Immunogenic presentation of viral and bacterial antigens: iscom and OMV as a basis for new vaccines

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Abbreviations

APC	antigen presenting cell
BPL	ß-propiolacton
BALT	bronchus associated lymphoid tissue
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMDP	dichloromethylene diphosphonate
DOC	deoxycholate
DTH	delayed type hypersensitivity
GALT	gut associated lymphoid tissue
GMP	good manufacturing practice
G-protein	glycoprotein
HIV	human immunodeficiency virus
i.c.	intracerebrally
IFN	interferon
Il	interleukin
i.m.	intramuscularly
i.n.	intranasally
i.p.	intraperitoneally
i.v.	intraveneously
iscom	immune stimulating complex
LPS	lipopolysaccharide
MALT	mucosa associated lymphoid tissue
MHC	major histocompatability complex
MMM	marginal metallophilic macrophages
MPL	monophosphoryl lipid A
MZM	marginal zone macrophages
NALT	nasal associated lymphoid tissue
N-protein	nucleoprotein
NSE	non specific esterase
OMP	outer membrane protein
OMV	outer membrane vesicle
OVA	ovalbumin
PAGE	poly acryl amide electrophoresis
PD50	dose of antigen protecting 50% of animals
RPM	red pulp macrophages
RSV	respiratory syncitial virus
RV	rabies virus
s.c.	subcutanously
SDS	sodium dodecyl sulphate
Th1	T helper cell type 1
Th2	T helper cell type 2
TNF-α	tumour necrosis factor-α
VNAb	virus neutralizing antibodies
WHO	World Health Organization

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I.1 Introduction

During life the body is challenged by a wide variety of infectious agents. To combat and constrain infections with these agents the immune system uses a complex network of defence mechanisms. One of these is the ability to respond in a specific way (adaptive immunity) to unique structures (antigens) of the agent or its products. Interestingly, many agents have developed ways to escape from, or suppress specific and non-specific immune effector mechanisms. The principle of vaccination, which was introduced more than 200 years ago by Edward Jenner (Willis, 1997), utilizes the ability of the immune system to develop a specific immune and/or memory response, before the body is exposed to the pathogen, thereby mimicking specific immunity induced by infection.

The immune system

For the immediate response against many pathogens which enter the body, the innate immune response is of utmost importance (cf Brown et al., 1994).

Innate immunity can be divided into three major functions: barrier, humoral and cellular functions.

Barrier functions include: surface epithelia (divided in: mechanical, chemical and microbiologically active barriers), macrophages circulating in blood and lymph, filtering and removing non-self particulate matter, natural polyclonal IgA (in external body fluids and at mucosal surfaces) to bind to invading pathogens, and massive storage of cytokine molecules in the skin released upon contact with penetrating substances.

Humoral functions include: the alternative pathway of complement activation in the blood which functions without the interference of antibodies; acute phase proteins in the liver; chemoattractants and certain cytokines like interferons, interleukins and TNF-α.

Cellular functions include: macrophages, dendritic cells, neutrophils, NK cells which kill cells harbouring intracellular pathogens and tumor cells, CD5+ B-cells producing antibodies to common bacterial products (mainly IgM, T-cell independent, no class switching or somatic hypermutation (Carroll and Prodeus, 1998)), $\gamma\delta$-T-cells found in mucosal surface epithelia probably recognising alterations in the (infected) epithelium, and direct activation of T-cells by certain cell surface molecules for which the ligands are not known (Doherty et al., 1992; Welsh et al., 1997).

It should be noted that although most of these innate functions are considered non specific, they may increase substantially when effector cells of the specific immune sytem are stimulated. In conclusion, numerous cells and factors which are primarily involved in adaptive responses can also play a role in innate immunity (Skoner et al., 1996; Medzhitov and Janeway, 1997). Innate immunity works almost immediately, upon contact with what is perceived to be "non-self". It does not utilize and hence can not be influenced by active immunization. However knowledge of this system is

particularly important because it may directly influence the outcome of vaccination since certain adjuvants may influence the complex interaction between antigen, effectors of the specific immune system, and effectors of the innate immune system (Chin and San Gil, 1998). For example antigen processing and presentation is largely mediated by cells of the innate immune system and also localized specific immune effector mechanisms take advantage of components of the innate immune system (van Rooijen and Sanders, 1997).

