thank Geert van Amerongen and Nico Schmidt for biotechnical assistance and Ellen Hulskotte for the SIV envelope iscoms. We also thank Rob van Herwijnen from the European Veterinary Laboratory for the SU and TM peptide ELISA, and we thank Conny Kruyssen for help in preparing the manuscript.

This work was supported by grants from Mallinckrodt Veterinary Ltd., Harefield, United Kingdom, and the Dutch Advisory Council on Health Research (RGO grant 88-90/89028).

REFERENCES

1. Ackley, C. D., J. K. Yamamoto, N. Levy, N. C. Pedersen, and M. D. Cooper. 1990. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. J. Virol. 64:5652–5655.
2. Arthur, L. O., J. W. Bess, Jr., D. J. Waters, S. W. Pyle, J. C. Kelliher, P. L. Nara, K. Krohn, W. G. Robey, A. J. Langlois, R. C. Gallo, and P. J. Fischinger. 1989. Challenge of chimpanzees (*Pan troglodytes*) immunized with immunodeficiency virus envelope glycoprotein gp120. J. Virol. 63:5046–5053.
3. Barlough, J. E., C. D. Ackley, J. W. George, N. Levy, R. Acevedo, P. F. Moore, B. A. Rideout, M. D. Cooper, and N. C. Pedersen. 1991. Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections. J. Acquired Immune Defic. Syndr. 4:219–227.
4. Berman, P. W., T. J. Gregory, L. Riddle, C. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg. 1990. Protection of chimpanzees from infection of HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature (London) 345: 622–625.
5. Berman, P. W., J. E. Groopman, T. Gregory, P. R. Clapman, R. A. Weiss, R. Ferriani, L. Riddle, G. Shimanski, C. Lucas, L. A. Lasky, and J. W. Eichberg. 1988. Human immunodeficiency virus type 1 challenge of chimpanzees immunized with recombinant envelope glycoprotein gp120. Proc. Natl. Acad. Sci. USA 85:5200–5204.
6. Carlson, J. R., T. P. McGraw, E. Keddie, J. L. Yee, A. Rosenthal, A. J. Langlois, R. Dickover, R. Donovan, P. A. Luciw, M. B. Jennings, and M. B. Gardner. 1990. Vaccine protection of rhesus macaques against simian immunodeficiency virus. AIDS Res. Hum. Retroviruses 6:1239–1246.
7. Daniel, M. D., F. Kirchhof, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. Science 258:1938–1941.
8. De Ronde, A., J. G. Stam, P. Boers, H. Langedijk, R. Meloen, W. Hesselink, L. C. E. J. M. Keldermans, A. van Vliet, E. J. Verschoor, M. C. Horzinek, and H. F. Egberink. 1994. Antibody response in cats to the envelope proteins of feline immunodeficiency virus: identification of an immunodominant neutralization domain. Virology 198:257–264.
9. Dow, S. W., M. L. Poss, and E. A. Hoover. 1990. Feline immunodeficiency virus: a neurotropic lentivirus. J. Acquired Immune Defic. Syndr. 3:658–668.
10. Emini, E. A., W. A. Schleif, J. H. Nunberg, A. J. Conley, Y. Eda, S. Tokiyoshi, S. D. Putney, S. Matsushita, K. E. Cobb, C. M. Jett, J. W. Eichberg, and K. K. Murthy. 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature (London) 355:728–730.
11. Heeney, J. L., C. van Els, P. de Vries, P. ten Haaft, N. Otting, W. Koornstra, J. Boes, R. Dubbes, H. Niphuis, M. Dings, M. Cranage, S. Norley, M. Jonker, R. E. Bontrop, and A. D. M. E. Osterhaus. 1994. Major histocompatibility complex class I-associated vaccine protection from simian immunodeficiency virus-infected peripheral blood cells. J. Exp. Med. 180:769–774.
12. Hoffmann-Fezer, G., J. Thum, C. Ackley, M. Herbold, J. Mysliwietz, S. Thefeld, K. Hartmann, and W. Kraft. 1992. Decline in $CD4^+$ cell numbers in cats with naturally acquired feline immunodeficiency virus infection. J. Virol. 66:1484–1488.
13. Hohdatsu, T., R. Pu, B. A. Torres, S. Trujillo, M. B. Gardner, and J. K. Yamamoto. 1993. Passive antibody protection of cats against feline immunodeficiency virus infection. J. Virol. 67:2344–2348.
14. Homsey, J., M. Meyer, and J. A. Levy. 1990. Serum enhancement of human immunodeficiency virus (HIV) infection correlates with disease in HIV-infected individuals. J. Virol. 64:1437–1440.
15. Homsey, J., M. Meyer, M. Tateno, S. Clarkson, and J. A. Levy. 1989. The Fc and not the CD4 receptor mediates antibody enhancement of HIV infection in human cells. Science 244:1357–1360.
16. Hoshe, M. J., R. Osborne, G. Reid, J. C. Neil, and O. Jarrett. 1992. Enhancement after feline immunodeficiency vaccination. Vet. Immunol. Immunopathol. 35:191–197.
17. Hu, S. L., K. Abrams, G. N. Barber, P. Moran, J. M. Zarling, A. J. Langlois, L. Kuller, W. R. Morton, and R. E. Benveniste. 1992. Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. Science 255:456–459.
18. Ho, S. L., P. N. Fultz, H. M. McClure, J. W. Eichberg, E. K. Thomas, J. Zarling, M. C. Singhal, S. G. Kosowski, R. B. Swenson, D. C. Anderson, and G. Todaro. 1987. Effect of immunization with a vaccinia HIV *env* recombinant on HIV infection of chimpanzees. Nature (London) 328:721–723.
19. Hulskotte, E. G. J., G. F. Rimmelzwaan, J. Boes, M. L. Bosch, Y. L. Heeney, S. G. Norley, P. de Vries, and A. D. M. E. Osterhaus. Antigenicity and immunogenicity of recombinant envelope glycoproteins of $SIV_{mac}32H$ with different *in vivo* passage histories. Vaccine, in press.
20. Hurtrel, M., J.-P. Ganière, J.-F. Guelfi, L. Chakrabarti, M.-A. Maire, F. Gray, L. Montagnier, and B. Hurtrel. 1992. Comparison of early and late feline immunodeficiency virus encephalopathies. AIDS 6:399–406.
21. Johnson, P. R., D. C. Montefiori, S. Goldstein, T. E. Hamm, J. Zhou, S. Kitov, N. L. Haigwood, L. Misher, W. T. London, J. L. Gerin, A. Allison, R. H. Purcell, R. M. Chanock, and V. M. Hirsch. 1992. Inactivated whole SIV vaccine in macaques: evaluation of protective efficacy against challenge with cell-free virus or infected cells. AIDS Res. Hum. Retroviruses 8:1501–1505.
22. Kurth, R., D. Binninger, J. Ennen, J. Denner, S. Hartung, and S. Norley. 1991. The quest for an AIDS vaccine: the state of the art and current challenges. AIDS Res. Hum. Retroviruses 7:425–433.
23. Langlois, A. J., K. J. Weinhold, T. J. Matthews, M. L. Greenberg, and D. P. Bolognesi. 1992. The ability of certain SIV vaccines to provoke reactions against normal cells. Science 255:456–459.
24. Lewis, M. G., W. R. Elkins, E. McCuthan, R. E. Benveniste, C. Y. Lai, D. C. Montefiori, D. S. Burke, G. A. Eddy, and A. Shafferman. 1993. Passively transferred antibodies directed against conserved regions of SIV envelope protect macaques from SIV infection. Vaccine 11:1347–1355.
25. Lombardi, S., C. Garcelli, C. LaRosa, L. Zaccaro, S. Specter, G. Malvaldi, F. Tozzini, F. Esposito, and M. Bendinelli. 1993. Identification of a linear neutralization site within the third variable region of feline immunodeficiency virus envelope. J. Virol. 67:4742–4749.
26. Lombardi, S., C. Garzelli, M. Pistello, C. Massi, D. Matteucci, F. Baldinotti, G. Cammarota, L. Da Prato, P. Bandecchi, F. Tozzini, and M. Bendinelli. 1994. A neutralizing antibody-inducing peptide of the V3 domain of feline immunodeficiency virus envelope glycoprotein does not induce protective immunity. J. Virol. 68:8374–8379.
27. Montefiori, D. C., W. E. Robinson, Jr., V. Hirsch, A. Modliszewski, W. Mitchell, and P. R. Johnson. 1990. Antibody-dependent enhancement of simian immunodeficiency virus (SIV) infection in vitro by plasma from SIV-infected rhesus macaques. J. Virol. 64:113–119.
28. Osterhaus, A. D. M. E., P. de Vries, and J. Heeney. 1992. AIDS vaccine developments. Nature (London) 355:684–685.
29. Pedersen, N. C., E. W. Ho, M. L. Brown, and J. K. Yamamoto. 1987. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science 235:790–793.
30. Putkonen, P., R. Thorstensson, J. Albert, K. Hild, E. Norrby, P. Biberfeld, and G. Biberfeld. 1990. Infection of cynomolgus monkeys with HIV-2 protects against pathogenic consequences of a subsequent simian immunodeficiency virus infection. AIDS 4:783–789.
31. Putkonen, P., R. Thorstensson, L. Ghavamzadeh, J. Albert, K. Hild, G. Biberfeld, and E. Norrby. 1991. Prevention of HIV-2 and SIV_{sm} infection by passive

