A BRIEF HISTORY

VACCINE – From the latin vacca (cow)

Edward Jenner (1749-1823)
A BRIEF HISTORY

Louis Pasteur (1822-1895)

XV cent. - In China healthy people acquired immunity against smallpox by sniffing powdered smallpox pustules or by inserting them into small cuts in the skin (“variolation”).

1796 - Edward Jenner conducts the first clinical investigations on vaccination with cowpox and smallpox viruses.

1875-1930 - Louis Pasteur - first attenuation of vaccines.

1875-1930 - Robert Koch - methodology, etiology, hypersensitivity, postulates.

1875-1930 - Emil von Behring - antibodies and immunotherapy.

1875-1930 - Paul Ehrlich - specific receptor-ligand binding, specific chemotherapy, antibody quantification.

By 1929 - Humoral immunologic phenomena described; Immunotherapy dominates the field; Credible and useful vaccines (smallpox and rabies, killed and/or attenuated typhoid, shigella, cholera, plague, diphtheria, tetanus, pertussis, and tuberculosis)

VACCINE – From the latin vacca (cow)
### TYPES OF TRADITIONAL VACCINES

| Vaccines containing killed microorganisms | Previously virulent micro-organisms that have been killed with chemicals or heat (ex. vaccines against flu, cholera, bubonic plague, and hepatitis A). |

| Vaccines containing live, attenuated microorganisms | Live micro-organisms that have been cultivated under conditions that disable their virulent properties. They typically provoke more durable immunological responses and are the preferred type for healthy adults (ex. yellow fever, measles, rubella, and mumps). |

| Toxoid-based vaccines | Inactivated toxic compounds from micro-organisms in cases where these (rather than the micro-organism itself) cause illness (ex. tetanus and diphtheria). |

| Subunit vaccines | Rather than introducing a whole inactivated or attenuated micro-organism to an immune system, a fragment of it can create an immune response (ex. vaccine against HBV and vaccine against Human Papillomavirus (HPV). |

### INNOVATIVE VACCINES

| Conjugate Vaccines | Linking poorly immunogenic polysaccharide outer coats to proteins (e.g. toxins) so that the immune system can be led to recognize the polysaccharide as if it were a protein antigen (ex. *Haemophilus influenzae* type B vaccine). |

| Recombinant Vectors | Combining the physiology of one micro-organism and the DNA of the other, so that immunity can be created against diseases that have complex infection processes. |

| DNA Vaccination | It works by insertion (and expression, triggering immune system recognition) into human or animal cells, of viral or bacterial DNA. Some cells of the immune system that recognize the proteins expressed will mount an attack against these proteins and cells expressing them. |
VACCINES: BASIC FACTS

- Pure recombinant or synthetic antigens used in modern day vaccines are generally far less immunogenic than older style live or killed whole organism vaccines.

- There is a major need for improved and more powerful adjuvants for use in these vaccines.

- With few exceptions vaccines are delivered parenterally.

- The delivery of antigens at mucosal surfaces (nasal, oral, pulmonary, urogenital) stimulates protective immune responses in both systemic and mucosal compartments due to the dissemination of antigen-sensitised cells to other tissues.

VACCINE FORMULATION

**Antigens**
- Inactivated organisms
- Attenuated organisms
- Isolated and purified proteins, glycoproteins, and carbohydrates

**Immune Potentiators**
- Bacterial products
- Toxins and lipids
- Nucleic acids, cytokynes, peptides
- Peptidoglycans, hormones
- Carbohydrates

**Delivery Systems**
- Mineral salts
- Microparticles
- Emulsions
- Liposomes

**Vaccine Adjuvants**
### VACCINE ADJUVANTS

**Adjuvants**  
From the Latin *adjuvare*, meaning "to help". Component that increases specific immune responses to the antigen.

*A very special type of excipients*

Safely and effectively used since 1925 when they were discovered by G. Ramon who found that the antitoxin response to tetanus and diphtheria was increased by injecting these vaccines with additional compounds such as agar, tapioca, lecithin, starch oil, saponin, bread crumbs!