The major characteristics of **adaptive immunity** are the specific recognition of the pathogen, the induction and persistence of memory and the clonal selection of monospecific effector lymphocytes (for review see Han et al., 1997). Furthermore, adaptive immunity involves affinity maturation (Rajewsky, 1996) and by definition takes longer to develop than innate immunity. Typically, a primary response will take days to weeks to reach its peak, whereas the emergence of a secondary or memory response is a matter of days. This branch of the immune system is divided into two arms, the humoral and the cellular part between which a close interaction exists.
The **humoral response** relies on the production of antibodies by plasma cells derived from (differentiated) B-cells. The Fab (fragment antigen binding) part of antibodies recognises conformational and linear determinants (B-cell epitopes) of microorganisms and viruses. Depending on the immunoglobulin isotype (IgA1,2,D,E,G1-4,M) antibodies can trigger a vast number of effector mechanisms designated to inactivate, neutralize and/or remove the microorganism and its products. It should be noted that major differences between the presence, properties and effector functions of different isotypes exist between different animal species, including man.
When antibodies bind to antigens their "tail" (Fc part) undergoes conformational changes. Furthermore, binding involves aggregation of antibodies on the pathogen. These two phenomena result in recognition of bound (and not free) antibody by specific Fc-receptors, with distinct receptors for several effector mechanisms. Effector mechanisms thus triggered include: inhibition of infectivity and neutralisation of toxins (high affinity IgM, IgG, IgA) opsonisation and enhanced phagocytosis (IgG, IgA), release of bactericidal agents by phagocytes (IgG1,2), classical pathway complement activation (IgM, IgG1,2,3, IgA), antibody dependent cellular cytotoxicity (ADCC, IgG1,3), NK activation (IgG1,3), sensitisation of neutrophils (IgG), mast cells/basophils (IgE) and eosinophils (IgG, IgE). A given pathogen (or fragment thereof) can be recognised by several antibodies of different specificities and this cross-linking will form a network called an immunecomplex. Immunecomplexes can be easily removed from blood and lymph by liver, spleen, kidney and lymph nodes, thereby speeding up the elimination process. Furthermore, immunecomplexes are trapped on follicular cells of spleen and lymph nodes which results in the induction and persistence of memory which is known to be antigen dependent (van den Eertwegh et al., 1992; Ridge et al., 1998). It should be noted that antibody binding may also have major effects on the conformation of the antigen. This may be an intrinsic part of the

mechanism that leads to neutralization but in certain cases also to antibody mediated enhancement of infectivity (Siebelink et al., 1995; Schutten et al., 1994; Huisman et al., 1998). Such mechanisms have been shown to play an important role in anti-viral immunity.

In **cellular responses** , T-cells (mainly those of the CD4+ phenotype) play an important role in regulating overall B- and T-cell functions. For instance they trigger B-cells to produce antibodies through a cognate interaction involving antigen, class II MHC and CD40-CD40 ligand (Janeway et al., 1994; Laman et al., 1996). Activated T-cells (usually those of the CD8+ phenotype) can also be directly involved in the elimination of cells infected with intracellular microorganisms. This cellular response depends on direct interactions between cytotoxic T-cells (CTL) and cells of the host bearing the antigen they recognise. For both pathways it is essential that small peptides are liberated, by correct processing in antigen presenting cells, from the target substance. The induction of CTL responses requires that the antigens are processed and that the resulting peptides (usually 9-mers) are routed via the cytoplasm and associate with class I molecules (Bjorkman et al., 1987; Neefjes and Ploegh, 1992; Yewdell and Bennink, 1992; Zhang et al., 1992). These MHC -peptide complexes are then expressed on the surface of the cells where they are recognized by cytotoxic T-cells (Townsend et al., 1986). Live viral vaccines or infection induce good CTL responses because synthesis of antigens takes place intracellularly. Inactivated vaccines are processed via the the endogenous processing pathway. Although most cells express MHC class I, professional antigen presenting cells and most likely dendritic cells are involved in the induction of CTL (Macatonia et al., 1989).

In recent years it has become clear that many of the activities of both CD4+ and CD8+ T-cells are related to their cytokine profile which allows a division into e.g. Th1 versus Th2 type responses (Audibert and Lise, 1993; Seder and Paul, 1994; Wood and Seow, 1996; Mosman and Sad, 1996; Constant and Bottomly, 1997). In mice Th1 type responses are related to the expression of interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin (LT). Th2 type responses are characterised by the expression of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Human T-cell subsets produce similar patterns but the expression of particular lymphokines like IL-2, IL-5, IL-6, IL-10 and IL-13 is not as restricted as it is in mouse T-cells.
The expression of the lymphokines correlates well with the functions of the individual subsets in that Th1 are mainly involved in cell mediated inflammatory reactions, cytotoxicity and DTH reactions. Th2 on the other hand tends to be more involved in providing help in antibody responses. Th2 lymphokine profiles are typical for strong antibody responses and allergic reactions. Some of the lymphokines that are produced by either Th1 or Th2 are inhibitory for the reciprocal subset. Th1 and Th2 originate from a single precursor cell that initially produces mainly IL-2 after stimulation with antigen but later differentiates in either Th1 or Th2 cells. However, depending on the

pathogen/antigen studied the effects of individual Th subsets may greatly vary. For instance Th1 subsets can protect against disease in some situations (Lehmann and Alber, 1998; Sjolander et al., 1998) but actually ameliorate pathology in others (Lucey et al., 1996). At the same time it is well known that Th2 cures disease in most helminthic and some viral infections whereas it exacerbates in others (Mossman, 1996). Therefore it can be assumed, and is generally accepted, that other factors, characteristic for the pathogen or dose studied, are also of importance in deciding the outcome of both infection and vaccination. In this thesis a comparison between two different vaccine modalities is made and in view of the contradictory results (and minimal predictive value) described in the studies referenced above, only minimal attention is paid to the role of cytokine profiles after immunization and adjuvation (Audibert and Lise, 1993).