immunization in cynomolgus monkeys. Nature (London) 352:436–438.
32. Putkonen, P., R. Thorstensson, L. Walther, J. Albert, L. Akerblom, O. Granquist, G. Wadell, E. Norrby, and G. Biberfeld. 1991. Vaccine protection against HIV-2 infection in cynomolgus monkeys. AIDS Res. Hum. Retroviruses 7:271–277.
33. Rimmelzwaan, G. F., K. H. J. Siebelink, R. C. Hulsman, B. Moss, M. J. Francis, and A. D. M. E. Osterhaus. 1994. Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation of the surface glycoprotein in immune-stimulating complexes. J. Gen. Virol. 75:2097–2102.
34. Robinson, W. E., Jr., T. Kawamura, D. Lake, Y. Masuho, W. M. Mitchell, and E. M. Hersch. 1990. Antibodies to the primary immunodominant domain of human immunodeficiency virus type 1 (HIV-1) glycoprotein gp41 enhance HIV-1 infection in vitro. J. Virol. 64:5301–5305.
35. Robinson, W. E., Jr., D. C. Montefiori, W. M. Mitchell, A. M. Prince, H. J. Alter, G. R. Dreesman, and J. W. Eichberg. 1989. Antibody-dependent enhancement of human immunodeficiency virus type 1 (HIV-1) infection *in vitro* by serum from HIV-1 infected and passively immunized chimpanzees. Proc. Natl. Acad. Sci. USA 86:4710–4714.
36. Schlienger, K., D. C. Montefiori, M. Mancini, Y. Rivière, P. Tiollais, and M. Michel. 1994. Vaccine-induced neutralizing antibodies directed in part to the simian immunodeficiency virus (SIV) V2 domain were unable to protect rhesus monkeys from SIV experimental challenge. J. Virol. 68:6578–6588.
37. Schutten, M., A. C. Andeweg, M. L. Bosch, and A. D. M. E. Osterhaus. 1994. Enhancement of infectivity of a non-syncytium inducing HIV-1 by sCD4 and by human antibodies that neutralize syncytium inducing HIV-1. Scand. J. Immunol. 41:18–22.
38. Shaferman, A., P. B. Jahrling, R. E. Benveniste, M. G. Lewis, T. J. Phipps, F. Eden-McCutchan, J. Sadoff, G. A. Eddy, and D. S. Burke. 1991. Protection of macaques with a simian immunodeficiency virus envelope peptide vaccine based on conserved human immunodeficiency virus type 1 sequences. Proc. Natl. Acad. Sci. USA 88:7126–7130.
39. Shelton, G. H., M. L. Linenberger, C. K. Grant, and J. L. Abkowitz. 1990. Hematologic manifestations of feline immunodeficiency virus infection. Blood 76:1104–1109.
40. Siebelink, C. H. J., R. W. Windrich, I. Chu, J. Groen, K. Weijer, F. G. C. M. Uytdehaag, and A. D. M. E. Osterhaus. 1990. An enzyme-linked immunosorbent assay (ELISA) for the detection of feline immunodeficiency virus (FIV) antigen in cell culture and FIV specific antibodies in feline serum. Dev. Biol. Stand. 72:189–196.
41. Siebelink, K. H. J., I. Chu, G. F. Rimmelzwaan, K. Weijer, R. van Herwijnen, P. Knell, H. F. Egberink, M. L. Bosch, and A. D. M. E. Osterhaus. 1990. Feline immunodeficiency virus (FIV) infection in the cat as a model for HIV infection in man: FIV-induced impairment of immune function. AIDS Res. Hum. Retroviruses 6:1373–1378.
42. Siebelink, K. H. J., I.-H. Chu, G. F. Rimmelzwaan, K. Weijer, A. D. M. E. Osterhaus, and M. L. Bosch. 1992. Isolation and partial characterization of infectious molecular clones of feline immunodeficiency virus obtained directly from bone marrow DNA of a naturally infected cat. J. Virol. 66:1091–1097.
43. Siebelink, K. H. J., J. A. Karlas, G. F. Rimmelzwaan, A. D. M. E. Osterhaus, and M. L. Bosch. A determinant of feline immunodeficiency virus involved in CrFK tropism. Vet. Immunol. Immunopathol., in press.
44. Siebelink, K. H. J., G. F. Rimmelzwaan, M. L. Bosch, R. H. Meloen, and A. D. M. E. Osterhaus. 1993. A single amino acid substitution in hypervariable region 5 of the envelope protein of feline immunodeficiency virus allows escape from virus neutralization. J. Virol. 67:2202–2208.
45. Sparger, E. E., P. A. Luciw, J. H. Elder, J. K. Yamamoto, L. J. Lowenstine, and N. C. Pedersen. 1989. Feline immunodeficiency virus is a lentivirus associated with an AIDS-like disease in cats. AIDS 3:S43–S49.
46. Stott, E. J. 1991. Anti-cell antibody in macaques. Nature (London) 353:393.
47. Takeda, A., and F. A. Ennis. 1990. FcR-mediated enhancement of HIV-1 infection by antibody. AIDS Res. Hum. Retroviruses 6:999–1004.
48. Verschoor, E. J., A. L. W. van Vliet, H. F. Egberink, W. Hesselink, W. E. van Alphen, I. Joosten, C. J. P. Boog, M. C. Horzinek, and A. de Ronde. Vaccination against feline immunodeficiency virus using fixed infected cells. Vet. Immunol. Immunopathol., in press.
49. Yamamoto, J. K., T. Hohdatsu, R. A. Olmsted, R. Pu, H. Loule, H. A. Zochlinski, V. Acevedo, H. M. Johnson, G. A. Soulds, and M. B. Gardner. 1993. Experimental vaccine protection against homologous and heterologous strains of feline immunodeficiency virus. J. Virol. 67:601–605.
50. Yamamoto, J. K., T. Okuda, C. D. Ackley, H. Loule, E. Pembroke, H. Zochlinski, R. J. Munn, and M. B. Gardner. 1991. Experimental vaccine protection against feline immunodeficiency virus. AIDS Res. Hum. Retroviruses 7:911–922.
51. Yamamoto, J. K., E. Sparger, E. W. Ho, P. R. Andersen, T. P. O'Connor, C. P. Mandell, L. Lowenstine, R. Munn, and N. C. Pedersen. 1988. Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. Am. J. Vet. Res. 49:1246–1258.