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### VACCINE ADJUVANTS: REQUIRED PROPERTIES

- Chemically pure and defined composition
- Act only to potentiate the vaccine
- Non-toxic or has a negligible toxicity at the dose range for effective adjuvanticity
- Stimulates a strong humoral and/or T cell immune response
- Provides good immunological memory or long-term immunity
- Does not induce autoimmunity
- Non-mutagenic, carcinogenic or teratogenic
- Non-pyrogenic
- Stable under broad ranges of storage time, temperature, and pH
- Biodegradable/biocompatible

*Multi enim sunt vocati, pauci vero electi*  
St. Matthew’s Gospel, 22, 14
## VACCINE ADJUVANTS

### PARTICULATE ADJUVANTS
- Aluminium salts
- W/O and O/W emulsions
- Immunostimulating complexes (ISCOMs)
- Liposomes
- Microparticles and nanoparticles
- Proteasomes/virosomes (IRIVs)

### NON-PARTICULATE ADJUVANTS
- Muramyl-dipeptide (MDP)
- Non-ionic block copolymers (POE, POP)
- Saponins (Quil-A, Quil-21)
- Lipid A
- Cytokines (IL1, IL2, IL4, γ-INF)
- Carbohydrate polymers (mannan, glucan)
- Bacterial toxins (CTB, LTB)

### ADJUVANT COMBINATIONS
- Freund’s adjuvant
- Chiron MF59
- Syntex adjuvant formulation (SAF)

---

## VACCINE ADJUVANTS: MODE OF ACTION

<table>
<thead>
<tr>
<th>Mode of Action</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunomodulation</td>
<td>Generally small molecules or proteins which modify the cytokine network.</td>
</tr>
<tr>
<td>Presentation</td>
<td>Generally amphipatic molecules or complexes which interact with antigen in its native form.</td>
</tr>
<tr>
<td>CTL induction</td>
<td>Particles which can entrap the antigen and fuse or disrupt cell membranes; w/o emulsions for direct attachment of peptide to cell surface MHC-1.</td>
</tr>
<tr>
<td>Targeting</td>
<td>Particulate carriers which entrap the antigen and interact with APCs. Carbohydrates that target lectin receptors on APCs.</td>
</tr>
<tr>
<td>Depot generation</td>
<td>Microsphere, nanoparticles, w/o emulsions, alum.</td>
</tr>
</tbody>
</table>
VACCINE ADJUVANTS

Adjuvants licensed for human use

- Aluminium salts (aluminum hydroxide, aluminum phosphate)
- Calcium phosphate
- MF 59 (squalene/water emulsion)
- IRIVs (immunostimulating reconstituted influenza virosomes)

---

MICROSHEPHERES AS IMMUNOLOGICAL ADJUVANTS

Immune response to i.m microencapsulated TT

![Graph showing immune response over time](image)

Almeida and Alpar (1994). 2nd EUFEPS Congress
MICROSPHERES AS IMMUNOLOGICAL ADJUVANTS

Immune response to i.m. microencapsulated TT
influence of particle size

MUCOSAL VACCINES
MUCOSAL VACCINES

THE COMMON MUCOSAL IMMUNE SYSTEM

ANTIGEN PROCESSING AFTER STIMULATION OF THE GALT

PARTICLE UPTAKE AT THE NALT AND ANTIGEN PROCESSING
VACCINE ADJUVANTS

PARTICULATE ADJUVANTS
- Aluminium salts
- W/O and O/W emulsions
- Immunostimulating complexes (ISCOMs)
- Liposomes
- Microparticles and nanoparticles
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ADJUVANT COMBINATIONS
- Freund's adjuvant
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- Syntex adjuvant formulation (SAF)