Anti-viral immunity

Specific protection against viruses can be mediated both by antibodies and by CTL responses. Antibodies that are generated either in response to infection or after immunization can neutralize the virus in several ways. A possible mechanism of virus neutralization is the direct blocking of the virus/receptor interaction (i.e. CD4 binding in the case of HIV). Conformational changes in the antigen can occur due to binding of the antibody that changes the target antigen to a non-functional form. Antibodies may also interfere with post entry events that prevent multiplication or spreading of the virus. Another mechanism by which antibodies help to clear infection is antibody mediated cellular cytotoxicity (ADCC). It has been shown *in vitro* that virus specific antibodies induced by immunization can also enhance infection thus leading to a more rapid development of disease instead of protection (Robinson et al.,1989; Siebelink et al., 1995). The same antibodies can be neutralizing or enhancing infectivity depending on the phenotype of the virus involved (Schutten et al., 1994). These enhancing antibodies can be a major concern in efficient vaccine development.

Furthermore maternal antibodies present at the time of infection or immunization may interfere with the outcome of vaccination and disease. It has been demonstrated that immunization with live vaccinia virus in the presence of maternal antibodies did not interfere with the induction of anti vaccinia virus immunity (Fenner et al.,1989). Proteins that are expressed in vaccinia virus however induce lower responses in the presence of maternal or passively acquired antibodies as was shown for measles and RSV proteins (Murphy et al., 1988; van Binnendijk et al., 1997; Osterhaus et al., 1998). In a study in macaques it was shown that passively transferred antibodies suppressed both humoral and cellular measles specific immune responses after immunization with attenuated virus or recombinant vaccinia virus expressing F and H proteins. An experimental iscom vaccine containing H and F proteins was not influenced by preexisting measles virus specific antibodies (van Binnendijk et al., 1997; Osterhaus et al., 1998).

Because viruses replicate intracellularly cytotoxic CD4/CD8 T-cells are considered important for the protection against some viral pathogens.
Vaccines that aim at protection against viral disease should therefore preferably be able to generate specific CTL responses. It has been speculated that the absence of a functional T-cell response to measles virus after immunization with an inactivated vaccine is responsible for atypical measles syndrome (UytdeHaag et al., 1994).

Anti-bacterial immunity
Protective immune responses against extracellullar bacteria are usually controlled by antibodies which may act via several mechanisms. Antibodies can bind to bacterial cell surface antigens or toxins and can block attachment receptors on host surfaces. A second mechanism, termed opsonization, acts via enhancement of phagocytosis by macrophages and neutrophils. After ingestion these bacteria are destroyed. Antibodies can also kill bacteria directly or indirectly by activating complement. Surface antigens are usually the target of these antibodies, except in the case of toxins. Bacterial capsular polysaccharides are weakly immunogenic and can effectively protect bacteria by shielding more immunogenic surface structures. The chemical structure of polysaccharides has an impact on the virulence of encapsulated bacteria, i.e. some polysaccharides make bacteria more virulent than others. This probably relates to the interaction with complement, i.e. CPS containing sialic acid (Carroll, 1998).
To combat infections with intracellular bacteria antibodies are less effective. Once the bacterium has invaded cells a specific CTL response is required to kill these cells and the bacteria they contain. Another mechanism by which intracellular bacteria can be removed is by the production of cytokines that either downregulate bacterial replication or enhances the ability of phagocytosing cells to kill ingested bacteria. In this thesis, where a meningococcal vaccine candidate is studied, only the formation of protein specific antibodies that are able to lyse bacteria in the presence of complement is studied. This is considered an important mechanism in the development of protection against meningococcal disease (for review see Hart and Rogers, 1993).