CHAPTER 8

Summarizing discussion

Despite numerous efforts a safe and effective vaccine for the prevention of HIV-1 infection has not yet been developed. A number of lentivirus characteristics hinder the development of effective vaccines against these viruses. Their extreme variability, their persistence as provirus, which remains hidden for the immune system, and their initial replication at the mucosal sites are some of the major problems that an effective vaccine has to deal with. A vaccine that would induce an efficient local immune response would abrogate or limit viral replication at an early stage, thereby facilitating the task for the systemic immune system. Virus particles escaping the local immune response should preferably encounter a strong specific systemic response, which ideally should include effective VN antibodies and CTL which would allow the killing of infected cells early in the viral replication cycle. To reduce the emergence of escape mutants it would be advantageous if antiviral immunity would be based on a variety of preferably conserved epitopes.

Our main efforts have focused on the development of lentivirus vaccines based on attenuated recombinant *Salmonella* strains. Attenuated *Salmonella* strains used as carrier show promise as lentivirus vaccines as they are able to induce specific local and systemic immune responses including class I restricted CTL responses. They have the capacity to express large heterologous genes enabling them to present a large number of viral B and T cell epitopes. Furthermore, they will be inexpensive to produce and easy to administer (orally), which are both important features, especially for third world countries where an HIV vaccine is most needed. However, a major obstacle in the development of vaccines based on live recombinant *Salmonella* strains, is that the amount of heterologous antigen required to induce an adequate immune response is often toxic for the recombinant bacteria. Therefore an expression system was developed that allows the continuous production of antigen by a bacterial population in spite of toxicity for individual producing bacteria. The system is based on a temperature inducible P_L promoter which inverts at random. This inversion is the result of recombination between two inverted repeats that border the promoter, by the site-specific recombinase Gin. The gene of interest is only expressed in one orientation of the promoter (ON-orientation), but not in the other (OFF-orientation). Provided the inversion frequency of the P_L promoter is low enough, only a minor part of the bacterial population will produce toxic levels of antigen, leaving the major part of the population unaffected. Unaffected by antigen production, the non-producing population may disseminate within the host and segregate new antigen producers. In this way antigen production can continue *in vivo* until the *S.*

typhimurium aroA mutant is cleared. In chapter 2 this expression system is evaluated using the Cholera toxin B subunit (CtxB) as a model antigen. Plasmid pYZ17 contains the *ctxB* gene under control of the invertible P_L promoter. Restriction enzyme digest on pYZ17 DNA revealed that the fast majority of the P_L promoters were in the OFF position, demonstrating selection against the ON form. BALB/c mice orally immunized with the *Salmonella typhimurium aroA* vaccine strain SL3261 harbouring pYZ17, induced high titres of CtxB specific IgG and IgA serum antibodies as well as secretory IgA in the gut. Nearly 100% of the bacteria stably maintained pYZ17 for several weeks *in vivo*.

