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Double mutant Salmonella microorganisms help prevent reactivity of the microorganism while maintaining the effectiveness of the microorganism to elicit an immune response. Various specific combinations of mutants are beneficial.

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ATTENUATED MICROORGANISMS FOR THE TREATMENT OF INFECTION

Field of the Invention

This invention relates to attenuated microorganisms that can be used in vaccine compositions for the prevention or treatment of bacterial or viral infections.

Background to the Invention

It is well established that live attenuated micro-organisms are highly effective vaccines; immune responses elicited by such vaccines are often of greater magnitude and of longer duration than those produced by non-replicating immunogens. One explanation for this may be that live attenuated strains establish limited infections in the host and mimic the early stages of natural infection. In addition, unlike killed preparations, live vaccines are able to induce potent cell-mediated responses which may be connected with their ability to replicate in antigen-presenting cells, such as macrophages.

There has been a long history of the use of live attenuated *Salmonella* vaccines as safe and effective vaccines for the prevention of salmonellosis in animals and humans. Indeed, the live attenuated oral typhoid vaccine, Ty21a (Vivotif), manufactured by the Swiss Serum Vaccine Institute, has proved to be a very successful vaccine for the prevention of typhoid fever and has been licensed in many countries including the US and Europe.

However, the attenuation of this strain was achieved using chemical mutagenesis techniques and the basis of attenuation of the strain is not fully understood. Because of this, the vaccine is not ideal in terms of the number of doses (currently four) and the number of live organisms that have to be given at each dose.

Modern molecular biology techniques, coupled with the increasing knowledge of *Salmonella* pathogenesis, has led to the identification of several genes that are essential for the *in vivo* growth and survival of the organisms. This has provided new gene targets for attenuation, leading to the concept that future vaccine strains can be 'rationally' attenuated by introducing defined non-reverting mutations into selected genes known to be involved in virulence. This will facilitate the development of improved vaccines, particularly in terms of the immunogenicity and therefore the number of doses that have to be given.

Although many attenuated strains of *Salmonella* are now known, few have qualified as potential vaccine candidates for use in humans. This may be due in part

to the need to balance the immunogenicity of the vaccine with the possibility of the *Salmonella* microorganism becoming reactive.

It is clear that the selection of appropriate targets for attenuation which will result in a suitable vaccine candidate, is not straightforward and cannot easily be predicted. Many factors may influence the suitability of the attenuated strain as an appropriate vaccine, and there is much research being carried out to identify suitable strains. For example, many attenuated strains tested as vaccine candidates lead to vaccinemia or abscesses in the patient.

It is therefore desirable to develop a vaccine having a high degree of immunogenicity with reduced possibility of the microorganism strain reverting to an reactive form.

Summary of the Invention

The present invention is based on the finding that several combinations of attenuating mutations introduced into a *Salmonella* microorganism can produce a vaccine having a high degree of immunogenicity and a low risk of the microorganism reverting to a reactive form. The resulting vaccine strains exhibit good side-effect profiles.

According to a first aspect of the invention, a *Salmonella* microorganism has an attenuating mutation which disrupts the expression of a gene located within the Spi2 pathogenicity island, and a further mutation which disrupts the expression of any of the genes *clpP*, *ompR*, *sifA*, *sseC* or *ssaB*.

According to a second aspect of the invention, a *Salmonella* microorganism has an attenuating mutation which disrupts the expression of an *aro* gene, and a further mutation which disrupts the expression of any of the genes *clpP* or *sifA*.

The *Salmonella* microorganisms may be used in the manufacture of a medicament for intravenous or oral delivery for the treatment of a bacterial or viral infection, e.g. for the treatment of typhoid.

Description of the Invention

The microorganisms and vaccine compositions of the present invention may be prepared by known techniques.

The choice of particular *Salmonella* microorganism and the selection of the appropriate mutation, can be made by the skilled person without undue experimentation. A preferred microorganism is *Salmonella typhimurium*.

A first set of mutants comprises a first mutation in a gene located within the region of the *Salmonella* pathogenicity island two (Spi2); this region is disclosed in WO-A-9617951.