Vaccines against infectious diseases
Since the introduction of the principle of vaccination about 200 years ago by Edward Jenner, the use of vaccines against many infectious diseases has led to their control in the developed world. Vaccination is amongst the most cost effective ways to control infectious diseases. The most striking example is the eradication of smallpox from the world, by the end of the seventies, with a vaccination approach that was originally introduced by Jenner. Similarly it may be expected that by the year 2000 poliomyelitis will effectively be eradicated (Tangermann et al., 1997) and possibly also measles by the year 2010. These tremendous successes have sofar all been achieved with classical live attenuated vaccines.
With the advent of modern immunological and molecular biological techniques other principles of vaccination have been developed over the last decades. These include

subunit vaccines which make use of only those parts of the infectious agent which carry the B- and T-cell epitopes of the pathogen, relevant for the induction of the desired immune response. Those subunits may be purified directly from the agent or alternatively be produced by recombinant DNA techniques or peptide synthesis. The first registered vaccine for human use, based on one of these principles is the subunit hepatitis B vaccine produced in yeast (Hilleman et al., 1987). Alternatively vectors, including viruses and bacteria which express the heterologous subunit antigen can also be used to present these antigenic moieties to the immune system (e.g. pox-, adeno-, and herpesviruses, *Salmonella* spp., *Mycobacterium* spp.). It has also been suggested that components of the host immune system itself -anti idiotypic or anti clonotypic structures- could be exploited as surrogate antigens, a concept which is based on the idiotype network interactions originally described by Jerne (Jerne, 1974). Sofar this approach has not led to any practical vaccine candidates.
Recently a novel approach was introduced exploiting the direct use of nucleic acids , encoding the antigen of interest. Generally for vaccines not based on the principle of active replication, adjuvants or adjuvant systems are required in order to obtain an adequate immune response (Jennings et al., 1998; Allison, 1997; Audibert and Lise, 1993; Gupta et al., 1995).
In deciding which of the approaches to select for the development or use of a vaccine for a particular infectious disease, not only technical feasabilities but also the minimal requirements for the induction of protective immunity should be considered. It is interesting to note that for some of the infectious diseases for which vaccines are not available but most urgently needed today, like AIDS, malaria and tuberculosis, correlates of protective immunity and immune pathogenesis are still largely unknown.
General criteria of the "ideal vaccine" against all infectious diseases are therefore hard to define. However it may still be stated that the "ideal vaccine" should generally meet the following requirements: it should induce a long lasting antibody response involving antibodies with the appropriate biological activities. It should induce long lasting T-cell responses involving cells with the appropriate phenotypes and effector functions. Preferably these antibody and T-cell responses and/or the corresponding memory responses should also be induced in the presence of pre-existing or maternal antibodies. Another important aspect is that for certain pathogens not only systemic immunity but also, or rather an effective immunity at the site of entry is required. Therefore the induction of mucosal immunity may be a major issue (McGhee et al., 1993). Collectively the induced immune responses should lead to a long lasting protection against all the antigenic variants of the infectious agent. Furthermore, production procedures should allow large scale cost effective production, under GMP conditions, of a product stable for prolonged periods of storage and transport that can be produced at a reasonable price. Finally it should be realized that apart from efficacy criteria the most important requirement for a vaccine is its overall safety.

Routes of administration

Usually vaccines are injected intramuscularly or subcutaneously to induce a systemic immune response. After immunization via this route antigens are taken up by antigen presenting cells and these cells migrate to draining lymph nodes and spleen where immune responses develop. This route of administration of vaccines is well accepted and most studies involving adjuvant research, study this type of administration . Many pathogens however enter the host via mucosal surfaces that are in direct contact with the external environment. Protection against infection with these pathogens should therefore preferably occur at these mucosal surfaces, and induction of a mucosal immune response should therefore, in some cases, be a target of vaccine development (McGhee et al., 1992 and 1993). The immune response at the mucosal sites takes place in a system that is generally termed mucosa associated lymphoid tissue (MALT). Protective immune responses against pathogens that invade the upper respiratory tract will have to develop in the nasal associated lymphoid tissue (NALT) and the bronchus associated lymphoid tissue (BALT). In close proximity with the intestinal mucosa lies the gut associated lymphoid tissue (GALT). Immune responses in the GALT and the NALT are initiated by uptake and transcytosis of antigen from the external environment by M-cells. These M-cells transport antigen to the underlying area where macrophages , dendritic cells, B-cells and T-cells can be found. Secretory IgA (sIgA) is an important humoral defence mechanism at mucosal surfaces (Lamm, 1997). This dimer of IgA is secreted by IgA specific plasma cells and is actively transported to external secretions. Cellular responses can also develop in the MALT. Dendritic cells (DC) are also important for antigen sampling at mucosal surfaces. In the respiratory tract, a submucosal network of DC's functions as a surveillance system to monitor inhaled antigens , and cross regulation takes place between DC's, alveolar macrophages and T-cells.

Ample research has been focussed on developing vaccines that can be easily administered. Targeting of antigens to the MALT requires special adjuvants or formulations. Cholera toxin (CT) and its B subunit (CTB) are widely studied as adjuvants to enhance mucosal immune responses. Mucosal adjuvants should facilitate antigen/receptor interaction to target antigens to immune cells in the gut. To generate local immune responses in the gut , live (replicating) vaccine formulations that can be administered orally are ideal (e.g. oral polio vaccine). Live vectors have been used to express foreign antigens to induce immune responses against these antigens.