In a single CtxB producing cell, on average only a fraction of the P_L promoters will be in the ON orientation. Therefore only a fraction of the *ctxB* gene copies will be expressed. We anticipated that antigen production could be increased considerably if a single promoter inversion would result in the expression of all the copies of the gene of interest. Therefore an improved invertible promoter expression system was developed and evaluated (chapter 3). This improved system consists of two different plasmids in one bacterial cell. One plasmid containing the gene of interest under control of a T7 promoter and the other containing the T7 RNA polymerase gene under control of the invertible P_L promoter. In this configuration a single promoter inversion results in the production of T7 RNA polymerase, which in turn will direct expression of all the gene copies under control of the T7 promoter. Using this two-plasmid system, much higher levels of CtxB could be obtained than with pYZ17. The higher production levels resulted indeed in enhanced CtxB specific serum IgG and IgA responses and sIgA responses in the gut of BALB/c mice after oral administration. It also induced enhanced CtxB specific serum IgG responses following i.p. immunization.

Following a promoter inversion, it will take longer before toxic production levels are obtained for a weakly toxic antigen than for more toxic antigens. Weakly toxic antigens will therefore allow a larger time span before cell division becomes impossible. In other words, weakly toxic antigens will tolerate a higher inversion frequency than more toxic antigens. To achieve optimal antigen expression, that is the maximum expression level that allows reasonably stable maintenance of the expression system, a series of different invertible promoter plasmids (pIP1 to pIP5, see Fig. 1 of chapter 3) was constructed. These plasmids differed in the relative orientation of *gin* and *cI857*, the composition of the untranslated leader of *gin*, or their copy numbers, which can all in their own way affect the inversion frequency. Different inversion frequencies may be reflected in different percentages of antigen

expressing cells and thereby in different amounts of antigen produced by the bacterial population as a whole. This was initially demonstrated for expression of the gene fusion between gene-10 of bacteriophage T7 and a 580 bp *env* fragment from HIV-1. Of the intermediate copy number plasmids pIP1 to -3, the highest expression of this gene fusion was revealed by pIP1, followed by pIP2 and finally pIP3. As expected, the low copy number plasmids pIP4 and pIP5 revealed less antigen production than their higher copy number counterparts pIP2 and pIP3, respectively. Similar observations were made for the maltose binding protein (MBP) production obtained with pIP3 and pIP5 (chapter 3) and production of the MBP-Gag FIV fusion protein obtained with pIP2 to -5. (chapter 5).

The two plasmid system can be used to produce toxic antigens. This was demonstrated for the gene-10-env fusion protein. When induction of T7 RNA polymerase is under control of a fixed P_L-promoter, so that protein production upon temperature induction will occur in all the cells of a bacterial population, colonies could only be recovered when the P_L promoter was not induced. However, when expression of the T7 RNA polymerase gene was under control of the invertible P_L-promoter, a comparable number of colonies was recovered at inducing and non-inducing temperatures. In spite of this toxic antigen production, most combinations of pET3xa-env with the different pIP vectors were essentially stably maintained *in vivo* , except for the combination with pIP1. This combination resulted in the highest antigen production levels, suggesting that the extreme instability observed with pIP1 might be the result of toxic antigen production in close to or more than 50% of the bacteria. The plasmid stability after growth of *S.typhimurium* in cell lines was determined and appeared to correlate with *in vivo* stability. Thus, an indication of plasmid stability *in vivo* may be obtained before animal experiments are initiated by determining the stability in cell lines.

Using the immunogenic maltose binding protein as a model antigen, it was demonstrated that the specific humoral response obtained after oral administration of SL3261 harbouring the invertible promoter system, was nearly forty times stronger than with a fixed promoter system (chapter 3, Fig. 7). This is most likely due to the higher viability of the bacterial population that employs the invertible promoter system. The non-producing part of this population will be able to actively infect the GALT and system lymphatics, meanwhile segregating new MBP producing bacteria. However, it is conceivable that the bacteria harbouring the fixed promoter system, become severely inhibited in their ability to invade the local and systemic lymphatics

soon after administration, as the vast majority of the bacteria loose viability within 45 minutes following exposure to a temperature of 37 ^{o}C *in vitro* .

Chapter 3 described to use of the invertible promoter system for the production of a viral (Env) and two bacterial (CtxB and MBP) proteins. The general applicability of the expression system for the continuous production of antigens at high levels, was further illustrated by the study described in chapter 4. In this study the system was used to express high levels of the Tam1-1 and Tams1-2 merozoite surface antigens of the parasite *Theileria annulata* by the *Salmonella* vaccine strain SL3261. Two days after infection of the macrophage cell line J774.16, the majority of these bacteria stably retained pIP5 in combination with the *Tam1-1* or *Tam1-2* containing plasmid. The stability in combination with pIP2 was about half of that observed for pIP5.

FIV infection of cats is considered a useful small-animal model for the evaluation of vaccine strategies relevant for controlling HIV-1 infections in humans. To evaluate the potential of recombinant *Salmonella* strains as candidate lentivirus vaccines, FIV antigens were expressed in SL3261 using the two plasmids system. Initially the ability of SL3261 as a carrier to present heterologous antigens to the feline immune system was determined. *Salmonella typhimurium aroA* strain SL3261 expressing the MBP-FIV Gag fusion protein and CtxB as a control, proved to be immunogenic in cats, as high specific serum antibody titres to *Salmonella* LPS, CtxB and MBP developed after a single immunization. Specific serum antibodies against Gag developed after a second i.p. immunization. No Gag specific antibodies were induced in the orally immunized cat, but this cat was primed for Gag by this immunization procedure as demonstrated by the faster development of Gag specific antibodies after FIV challenge of this cat compared to the control cats. It is unlikely that this faster antibody response is the result of enhancing antibodies acting at levels below the detection limit, as it is unlikely that antibodies against the capsid proteins will induce enhancement of infectivity. Also, the viral loads 33 days post challenge in the cat orally immunized with the MBP-Gag expressing *Salmonella* was not higher than that of the control cats. Although this study demonstrated the ability of *Salmonella* to present antigens to the feline immune system, the immunisation failed to protect the cats against a challenge with homologous molecular cloned FIV. The vaccination schedule was therefore extended. Two experimental FIV vaccines were tested, either alone or in combination. One vaccine (SL3261-FIV) incorporated SL3261 strains expressing Gag, as well as strains expressing part of the envelope protein (Env) of FIV. The other vaccine was composed of bacterial derived FIV-Gag and