Spi2 is one of two classical pathogenicity islands located on the *Salmonella* chromosome. Spi2 comprises several genes that encode a type III secretion system involved in transporting Spi2-encoded virulence-associated proteins (so-called effector proteins) outside of the *Salmonella* bacteria and potentially directly into target host cells such as macrophages. Part of Spi2 (the apparatus genes) encodes the secretion apparatus of the type III system. Spi2 is absolutely essential for the pathogenesis and virulence of *Salmonella* in the mouse, an observation now documented by several different groups around the world. *S. typhimurium* Spi2 mutants are highly attenuated in mice challenged by the oral, intravenous and intraperitoneal routes of administration.

In a preferred embodiment, the gene in the Spi2 region is an apparatus gene. Apparatus genes located within Spi2 are now well characterised; see for example Hensel *et al.*, Molecular Microbiology, (1997); 24(1): 155-167. Genes suitable for use in the present invention include *ssaV*, *ssaJ*, *ssaK*, *ssaL*, *ssaM*, *ssaO*, *ssaP*, *ssaQ*, *ssaR*, *ssaS*, *ssaT*, *ssaU* and *ssaH* genes.

The mutation in the Spi2 region does not necessarily have to be within a gene to disrupt the function. For example, a mutation in an upstream regulatory region may also disrupt gene expression, leading to attenuation. Mutations in an intergenic region may also be sufficient to disrupt gene function.

In a preferred embodiment of the invention, the Spi2 gene is *ssaV* and the further mutation disrupts any of *clpP*, *ompR*, *sifA* or *sseC*. In a separate preferred embodiment, the mutation disrupts *ssaT* and the further mutation disrupts *ssaB*.

The *clpP* gene is described in Gifford *et al.*, Gen. Microbiol., 1993; 139:913-920. The encoded protein is a stress-response protease.

The *ompR* gene is described in Chatfield *et al.*, Infection and Immunity, 1991; 59(1): 449-452. The encoded protein is a component of a two-component system (OmpR-EnvZ) with a global regulatory function, and is also a regulator for the two-component system *ssrA-ssrB* in Spi2 (Lee *et al.*, J. Bacteriol., 2000; 182(3): 771-781).

The *sseC* gene is described in Medina *et al.*, Infection and Immunity, 1999; 67(3): 1093-1099. The function of the encoded product is unknown.

The *ssaB* gene is described in Hensel, Molecular Microbiology, 2000; 36(5):1015-1023. The encoded product is a known substrate protein for Spi2, and interacts with normal endosomal trafficking in macrophages.

A second separate set of mutants comprise a first mutation that disrupts an *aro* gene. This mutation may be termed an "auxotrophic mutation" as the *aro* gene is essential in a biosynthetic pathway present in *Salmonella*, but not present in mammals. Therefore, the mutants cannot depend on metabolites found in the treated patient to circumvent the effect of the mutation. Suitable genes for the auxotrophic mutation, include *aro*A, *aro*C, *aro*D and *aro*E. In the preferred embodiment, *aroC* is disrupted.

The second mutation disrupts any of the *clpP* or *sifA* genes. *ClpP* is described above. The *sifA* gene is described in Stein *et al.*, Mol. Microbiol., 1996; 20(1):151-164 and Beuzon *et al.*, EMBO J., 2000; 19(13): 3235-3249. The *sifA* gene product is involved in the production in epithelial cells of lysosomal glycoprotein-containing structures.

The mutations may be introduced into the microorganism using any known technique. Preferably, the mutation is a deletion mutation, where disruption of the gene is caused by the excision of nucleic acids. Alternatively, mutations may be introduced by the insertion of nucleic acids or by point mutations. Methods for introducing the mutations into the specific regions will be apparent to the skilled person.

For example, gene deletions may be created by first amplifying the target gene plus flanking DNA using PCR and a high fidelity polymerase. The amplified product may then be cloned into a suitable cloning vector. PCR primers can be designed to delete the gene when used in inverse PCR, to generate an initial construct. The PCR primers may contain an *XbaI* site to introduce a new restriction site and thus provide a marker for the gene deletion. The deletion construct can then be transferred to a suicide vector for transfer to the Salmonella chromosome. This construct can be electroporated or conjugated into the desired strain, and recombinants containing the plasmid integrated into the chromosome at the homologous site (merodiploids), selected using an antibiotic resistance marker carried on the plasmid. The suicide vector may also contain the *sacB* gene that encodes the enzyme levan sucrase, which is toxic to most Gram-negative bacteria in the presence of sucrose. Sucrose selection may therefore be employed to isolate colonies where a second recombination event has occurred, resulting in loss of the plasmid from the chromosome. This second recombination event can result in two outcomes, re-generation of the wild-type allele

or generation of a deletion mutant. Colonies containing the deletion mutation may then be identified by colony-PCR and the deletion confirmed by Southern blot analysis.