For large scale immunization a technique which employs needle-less jet guns may be useful (Fournier, 1973; Mathei et al., 1997; Parent du Chatelet et al., 1997). These so called jet guns inject vaccines subcutaneously by a thin, high pressure nozzle which can be resterilised at the end of each section. Since no needle is involved that penetrates the skin the risks of viral transmission from one recipient to the other are much lower but not absent (Brink et al., 1986). These jet guns have several advantages over classical syringes with needles like; absence of risk of accidental puncture; possibility to immunize large groups of people rapidly since less manipulation is

required. Side effects are slightly higher than those observed with conventional techniques. When delivered by jet gun, vaccines were of equivalent or superior immunogenicity, as compared to the syringe technique (Mathei et al., 1997).

Adjuvants and adjuvant systems

Vaccines based on inactivated pathogens or subunit vaccines generally induce poor immune responses inactivated and subunit vaccines are considered to be much safer than attenuated vaccines since they contain no infectious agent at all. Limitations of these vaccines are that they generally induce lower immune responses than those following natural infections or vaccination with attenuated vaccines. There are a number of variables which determine the outcome of immunization like: dose, nature of the antigen, immunization schedule, route of administration, immune status of the host and the choice of adjuvant that is used .

An adjuvant is, by definition, a substance which, when given in combination with the antigen of interest, leads to a better immunity. However the effects of individual adjuvants may differ largely. The most important of these effects are as listed below:

1) *Antigen dose* may be lowered to get the same level of immunity/protection. This can be an advantage when antigen is a limiting factor for vaccine production. It is of economical interest when antigen is relatively expensive as compared with the adjuvant.

2) *Maximal level of immunity (plateau)* will be higher when the same amount of antigen is used. A higher maximal level of immunity is for instance an advantage in cases where the antigen alone is not protective, even at high doses, but where the combination with an adjuvant leads to protective levels of immunity.

3) *Time to reach a protective level of immunity* can be shorter (but also longer) when an adjuvant is used.

4) *Number of immunizations* can be reduced to reach the same level of immunity.

5) *Duration of immunity* can be prolonged when an adjuvant is used. Immunity levels tend to drop over time, even to a level where they are no longer protective. Booster immunizations can be required after some time to maintain a protective level of immunity. The use of an adjuvant has advantages if less booster immunizations are required.

6) *Nature of immunity* can be changed. Adjuvants may change the nature of immune responses. Inactivated antigens are generally poor inducers of cellular immunity but in combination with certain adjuvants this can be overcome. Moreover, adjuvants can influence the balance between Th1 and Th2 responses and can thus be used to control the desired type of immunity. To achieve this goal an extensive knowledge of the mechanisms by which adjuvants work is necessary.

7) More potent induction of memory the induction of protective immunity by vaccination in most cases primarily depends on an adequate response at B- and T-cell levels after infection. Consequently moieties that enhance the induction of such memory responses will increase protective immunity of vaccines and therefore may

also be considered to be adjuvants.
Few adjuvants have all of the above mentioned effects, and the effects are largely dependent on the mechanisms by which they influence the immune system (for review see Cox and Coulter, 1997).
Adjuvants are a heterogeneous group of compounds or mixtures of compounds. Until now approximately 100 different substances have been identified as adjuvants. A compendium giving an extensive overview of adjuvants that are currently available on the market has been recently published (Vogel and Powell, 1995). Since most adjuvants induce considerable side effects, not all substances can be readily applied in vaccines (Gupta et al., 1993).
As mentioned above different mechanisms play a role in the adjuvanticity of this heterogenous group of compounds (for review see Gupta and Siber, 1995; Gupta et al., 1993; Cox and Coulter, 1997). These mechanisms are listed below together with examples of adjuvants that can be grouped in this way.
1) *Depot formation* at the site of injection results in the sustained release of the antigen, a mechanism that exposes the immune system to the antigen for a prolonged period of time. Mineral compounds (e.g. alum), oil based adjuvants (Freund's complete and incomplete adjuvant (FCA and IFA)), liposomes and biodegradable polymer microspheres >10μm (so called slow release particles) all result in depot formation.
2) *Targeting of antigens* to cells involved in the immune system like, antigen presenting cells, B- or T-cells. Examples of these are liposomes, oil based adjuvants, small (<10μm), and non ionic block polymer surfactants. Small particles presenting antigens are favoured because they may interact easily with the cells of the immune system.
3) *Immunostimulators or immunomodulators* which influence immune cells to secrete specific cytokines as a result of which certain effector mechanisms are influenced. Compounds that act via this mechanism are FCA, muramyl dipeptide (MDP), lipopolysaccharide (LPS), monophosphoryl lipid-A (MPLA), saponins (e.g. Quil-A , QS-21), Pertussis toxin (PT) and cytokines. For this thesis LPS and Quil-A will be further addressed.
4)V*ehicle effect* which protects the antigen against degradation.
5) *Aggregation of antigens* certain modifications of antigens may also lead to enhanced immunogenicity. Aggregation of antigens generally results in an increase of their immunogenicity larger particle antigens are more efficiently recognized by the immune system, easier than soluble proteins (e.g. liposomes).
Most of the above mentioned mechanisms have been observed for iscom and OMV, immunogenic presentation in the framework of this thesis (see below).
Cytokines by themselves represent another type of adjuvant which acts by modulating the immune system directly. Several researchers have demonstrated that the addition of cytokines to antigens is beneficial for the generation of a protective immune response (for review see Dong et al., 1995; chapter II.6 this thesis). Factors complicating the use of cytokines as generally applicable adjuvants are species specificity and the fact that