recombinant vaccinia virus derived FIV-Env proteins incorporated in immune stimulating complexes (iscom-FIV). Cats of group A received four immunisations with SL3261-FIV and cats of group B two immunisations with SL3261-FIV, followed by two immunizations with iscom-FIV. Cats of group C were immunised twice with the CtxB expressing SL3261 strain (SL3261-CtxB) followed by two immunisations with iscom-FIV. The cats of group D, which served as a negative control, were immunized twice with SL3261-CtxB and twice with iscom in which the Gag and Env proteins of SIV had been incorporated (iscom-SIV). It was hoped that the SL3261 and iscom immunisation would complement each other. Both are able to induce class I restricted CTL, but may do so by different routes. The induction of CD8+ CTLs by iscoms is most likely a result of entry into the endogenous pathway of antigen processing and presentation[7], while SL3261 may have access to an alternative route of class I restricted presentation, described for particulate antigens (for review see ref. 4) that requires phagocytotic APCs. Furthermore, the glycosilated vaccinia derived Env protein in the iscom preparation, may present additional VN epitopes that are absent from the non-glycosilated Env produced by SL3261. Specific antibodies were induced much more efficiently by immunisation with iscom-FIV than by immunisation with SL3261-FIV. Repeated immunisations with the last, did not induce detectable Env-specific antibody responses and only weak Gag-specific responses. However, cats that had previously been immunized with SL3261-FIV were primed, as Gag-specific antibodies developed faster in these cats after immunisation with iscom-FIV, compared to cats previously immunized with SL3261-CtxB. Two weeks after the last immunisation all the cats were FIV-challenged. At this time point all the cats that had received iscom-FIV had developed considerable serum antibody responses to Gag and Env, while these responses were weak or undetectable in the cats immunized four times with SL3261-FIV. Seven weeks after FIV-challenge the Env-specific antibody responses had increased considerably in the cats of all groups, apart from the group immunised four times with SL3261. By sixteen weeks post challenge only one cat of this group had developed a considerable anti-Env response, comparable with that of the negative control cats. The overall virus load, as determined by the number of FIV infected PBMC and by PCR end-point titration, was lowest in group A at all time points. Taken together, these data strongly suggests that the cats immunized four times with SL3261-FIV were partially protected against FIV-challenge. It is unlikely that the reduced virus load in this group was a result of VN antibodies, as no significant Env-specific antibody titres were detected and only

weak Gag-specific titres. Furthermore, in spite of much higher specific serum antibody titres in the cats immunised with iscom-FIV, no reduced virus load was observed in these cats. It seems therefore more likely that a cellular response is at the basis of the observed reduced virus load. The cats of the previous study, immunized only with SL3261-Gag, were not protected against FIV-challenge, suggesting that SL3261-Env contributed to the observed partial protection. These results are in accordance with the observation that Env but not Gag-specific CTL responses correspond with protection against FIV infection[1]. Care should be taken in the interpretation of these results, however, as the group sizes were small and the used immunisation schedules not identical.

In chapter 7 the evaluation of different FIV envelope preparations incorporated in iscoms is described. Two different recombinant vaccinia virus derived glycosilated envelope proteins and a non-glycosilated bacterially produced b-galactosidase-Env fusion protein were compared. Both glycosilated proteins were identical except that one was expressed from an *env* gene in which the region encoding the cleavage site between the surface and the transmembrane proteins, had been deleted to facilitate incorporation of the FIV SU protein into iscom. Env was prepared using a recombinant vaccinia virus expression system. The different envelope proteins incorporated into iscom, and the relevant control preparations - cleavage site deleted FIV glycoproteins mixed with Quil A, and a SIV iscom preparation - were used to immunise cats. The highest Env specific serum antibody responses and VN antibody responses were induced by the iscoms preparations that contained the vaccinia virus derived envelope proteins, with no significant difference between the envelope proteins with and without cleavage site. In accordance with earlier studies[3] the responses were considerably weaker when Env was not incorporated in iscom, but just mixed with Quil A. The b-galactosidase-Env fusion protein iscom preparation was least effective in the induction of Env-specific antibodies and failed to induce detectable levels of VN antibodies. All vaccine preparations failed to protect cats against FIV challenge. More striking, the preparations that induced VN antibodies detectable in the CrFK VN assay even enhanced FIV infectivity. This enhancement effect could be transferred with plasma of the vaccinated cats, indicating that it was mediated by antibodies.

Although iscom-FIV induced antibodies much more effectively than SL3261-FIV, it had no protective effect against FIV infection of cats. SL3261-FIV, on the other hand, did not induce detectable antibody responses to Env and induced only weak responses to Gag, but the data suggest that cats immunised four times with this vaccine were partly protected against FIV infection. A

number of studies support the view that the use of attenuated *Salmonella* strains as carries for heterologous antigens favours the induction of a Type-1 immune response[8], which is characterised by a dominant cellular response, as opposed to a Type-2 response which favours the development of antibody responses. In HIV-1 infected individuals the switch from a dominant Type-1 immune response to a dominant Type-2 response is associated with a fast progression towards AIDS. It may be speculated that the immunization with the Env- and Gag-expressing SL3261 strains has driven the immune system to the more favourable Type-1 response, while the immunization with iscom-FIV, directed the immune system to the less favourable Type-2 response. This hypothesis is in accordance with the relatively low cell-associated virus load and with the weak induction of antibodies against SU1 and SU3 in the cats immunized four times with the Gag- and Env-expressing SL3261 strains upon FIV challenge. It would also explain why the antibody response to Gag following the first iscom-FIV immunization is faster, but not stronger in cats primed with SL3261-FIV (group C) compared to unprimed cats (group B). The second iscom-FIV immunisation may have driven the immune system further toward a Type-2 response, thereby undoing any potentially protective effect from the SL3261-FIV priming.