CARRIER SYSTEMS/ADJUVANTS FOR MUCOSALLY ADMINISTERED ANTIGENS
- Microspheres and nanoparticles
- Liposomes
- Virosomes (IRIVs)
- Mutant live bacteria
- Bacterial invasins
- Lectins
- Bioadhesive/mucoadhesive polymers
- E. coli heat-labile enterotoxin B subunit
- Cholera toxin B subunit (CTB)
- Monoclonal antibodies
- Vitamin B12
- Vitamin E
- Glycodeoxycholic acid
- poly-ornithine
- Dimethyl-β-cyclodextrin
- Spermine
CYCLODEXTRINS AS PHARMACEUTICAL EXCIPIENTS (1)

- Formation of inclusion complexes with various molecules.
- Modification of physical and chemical properties of incorporated guest compounds.
- Stabilization of drugs and excipients during storage or processing.
- Taste and odour masking.
- Conversion of liquid materials to dry form.
- Improvement of drug solubility in water.
- Emulsification of drugs.
- Controlled release of drugs.
CYCLODEXTRINS AS PHARMACEUTICAL EXCIPIENTS (2)

- Peptide and protein carriers
  - Stabilization (chemical chaperones).
  - Protection against enzymatic degradation
  - Chemical modification of enzymes.

CYDs as vaccine adjuvants?

CyDs ACT AS STABILISING AGENTS FOR MICROENCAPSULATED TT

- Tetanus Toxoid (TT) - 150 KDa
- PLGA 50:50 microspheres
- Spray-drying of w/o emulsions

<table>
<thead>
<tr>
<th>Type Content (%)</th>
<th>Fluorimetry (ELISA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose 15</td>
<td>93.5 5.8</td>
</tr>
<tr>
<td>BSA 5</td>
<td>17.9</td>
</tr>
<tr>
<td>α-CD 10</td>
<td>75.7 3.3</td>
</tr>
<tr>
<td>β-CD 15</td>
<td>76.2 6.7</td>
</tr>
<tr>
<td>γ-HPCD 15</td>
<td>90.0 10.3</td>
</tr>
</tbody>
</table>

Johansen et al. (1998), Pharm Res 15: 1103-1110
HP-β-CyD REDUCES HAEMOLYTIC ACTIVITY OF SAPONIN ADJUVANTS

- Evaluation of the safety and tolerance of saponin adjuvant QS-21
- *In vitro* haemolysis assay

![Graph showing haemolysis assay results](image1)

Waite et al. (2001). Vaccine 19: 3957-3967

HP-β-CyDs AS PAIN REDUCING AGENTS IN VACCINE I.M. VACCINES

- Evaluation of the safety and tolerance of saponin adjuvant QS-21
- Double-blind, randomised trial
- 15 human volunteers

![Graph showing pain reduction](image2)

**SULFOLIPO-CyDs AS A VACCINE ADJUVANT**

- Inclusion of sulfo-lipo CyDs in a O/W emulsion (squalane in water):
  - ↑ physical stability of the formulation;
  - ↑ adjuvanticity
  - ↓ reactogenicity

![Diagram](image)

**Reactogenicity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL-CyDs/W</td>
<td>0</td>
</tr>
<tr>
<td>SL-CyDs/W + Adjuvant</td>
<td>0</td>
</tr>
<tr>
<td>W/O + Adjuvant</td>
<td>0</td>
</tr>
<tr>
<td>W/O</td>
<td>0</td>
</tr>
</tbody>
</table>

**SULFOLIPO-CyDs AS A VACCINE ADJUVANT**

- Sulfo-lipo CyDs/squalane/W
- Inactivated Bovine Herpes Virus Type 1 (BHV-1)
- Calves
- I.m immunisation at days 0, 46 and 226

![Graph](image)

**Immunisation**

**Challenge**

![Graph](image)
SULFOLIPO-CyDs AS A VACCINE ADJUVANT

• Sulfo-lipo CyDs/squalane/W
• Pseudorabies virus DNA
• Pigs
• S.c immunisation at days 0, 28, 56, and 84


CYCLODEXTRINS AS PHARMACEUTICAL EXCIPIENTS (2)

• Peptide and protein carriers
  ✓ Stabilization (chemical chaperones).
  ✓ Protection against enzymatic degradation
  ✓ Chemical modification of enzymes.