the cytokines have to be delivered to the appropriate cell at the right time. Moreover, most cytokines exert more than one effect on different cell subsets, both up and down regulating the immune response involved.

Sofar the only registered adjuvant for human application are aluminum salts. LPS which has adjuvant activities is intrinsically present in inactivated bacterial vaccines derived from gram negative bacteria and thus, although it is not registered as an adjuvant, it may play a role in such vaccines. Other adjuvants (QS-21, MPL, iscom) are tested in clinical trials but most of them are not considered safe enough for use in human vaccines. When adjuvants or adjuvant systems are considered for use in human vaccines the adjuvated vaccine should in principle approach the ideal vaccine.

characteristics	iscom	OMV
size	30-40 nm	10-150 nm
structure	rigid cage like structure	lipid bilayer
adjuvant	Quil-A	LPS
number of antigen copies per particle	minimal 3 required for induction of immune response	several hunderd copies per particle
conformation of antigen	as in membraneous environment	as in outer membrane of bacteria
viral antigens	+	-
bacterial antigens	+	+
parasite antigens	+	-
peptide antigens	+	+
lipid composition	cholesterol/PE/PC	as in outer membrane of bacteria
toxicity	mild haemolytic effect	mild pyrogenicity due to presence of LPS

table 1: characteristics of the iscom and OMV antigen presenting structures. +: tested and immunogenic. -: not tested

The adjuvant systems described in this thesis, iscom and bacterial outer membrane vesicles (OMV), combine several mechanisms to enhance the immunogenicity of the incorporated antigens. Both iscom and OMV contain multiple copies of (protein) antigens. Iscom contain Quil-A derived glycosides (triterpenoids) as a built-in adjuvant in the same particle as the antigen. OMV possesses intrinsic adjuvant activity due to

the presence of bacterial LPS. Iscom (⌀40nm) and OMV (⌀10-100 nm) are comparable in size. Some of the main characteristics of iscom and OMV are summarized in table 1.

Adjuvant activity of Quil-A

Quil-A is a crude extract from the bark of *Quillaja saponaria Molina* which shows a potent adjuvant activity and is widely used as an adjuvant in veterinary vaccines, particularly against foot-and mouth-disease. The adjuvant activity is due to the presence of a mixture of triterpene glycosides (Dalsgaard, 1978). The structure of Quil-A saponins has been further characterized and typical structures found in the mixture are given in figure 1 (Higushi et al., 1987; van Setten et al., 1995; Kensil et al., 1995).

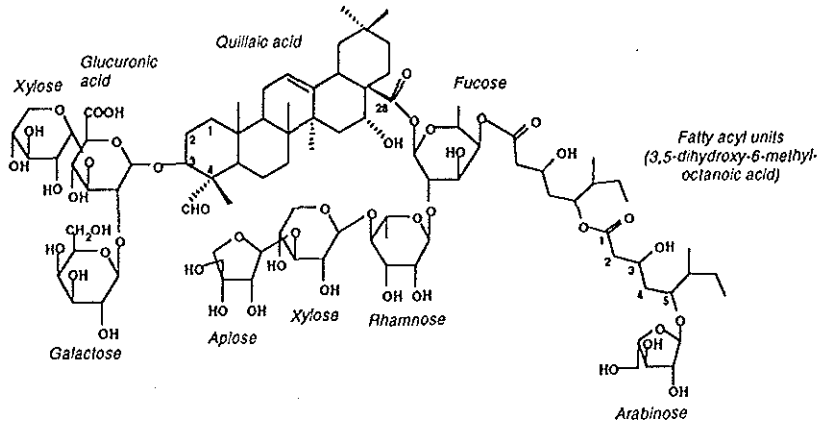

figure 1: Structure of QS-21. This structure is representative for the triterpene glycoside structure that can be found in the QuilA saponin mixture. The aglycon structure, the central Quillaic acid, is glycosylated at positions 3 and 28. The structure is amphipathic due to the presence of the hydrophobic domains (triterpenes and fatty acids) and hydrophilic domains (glycosides). (taken from Kensil et al., 1995)

Quil-A is part of the iscom structure and it is generally accepted that Quil-A is responsible for the observed adjuvant activity of iscom (Morein et al., 1984; Rimmelzwaan and Osterhaus, 1995; this thesis).