Concluding remarks

The invertible promoter system described in this thesis is very universal. In principle it allows the continuous expression of every gene that can be expressed with the T7 RNA polymerase/T7 promoter systems described by Studier et al.[5] and Tabor[6], even if gene expression is toxic. In fact, due to the high transcription levels obtained, expression of most genes employing the T7 RNA polymerase system will be toxic for the expressing bacteria[5]. In its current form the invertible promoter system should be considered as a prototype. The antibiotic resistance genes present on the plasmids, make its use in a human vaccine unacceptable. Only low copy numbers of the T7 RNA polymerase gene are needed. Therefore the T7 RNA, polymerase gene and its invertible promoter can be integrated into the chromosome, obviating the need of antibiotic selection pressure to stably maintain the gene. To obtain high production levels of the antigen of interest, the corresponding gene should be present on a high copy number plasmid. To allow stable maintenance of this plasmid in the absence of antibiotic selection pressure, the ampicillin resistance gene can be replaced by an essential genes that has been deleted in the carrier strain. For this purpose the well characterised *asd*-system described by

Nakayama et al.[2] can be used. Both modifications have the additional advantage that they will further stabilise the expression system.

Although none of the vaccine strategies tested in this study fully protected cats against FIV infection upon challenge, the reduced virus load observed in cats immunized four times with SL3261-FIV compared to control cats is encouraging. More extended vaccine studies using attenuated *Salmonella* strains as carriers will have to demonstrate the true significance of this approach for lentivirus vaccine development. Whatever the outcome of these studies, the ability of the invertible promoter system to allow the abundant expression of (toxic) heterologous antigens by recombinant *Salmonella* strains, may greatly facilitate the evaluation of these live carriers as candidate vaccines against a variety of other pathogens.

REFERENCES

1. **Flynn, J.N., Keating, P., Hosie, M.J., Mackett, M., Stephens, E.B., Beatty, J.A., Neil, J.C. & Jarrett, O.** (1996). Env-specific CTL predominate in cats protected from feline immunodeficiency virus infection by vaccination. *Journal of Immunology* **157**, 3658-3665.

2. **Nakayama K., Kelly S.M. and Curtiss R.** (1988). Construction of an *asd*$^+$ expression-cloning vector: Stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**:693-697.

3. **Rimmelzwaan G.F., Siebelink K.H.J., Huisman R.C., Moss B., Francis M.J. and Osterhaus A.D.M.E.** (1994). Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation in immune-stimulating complexes. *Jounal of General Virology* **75**, 2097-2102.

4. **Rock K.L.** (1996). A new foreign policy: MHC classI molecules monitor the outside world. *Immunology Today* **17**, 131-137

5. **Studier F.W., Rosenberg A.H., Dunn J.J., and Dubendorff J.W.** (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.***189**, 60-89.

6. **Tabor S.** (1990). Expression using the T7 RNA polymerase/ promoter

system, p. 16.2.1-16.2.11 *In* F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed.) Current protocols in Molecular Biology. Greene Publishing and Wiley-Interscience, New York.

7. **Van Binnendijk, R.S., Van Baalen C.A., Poelen M.C.M., De Vries P., Boes J., Cerundolo V., Osterhaus A.D.M.E. and UytdeHaag F.G.C.M.** (1992). Measles virus transmembrane fusion protein synthesized *de novo* or presented in iscom is endogenously processed for HLA class I and class II restricted cytotoxic T cell recognition . *J. Exp. Med.* **176**, 119-128.

8. **Yang D.M., Fairweather N., Button L.L., McMaster W.R., Kahl L.P., and Liew F.Y.** (1990). Oral *Salmonella typhimurium* (AroA-) vaccine expressing a major Leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against Leishmaniasis. *Journal of Immunology* **145**, 2281-2285.

Samenvatting

Ondanks grote inspanning is er tot op heden nog geen veilig en effectief vaccin ter voorkoming van HIV-1 infecties ontwikkeld. Een aantal eigenschappen van lentivirussen, zoals hun extreme variabiliteit en hun vermogen om als provirus "onzichtbaar" voor het immuunsysteem te persisteren, bemoeilijken de ontwikkeling van een dergelijk vaccin. Een vaccin dat in staat is een effektieve mucosale immuunrespons te induceren, zal in een vroeg stadium replicatie van het binnenkomende virus kunnen beperken of zelfs voorkomen. Een sterke systemische immuunrespons, bestaande uit cytotoxische T- cellen (CTL) die een geïnfecteerde cel vroeg in de replicatiecyclus kunnen vernietigen, alsmede virus-neutraliserende (VN) antilichamen, moeten dan de virussen uitschakelen die aan de mucosale immuunrespons ontsnapt zijn. Om het onstaan van zogenaamde "escape"-mutanten zoveel mogelijk te voorkomen, zou de antivirale immuniteit op een aantal, bij voorkeur geconserveerde, epitopen gebaseerd dienen te zijn.

Het in dit proefschrift beschreven onderzoek heeft zich allereerst geconcentreerd op de ontwikkeling van lentivirusvaccins gebaseerd op geattenueerde recombinant *Salmonella* stammen. Deze stammen koloniseren na orale toediening de lymfoïde weefsels van het darmkanaal (de GALT) en vervolgens de systemische lymphoïde organen. Geattenueerde *Salmonella* stammen zijn veelbelovend als "dragers" voor lentivirus-antigenen, omdat zij in staat zijn specifieke lokale en systemische immuunresponsen, waaronder klasse I gerestrikteerde CTLs, te induceren. Ook zijn ze in staat grote heterologe genen, met veel virale B en T cel epitopen, tot expressie te brengen. Verder zullen dit soort vaccins relatief goedkoop geproduceerd en eenvoudig (oraal) toegediend kunnen worden. Deze laatste twee eigenschappen zijn met name van belang voor Derde-Wereld landen, waar een HIV-vaccin het meest nodig is. Om een voldoende sterke specifieke immuunrespons tegen het heterologe antigeen te induceren moet doorgaans echter veel van het antigeen door de bacteriën geproduceerd worden. De produktie van deze hoeveelheden is vaak toxisch voor de bacteriën, hetgeen resulteert in een te sterke attenuatie van de bacteriën en/of instabiliteit van de antigeenproduktie. Beiden resulteren in de presentatie van suboptimale hoeveelheden antigeen aan het immuunsysteem. Er is daarom een expressiesysteem ontwikkeld dat een bacteriële populatie in staat stelt voortdurend grote hoeveelheden heteroloog antigeen te produceren, ondanks dat dit toxisch is voor de individuele producerende bacteriën. Het systeem is gebaseerd op een temperatuur-induceerbare P_L promoter die van oriëntatie kan veranderen. Deze inversie is het resultaat van recombinatie tussen twee "inverted