• Absorption enhancers at the mucosal sites (mainly nasal)

CYcDs as mucosal vaccine adjuvants?
**IMMUNE RESPONSE OVA-CyD COMPLEXES**

- Ovalbumin (OVA) - 43 KDa
- B6D2F1 mice
- Oral administration at days 1 and 14
- S.c. administration at day 1

**CyDs AS PENETRATION ENHANCERS FOR NASALLY ADMINISTERED TT**

- Tetanus Toxoid (TT) - 150 KDa
- BALB/c mice
- I.n. administration at days 1 and 49
- Dimethyl-β-cyclodextrin (CYC)
- Glycodeoxycholic acid (BS)
CyDs AS PENETRATION ENHANCERS FOR NASALLY ADMINISTERED DT

- Diphtheria toxoid (DT) - 65 KDa
- BALB/c mice
- I.n. administration at days 1 and 49
- Dimethyl-β-cyclodextrin (CYC)
- Glycodeoxycholic acid (BS)

ANTIGEN UPTAKE AT THE NALT AND ANTIGEN PROCESSING

- Mucosal response
- SIgA
- Systemic response (possibly via the spleen)
- PCLN
- SCLN

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A.J. Almeida, 2007
SOME REMARKS ON CyDs AS VACCINE CARRIERS

- CyDs play an important role as chemical chaperones, thus preserving antigen integrity and immunogenicity during storage or processing, which is certainly useful in vaccine formulation.
- The inclusion of CyDs in emulsion or saponin-adjuvanted parenteral vaccines reduces reactogenicity.
- CyDs may also control the rate of antigen release from microsphere vaccine formulations, thus influencing the depot effect.
- CyDs may be included in suitable vaccine formulations for immunisation purposes upon uptake at mucosal sites.
- Antigen uptake and translocation across the nasal mucosa may be significantly increased by CyDs, resulting in high specific systemic humoral immune responses.
- The role of CyDs as vaccine components is far from being fully understood. Further understanding would promote the optimisation of vaccine delivery systems.

MUCOSAL VACCINATION AGAINST STRANGLES
BACKGROUND

PLGA

PCL/Chi

SLN

SLM/SCF

SLN/SCF

KYIONO AND FUKUYAMA (2004) NAT. REV. IMMUNOL.

A.J. Almeida, 2007

STRANGLES

• Infection of the respiratory tract of equidae caused by nasopharyngeal infection by *Streptococcus equi* subsp. *equi* that spreads rapidly to the lymph nodes of the head.

• Characterised by an acute, febrile, suppurative, retropharyngeal and submandibular lymphadenitis, with abscess capsule formation that will drain pus to the nearest site of egress (skin or upper respiratory tract mucosa).

• Abscesses can form in other body organs and their rupture may be fatal.

• Morbidity may be as high as 100% and infection may be fatal.

• M-like protein is believed to induce both local and systemic immunity.
The Nasal Route of Immunisation

- Antigens delivered intranasally avoid the proteolytic and acidic environment of the stomach, encountered by orally administered antigens.

- Immune responses elicited by intranasal vaccination are generally stronger than those induced by the same antigens delivered orally.

- Systemic immune responses, are generally easier to achieve by intranasal delivery than by oral delivery.

- The respiratory tract is less colonised by commensal microorganisms than the gut, thereby decreasing interference of vaccine-strain uptake by ecological competition.

Immunisation Against Strangles

<table>
<thead>
<tr>
<th>Name</th>
<th>Antigen</th>
<th>Adjuvant</th>
<th>Route</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivac®</td>
<td>S. equi</td>
<td>lysate</td>
<td>i.m.</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Strepvax II®</td>
<td>S. equi</td>
<td>SeM-based Aluminium</td>
<td>i.m.</td>
<td>Boehringer Ingelheim</td>
</tr>
<tr>
<td>Strepguard®</td>
<td>S. equi</td>
<td>SeM-based Havlogen®</td>
<td>i.m.</td>
<td>Intervet</td>
</tr>
<tr>
<td>Equilis Strep E®</td>
<td>S. equi</td>
<td>Live mutant</td>
<td>submucosal</td>
<td>Intervet</td>
</tr>
<tr>
<td>Pinnacle®</td>
<td>S. equi</td>
<td>Live attenuated</td>
<td>Intranasal</td>
<td>Fort Dodge</td>
</tr>
</tbody>
</table>