Quil-A saponins are amphipathic molecules due to the presence of hydrophobic domains (triterpene and fatty acids) and hydrophilic domains (glycosides). The structure of QS-21, a fraction of Quil-A saponins with high adjuvant activity is given in figure 1. Crude Quil-A is relatively toxic, probably due to its haemolytic activity. It forms micelles in PBS (Morein et al., 1984; Kersten et al., 1991) and it is not known whether the immuno- active form of Quil-A is monomeric or multimeric. Quil-A saponins have been purified to identify the components that are relevant for adjuvant

activity and to separate toxic and immunostimulating components. Kersten et al. (1991) showed by HPLC analysis that crude Quil-A is composed of 24 peaks which was later confirmed by Kensil et al. (1991). Individual purified saponins were shown to possess different levels of adjuvant activity and iscom forming properties. A fraction termed QS-21 was isolated that combined potent adjuvant activity, low toxicity and availability in relatively high amounts (Kensil et al., 1995). Many studies have been performed with QS-21 which enhances both humoral and cellular immune responses. The induction of class 1 restricted CTL against protein antigens is claimed using QS-21 as an adjuvant (Newman et al., 1992; Shirai et al., 1994). QS-21 has been tested in phase 1 studies.
In a separate study Quil-A was purified in crude fractions (Behboudi et al., 1995). This preparation could be used for the preparation of functional iscoms and opens perspectives for a safer iscom vaccine.

Adjuvant activity of LPS and LPS derivatives
The bacterial LPS is a component of the bacterial outer membrane. Moreover, LPS is a structural component of the OMV and, although it is considered a toxic molecule, it is at the same time being studied as a possible vaccine adjuvant (Johnson et al., 1956). The structure of meningococcal LPS is given in figure 2.
The early findings that the addition of whole cell pertussis vaccine or other gram negative bacteria to tetanus or diphtheria toxoid increased immunogenicity of these vaccines led to the conclusion that these bacteria possessed adjuvant characteristics. The responsible factor for this appeared to be the bacterial LPS which also proved to be responsible for the endotoxin activity of bacterial vaccines. Initially LPS-protein complexes were held responsible for the adjuvant activity but the nitrogen content could be reduced to as low as 0.6 % without loss of adjuvant activity, indicating that the protein was not essential for adjuvanticity. Both humoral and cell mediated immunity could be elevated markedly by LPS. Chemical modification of *Salmonella minnesota* R595 LPS results in a form which contains only one phosphate group, and which lacks the sugar chains, the so-called monophosphoryl lipid-A (MPLA) (Ribi et al., 1986; cf Ulrich and Myers, 1995). MPLA still possesses adjuvant activity but much lower toxicity.
Some bacterial strains, *Neisseriae* among others, shed vesicular structures of their outer membrane, so called blebs (10-100nm). The biological function of these blebs is unknown but it has been speculated that the bacteria use these blebs to fool the immune system. Blebs may bind antibodies and complement and in this way some of the invading pathogenic bacteria can possibly survive in a hostile environment. These blebs contain antigens and LPS that are normally present in the outer membrane of the bacterium and that could function as a target for protective antibodies. Blebbing can be promoted by the addition of deoxycholate (DOC) which results in the massive formation of OMV and at the same time in the complete inactivation of the bacterium.