In addition to the two mutations, the *Salmonella* microorganism may also comprise heterologous antigens. The attenuated microorganism can therefore act as a delivery vehicle for administering antigens against other bacterial or viral infections. Antigens which are suitable for use in this way will be apparent to the skilled person and include:

Pathogenic *E. coli* antigens, i.e. ETEC

Hepatitis A, B and C antigens

Lime disease antigens

Vibrio cholera antigens

Helicobacter antigens

Herpes Simplex virus antigens

Human papilloma virus antigens

This system also has the potential to deliver therapeutic proteins, peptides or nucleic acids for the treatment of patients, e.g. patients infected with hepatitis. Cytokines are an example of suitable therapeutic proteins which may be delivered by the mutant microorganisms. Methods for the delivery of heterologous antigens or therapeutic proteins using the vaccine compositions will be apparent to the skilled person.

Vaccines made using the microorganisms of the invention have application to the treatment of infections in human patients and in the treatment of veterinary infections.

The double mutation provides an effective means to attenuate the microorganism to provide a safe vaccine candidate.

The vaccine compositions provide effective protection even in immuno-compromised patients, and importantly offer a low risk in developing spleen abscesses. Spleen abscesses have been identified using vaccines based on a single mutation, and therefore the present compositions may offer a substantial benefit to patients.

To formulate the vaccine compositions, the mutant microorganisms may be present in a composition together with any suitable pharmaceutically acceptable adjuvant, diluent or excipient. Suitable formulations will be apparent to the skilled person. The formulations may be developed for any suitable means of administration. Preferred administration is via the oral or intravenous routes and the vaccines are live attenuated *Salmonella* microorganisms. The number of microorganisms that are

required to be present in the formulations can be determined and optimised by the skilled person. However, in general, a patient may be administered approximately 10^7-10^{10} CFUs of the microorganism, preferably approximately 10^8-10^9 CFUs per single dosage unit.

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CLAIMS

1. A *Salmonella* microorganism having an attenuating mutation which disrupts the expression of a gene located within the Spi2 pathogenicity island, and a further mutation which disrupts the expression of any of the genes *clpP*, *ompR*, *sifA*, *sseC* and *ssaB*.

2. A *Salmonella* microorganism having an attenuating mutation which disrupts the expression of an *aro* gene, and a further mutation which disrupts the expression of any of the genes *clpP* and *sifA*.

3. A microorganism according to claim 2, wherein the *aro* gene is *aro*C.

4. A microorganism according to claim 1, wherein the Spi2 gene is *ssaV*, and the further mutation disrupts *clpP*, *ompR*, sifA or *sseC*.

5. A microorganism according to claim 1, wherein the Spi2 gene is *ssaT,* and the further mutation disrupts *ssaB*.

6. A microorganism according to any preceding claim, which further comprises a heterologous antigen or a therapeutic protein.

7. A microorganism according to claim 6, wherein the antigen is a hepatitis A, B or C antigen.

8. A microorganism according to any preceding claim, wherein the microorganism is *Salmonella typhi Ty2*.

9. A microorganism according to any preceding claim, for use in therapy.

10. A vaccine composition comprising a microorganism according to any of claims 1 to 8, an adjuvant and a physiologically acceptable diluent.

11. A composition according to claim 10, comprising from 10^7-10^{10} CFUs of the microorganism per dosage unit.

12. A composition according to claim 11, comprising 10^8-10^9 CFUs of the microorganism per dosage unit.

13. Use of a microorganism as defined in any of claims 1 to 8, in the manufacture of a medicament for the treatment of systemic bacterial infection.

14. Use according to claim 13, wherein the infection is typhoid.