repeats" waartussen de P_L promoter gesitueerd is. Slechts in één oriëntatie van de P_L promoter (AAN-oriëntatie), maar niet in de andere (UIT-oriëntatie), zal het heterologe gen tot expressie komen. De essentie van het systeem is, dat slechts een minderheid van de bacteriën toxische hoeveelheden antigeen produceert. Hierdoor blijft het grootste deel van de populatie onaangetast. Deze niet producerende bacteriën zijn in staat de infectie voort te zetten en zullen door promoter inversies voortdurend nieuwe antigeen producerende bacteriën voortbrengen. Op deze wijze kan de presentatie van antigeen aan het immuunsysteem voortgezet worden, tot de geattenueerde*Salmonella* stam uiteindelijk uit het lichaam verdwijnt. In hoofdstuk 2 van dit proefschrift is de B subunit van Cholera toxine (CtxB) gebruikt om dit expressiesysteem te evalueren. Plasmide pYZ17 bezit het *ctxB* gen onder de controle van de inverteerbare P_L promoter. Restriktieënzym analyse van pYZ17 DNA wees uit dat het merendeel van de P_L promoters zich in de UIT-oriëntatie bevond. Er vindt dus selectie plaats tegen de AAN-oriëntatie. Een *Salmonella* vaccin-stam dat pYZ17 bevatte werd vervolgens via een maagsonde aan BALB/c muizen toegediend. Deze muizen ontwikkelden hoge titers CtxB-specifiek IgG en IgA in hun sera en tevens secretoir IgA in de darmen. Bijna 100% van de bacteriën behield pYZ17 gedurende enkele weken *in vivo*.

In een CtxB producerende bacterie zal doorgaans slechts een deel van de P_L promoters de AAN-orientatie hebben. Daarom zal slechts een deel van de *ctxB* genen in zo'n bacterie aan de CtxB produktie bijdragen. Als een enkele promoterinversie in de expressie van alle *ctxB* genen binnen die bacterie zou resulteren, dan zou de CtxB produktie aanzienlijk vergroot kunnen worden. In hoofdstuk 3 is een expressiesysteem beschreven dat dit mogelijk maakt. Dit verbeterde expressiesysteem bestaat uit twee verschillende plasmiden. Een van de plasmiden bezit het *ctxB* gen onder controle van een T7 promoter. Het andere plasmide bevat het T7 RNA polymerase gen onder controle van de inverteerbare P_L promoter. Door deze configuratie zal een enkele promoter inversie in een bacterie leiden tot de produktie van T7 RNA polymerase. Dit zal op zijn beurt resulteren in de expressie van alle *ctxB* genen binnen deze bacterie. Dit 2-plasmiden systeem resulteerde inderdaad in een veel hogere CtxB-produktie dan met pYZ17 verkregen kon worden. De hogere produktie leidde na orale toediening tot aanzienlijk hogere CtxB-specifieke IgG en IgA titers in de sera, en sIGA titers in de darmen van BALB/c muizen.

Om de algemene toepasbaarheid van het verbeterde inverteerbare promoter (IP) expressiesysteem te demonstreren, werden een ander bacteriëel antigeen (het maltose bindend proteïne (MBP)), een viraal antigeen (een deel van

het HIV-1 envelopeiwit gefuseerd met het gen-10 eiwit) en twee protozoaire antigenen (de Tam1-1 en Tam1-2 merozoïet oppervlakte antigenen van *Theileria annulata* (hoofdstuk 4)) tot expressie gebracht. Met het IP-systeem konden al deze genen continu en in grote hoeveelheden geproduceerd worden. Wordt expressie van T7 RNA polymerase echter gecontroleerd door een gefixeerde promoter, zodat antigeen produktie na temperatuur induktie in alle cellen van de bacteriële populatie plaats vindt, dan kunnen de bacteriën alleen groeien bij een temperatuur waarbij de PL promoter onderdrukt blijft.

Het immunogene MBP werd als model antigeen gebruikt om het voordeel van het IP-systeem voor vaccinatiedoeleinden te demonstreren. Na orale immunisatie van muizen werd een bijna 40 maal sterker MBP-specifieke antilichaam respons met het IP-systeem verkregen, dan met het gefixeerde promoter systeem. Vervolgens werd het IP-systeem gebruikt om het Gag eiwit van het feline immunodeficiëntie virus (FIV) als fusieëiwit met MBP tot expressie te brengen. *Salmonella* stammen die dit fusieëiwit of, als een controle, *ctxB* expresseerden bleken immunogeen in katten. Een enkele immunisatie resulteerde in hoge specifieke antilichaamresponsen tegen *Salmonella* LPS, CtxB en MBP. Tevens werden na een tweede i.p. immunisatie ook FIV Gag specifieke antilichamen geïnduceerd. Orale immunisatie induceerde geen Gag-specifieke antilichaam respons, maar resulteerde wel in Gag-specifieke "priming". Ofschoon dit experiment aantoonde dat *Salmonella* in staat is antigenen aan het immuunsysteem van katten te presenteren, beschermde de immunisatie de katten niet tegen een experimentele infectie met FIV. Het vaccinatieschema werd daarom uitgebreid. Naast Gag producerende *Salmonella* stammen werden tevens FIV-Env producerende stammen toegediend. Bovendien kregen de katten dit *Salmonella*-FIV vaccin zowel oraal als i.p. toegediend. Een ander vaccin bestond uit recombinant FIV-Gag en FIV-Env geïncorporeerd in immuun stimulerende complexen (iscom). Na FIV-infectie was alleen in de groep katten die vier maal met het *Salmonella*-FIV vaccin geïmmuniseerd was, de virustiter lager dan in de controle groep. Verder was na FIV-infectie de Env-specifieke antilichaamrespons in deze groep veel zwakker dan de overige groepen. Deze resultaten suggereren dat de vier immunisaties met *Salmonella*-FIV een zekere mate van bescherming tegen infectie met FIV induceerden.