Safety concerns, due to reactions including nasal discharge, abscessation of lymph nodes and other sites, allergic reactions, and purpura-like signs.
Regular vaccination program: 
*S. equi* M-like protein (SeM) rich extracts associated with nanoparticulate carriers.

---

**IMMUNISATION AGAINST STRANGLES**

Vaccine Adjuvants Tested

- Aluminium hydroxide (Hoffman et al, 1991)
- Freund’s complete adjuvant (Jean-François et al, 1991)
- Monophosphoryl lipid A / trehalose 6,6’-dimycolate (Timoney and Guan, 1996)
- Havlogen® (polyacrylate cross-linked with polyallylsucrose) (Sheoran et al, 1997)
- Sucrose acetate isobutyrate (Nally et al, 2001)
- Cholera toxin (Sheoran et al, 2002)
- *E. coli* heat-labile enterotoxin B subunit (Flock et al, 2004)
- PLGA microspheres (Azevedo et al, 2006)
- ISCOM’s (Flock et al, 2006)
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Production and characterisation of an effective mucosal vaccine against strangles, prepared by association of \textit{S. equi} antigens in micro / nanoparticles.

**IMMUNE RESPONSE AFTER NASAL DELIVERY OF S. equi ANTIGENS**

- Specific SIgA
- Oral route
- Nasal route
- Specific IgG
- i.m. route

**Streptococcus equi**

- Fermentation (30 l)
- Enzymatic extraction
- Inactivation (formaldehyde)
- Disruption
- Freeze-drying
- Subunit antigen (SeM)
- Characterisation (physical, chemical, pharmaceutical)
- Micro/Nanoparticles
- In vivo studies

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MICROENCAPSULATION OF *S. EQUI* ANTIGENS

Enzymatic extract (6.0 μm; 23% EE)

Disrupted *S. equi* (6.0 μm; 80% EE)

Whole-killed *S. equi* (6.0 μm; 100% EE)

---

SERUM IMMUNE RESPONSE AFTER NASAL DELIVERY OF *S. equi* ANTIGENS

Nasal route

![IgG Titre (OD 405 nm) vs Time (day)](chart1)

- WKSe
- PLGA-WKSe
- Se lysate
- PLGA-Se lysate

i.m. route

![IgG Titre (OD 405 nm) vs Time (day)](chart2)

(n=5 per group; pooled samples)
MUCOSAL IMMUNE RESPONSE UPON IMMUNISATION WITH PLGA-TT

- Tetanus Toxoid (TT) in PLGA microspheres
- BALB/c mice
- Intranasal, oral and im. Immunisation at days 1 and 49

PROTECTION AFTER NASAL DELIVERY OF S. EQUI ANTIGENS

Challenge with a virulent strain of *S. equi*  
(according to J.F. Timoney, U.S. Patent 5,183,659, 1993)

<table>
<thead>
<tr>
<th>Group</th>
<th>Preparation</th>
<th>Route</th>
<th>Morbidity (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WKSe-PLGA microspheres</td>
<td>nasal</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>WKSe-PLGA microspheres</td>
<td>i.m.</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Se-lysate-PLGA microspheres</td>
<td>nasal</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Se-lysate-PLGA microspheres</td>
<td>i.m.</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>Free WKSe</td>
<td>nasal</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Free WKSe</td>
<td>i.m.</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Free Se-lysate</td>
<td>nasal</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Free Se-lysate</td>
<td>i.m.</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
PROTEIN ADSORPTION ONTO MICRO / NANOPARTICLES

- Association to colloidal carriers avoiding harsh formulation procedures
- A way of increasing the loading of the antigen-containing nanoparticles.
- Protection from degradation after administration
- Avoid the possible degradation of protein antigens caused by the contact with organic solvents
- Slow-release of antigen