Het laatste deel van het proefschrift beschrijft de evaluatie van verschillende subunitvaccins gebaseerd op FIV-envelopeiwitten. Geglycosyleerd envelopeiwit met of zonder de klievingsplaats en een bacteriëel geproduceerd ongeglycosileerd FIV-envelop fusieëiwit, werden geïncorporeerd in iscoms of gemengd met QuilA als adjuvant. Al deze vaccins induceerden Env-specifieke

antilichamen in katten, maar er bestonden duidelijke verschillen tussen de responsen geïnduceerd door enerzijds de geglycosileerde eiwitten en anderzijds het ongeglycosileerde envelopeiwit. Zo induceerden uitsluitend de geglycosileerde eiwitten antilichamen die in staat waren FIV-infectie van een kattenier cellijn te neutraliseren. Verder herkenden de antilichamen geïnduceerd met de geglycosileerde eiwitten meer verschillende regio's van het envelopeiwit. Na infectie met FIV bleek in de katten die met de geglycosileerde eiwitten geïmmuniseerd waren, eerder virus in het bloed aantoonbaar, dan bij de katten van de andere groepen. Deze versnelde infectie kon met plasma van de gevaccineerde dieren worden overgebracht naar naïve katten.

Geen van de vaccinstrategiën beschreven in dit proefschrift was in staat katten volledig tegen een experimentele FIV infectie te beschermen. Het is echter bemoedigend te zien dat de virustiter na experimentele FIV-infectie lager was in de groep katten die vier maal met *Salmonella*-FIV geïmmuniseerd was, dan in de controle groep. Meer uitgebreide vaccinstudies waarin *Salmonella* stammen als dragers van lentivirus antigenen gebruikt worden zullen het uiteindelijke belang van deze benadering voor de ontwikkeling van lentivirus vaccins moeten demonstreren. Het vermogen van het inverteerbare promoter systeem om continu grote hoeveelheden (toxisch) antigeen door recombinante *Salmonella* stammen te laten produceren, zal de evaluatie van deze levende dragers als candidaat vaccins tegen lentivirussen en andere pathogenen aanzienlijk vereenvoudigen.

Dankwoord

Eindelijk is-ie dan af. Eigenlijk valt het schrijven van zo'n boekje best nog mee. Als je er maar een beetje de tijd voor neemt! En natuurlijk is ook de hulp van vele anderen onontbeerlijk. Een aantal mensen wil ik in dit kader niet onvermeld laten. Laat ik maar beginnen met Ab. Tenslotte is zo'n promoter toch de drijvende kracht achter een promotieonderzoek. Ab, je tomeloze energie is zeer inspirerend en zonder jouw motiverende woorden had dit boekje nog wel eens langer op zich kunnen laten wachten. Beste Frits, als co-promoter ben jij mijn grote steun geweest. Je interesse in het onderzoek, de tijd die je altijd voor mij wist vrij te maken, je goede raad en je integriteit waardeerde ik zeer (behalve wanneer dat inhield dat een prachtig resultaat nog maar een paar keer herhaald diende te worden in verband met de reproduceerbaarheid). Jos, mijn paranimf en voormalig "superanalist", bedank ik voor al het werk dat hij verzet heeft. Je humor en gezelligheid hebben enorm bijgedragen aan de goede tijd die ik op het RIVM heb gehad. Robin, bedankt voor het FIV envelop eiwit en de hulp bij het opwerken van het kattenbloed (we hebben 's avonds laat wat gelachen op het lab, hè). Kees, zonder al het werk dat jij al besteed had aan het opzetten van het FIV model was het natuurlijk nooit wat geworden met die kattenstudies. Willem bedank ik voor het afronden van de "infectious centre" tests, terwijl ik al in Schotland van het goede leven genoot. Het (komisch) duo Geert en Nico ben ik dankbaar voor de vakbekwame uitvoering van de kattenexperimenten. Ellen, je SIV-iscoms waren van top-kwaliteit! Leuk dat onze samenwerking in het afgelopen jaar nu vruchten lijkt af te werpen. Tony de Ronde en Rob van Herwijnen dank ik voor het uitvoeren van, respectievelijk, de FIV-Env en -Gag ELISA's. Frank, bedankt voor je grote inzet tijdens je stage. Sabine, your contribution to this thesis may have been modest, but your impact on me certainly wasn't! Your friendship is greatly appreciated. Christine, ik heb onze samenwerking zeer op prijs gesteld. Zonder jouw bijdrage was dit proefschrift een hoofdstuk korter geweest. I am grateful to Thomas Meyer and Yan Zheng-Xin of the Max Planck Institute in Tübingen for their hospitality during my stay in their laboratory and the fruitful collaboration on the phase-variable expression system. Of my present colleagues I would like to thank Alex for his help with the cover of this thesis and my supervisor Rick Randall for his patience and understanding in the past three years. Ron ben ik dankbaar voor het gebruik van zijn computer wanneer ik tijdens mijn "vakanties" in Nederland weer eens een poging deed dit proefschrift af te ronden. Verder wil ik alle collega's en ex-collega's van het voormalige IMB en van het MMB bedanken voor hun goede adviezen, de expressievectoren, chemicalien en enzymen die ik in de loop der jaren van ze "gebietst" heb en vooral ook voor de uitstekende werksfeer.

Himali, ik had vaak de indruk dat het schrijven van dit proefschrift voor jou nog een grotere beproeving was dan voor mij. Ik ben je dan ook dankbaar voor je liefdevolle geduld. Wat jij en mijn familie voor mij betekenen is niet in een dankwoord uit te drukken. Ik draag dit proefschrift dan ook aan jullie op.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 20 juni 1962 te Hilversum. In 1981 werd het VWO-B diploma behaald, waarna werd aangevangen met de studie Biologie aan de Rijks Universiteit Utrecht. In 1988 werd het doctoraal examen behaald, met de hoofdvakken Moleculaire Biologie (Prof. H.O. Voorma) en Microbiologie (Dr. J. Tommassen) en het bijvak Immunologie (Prof. Dr. A.D.M.E. Osterhaus). Na vier maanden werkzaam te zijn geweest op de afdeling tumorbiologie van het Nederlands Kanker Instituut in Amsterdam, werd in december 1989 het in dit proefschrift beschreven promotie onderzoek gestart aan het voormalig Laboratorium voor Immunobiologie van het Rijks Instituut voor de Volksgezondheid en het Milieu (RIVM) in Bilthoven. Sinds Augustus 1994 is de auteur werkzaam bij de School of Biomedical Sciences in St. Andrews (Schotland).