PROTEIN ADSORPTION ONTO PLA MICROSPHERES

Adsorption Procedure

1. Incubation in a shaking water bath (25ºC/o.n.)
2. Centrifugation, washing and drying
3. Recovered particles
PROTEIN ADSORPTION ONTO PLA MICROSPHERES

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IMMUNE RESPONSE AFTER INTRANASAL DELIVERY OF TETANUS TOXOID


Tetanus Toxoid

BSA

γ-Globulin

Adsorption Process

Equilibrium concentration (µg/ml)

Amount adsorbed (µg/mg)

Equilibrium concentration (µg/ml)

Equilibrium concentration (µg/ml)

Tetanus Toxoid

γ-Globulin

BSA

IMMUNE RESPONSE AFTER INTRANASAL DELIVERY OF TETANUS TOXOID

Immune Response (IgG titre)

Time (week)

PBS
Soluble TT (60 µg)
PLA-adsorbed TT (60 µg)
**IMMUNE RESPONSE TO ORALLY DELIVERED CTB**

Cholera toxin B subunit (0.8 μm PLA particles)

- **Free CTB** (10 μg/dose)
- **Adsorbed CTB** (0.25 μg/dose)

**Time (day)**

**IgG Titre (adsorbed CTB/free CTB)**

- **Adsorbed CTB (0.25 μg/dose)**
- **Free CTB (10 μg/dose)**

---

**PLA AND PCL NANOPARTICLES**

**Adsorption of S. equi Enzymatic Extract**

- **PLA/CS (+)** PLA/PVA (-)
- **VMD (nm)**
  - PLA/CS: 719.9±2.18
  - PLA/PVA: 414.4±3.57
- **Zeta potential (mV)**
  - PLA/CS: +21.2±1.23
  - PLA/PVA: -29.9±3.21
- **Loading efficiency (%)**
  - PLA/CS: 12.6±0.8
  - PLA/PVA: 14.9±0.9

**PVA/CS**

- **LV**
- **Total Protein amount (μg/mg particles)**
  - PVA/CS: 72 μg/mg particles
  - PVA/PVA: 96 μg/mg particles

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A.J. Almeida, 2007


POLY-ε-CAPROLACTONE MICROSPHERES

Adsorption of S. equi Enzymatic Extract

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (μm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-PCL/PVA</td>
<td>2.27±1.56</td>
<td>-31.2±2.0</td>
</tr>
<tr>
<td>(+)-PCL/chitosan</td>
<td>2.88±0.91</td>
<td>+37.2±1.7</td>
</tr>
</tbody>
</table>


PLA AND PCL NANOPARTICLES

Adsorption of S. equi Enzymatic Extract

Uptake of PCL/chitosan/FITC-BSA nanoparticles by macrophages (J774A1 cell line)

Immunisation studies
• S. equi enzymatic extract adsorbed onto PCL and PLA nanoparticles enhanced serum specific IgG, IgG1 and IgG2a as well as mucosal IgA responses.
• Antibodies and cytokine titers (IL-2, IL-4, IL-6, IFN-γ) predict protection.

CONCLUSIONS

• The successful development of mucosal particulate vaccines depends on the understanding of their physico-chemical and biological properties.

• Association of particulate systems with other adjuvants or absorption enhancers (e.g. CTB, CyDs) plays an important role is certainly useful in vaccine formulation.

• Further understanding the role absorption enhancers would promote the optimisation of particulate mucosal vaccine delivery systems.

• Microencapsulated S. equi lysates or whole-killed cells given by the intranasal and intramuscular caused full protection against a virulent strain upon experimental infection.

• Adsorption of S. equi enzymatic extract onto PCL microspheres enhanced serum specific IgG antibody responses, even 180 days after a single dose administration.

• The delivery of S. equi antigens is receiving further attention in order to fully understand the mechanisms responsible for their strong activity upon association to microspheres/nanoparticles.

A.J. Almeida, 2007