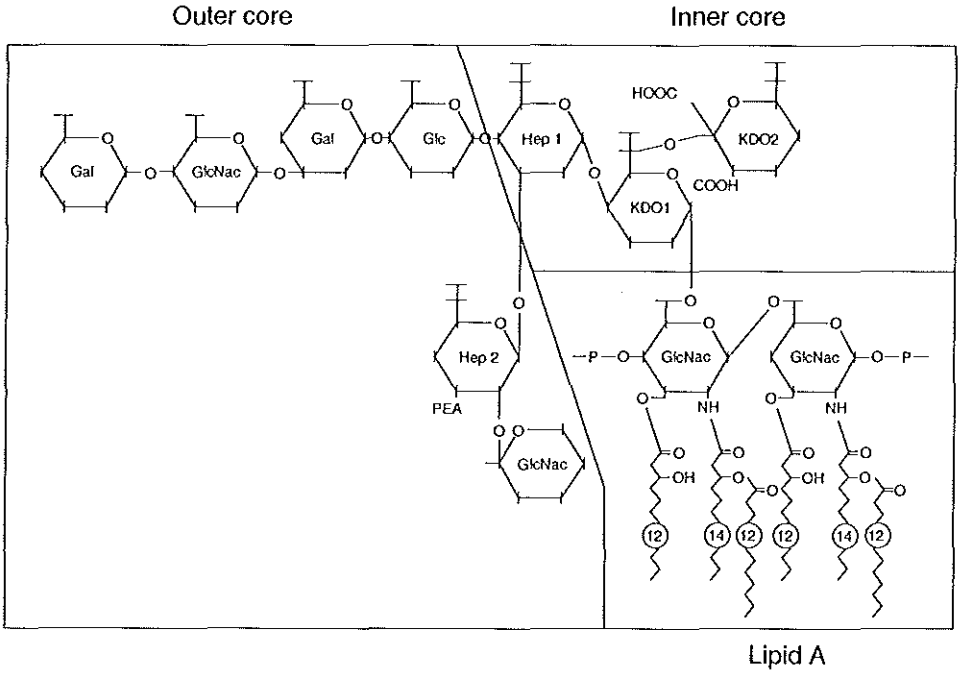

figure 2: Structure of meningococcal lipopolysaccharide of the L3,7 immunotype. Fatty acids attached to the lipid A part are inserted in the bacterial outer membrane. The inner core region contains the saccharides L-glycerero-D-manno-heptose (Hep 1) and 2-keto-3-deoxy-D-manno-octulosonic acid (KDO). The outer core consists of a lacto-N-tetraose unit and a phosphethanolamine containing group. (with permission taken from: T cell recognition of Neisseria meningitidis outer membrane proteins (1993) E. Wiertz, thesis, Utrecht)

The LPS content is reduced during down stream processing to a level where residual side effects are minimal. This approach is used to develop vaccines based on outer membrane protein antigens.

OMV are probably less suited for the presentation of most viral antigens but they are very interesting candidates for the presentation of bacterial antigens. The formation of vesicles containing bacterial outer membrane antigens (proteins and LPS) occurs spontaneously in some gram negative bacteria. Also the formation of large amount of OMV can be stimulated by the extraction of bacteria with a detergent, deoxycholate which may be beneficial for large scale production of OMV. Experimental vaccines described in this thesis are designed to induce responses against outer membrane proteins (OMP), that are known to induce bactericidal antibodies, which play an important role in the protection against infection and development of disease.

The iscom structure as an immune-enhancing moiety: experience with viral systems

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The iscom as an antigen-presenting structure

Extensive studies on fundamental mechanisms of protection induced by vaccination have led to insight into the components necessary for the induction of protective immunity. This has enabled vaccine researchers to focus on those components which are involved in the actual induction of immune responses and to exclude irrelevant, immunosuppressive or potentially harmful components from vaccines.

The "ideal" vaccine should meet the following requirements: the induction of a long-lasting antibody response of biologically active antibodies, which should be elicited even in the presence of maternal antibodies; functional T-cell responses which comprise both major histocompatibility complex (MHC) class-I- and class-II-restricted cytotoxic T cells (CTL) and helper T cells; long-lasting protection against infection with the pathogen. Furthermore, the production procedures must be suitable for large-scale production and the product must be stable during prolonged periods of storage and transport. Last but not least, it has to be safe and devoid of any undesirable side effects. New generations of vaccines will probably be composed of relevant antigen subunits either derived from the pathogen itself or prepared via molecular biological or synthetic techniques. However, vaccine formulations based on individual antigens or subunits bearing one or more B- and T-cell epitopes in a monomeric form have a major disadvantage: they are usually poorly immunogenic as compared to the original inactivated or attenuated pathogen. Therefore adjuvants or immune enhancers are needed to elicit the desired immune response. In general, adjuvants should contribute to the requirements stated above for the "ideal" vaccine. Most adjuvants however, only contribute to some of these, and concerns about undesirable side effects have largely prohibited their use in registered vaccines for humans.

In our laboratory, we have been especially interested in evaluating the potential of the immune-stimulating complex (iscom) as a carrier for viral proteins.

Iscom was first described in 1984 (Morein *et al.*, 1984). It is a cage-like structure, usually about 30 to 40 nm in diameter, composed of glycosides present in the adjuvant Quil-A, cholesterol, the immunizing (protein) antigen and also, in most cases, phospholipids. Although similar structures had been observed before (Horzinek *et al.*, 1973), it was not until the work of Morein *et al.* that their potential as an immune-enhancing moiety was recognized.

Quil-A is a crude extract from the bark of *Quillaja saponaria Molina* which has potent adjuvant activity and is widely used as an adjuvant in veterinary vaccines. The adjuvant activity is due to the presence of a mixture of triterpine glycosides (Dalsgaard, 1978).

Although antigens of different origin including viruses, bacteria and parasites have been incorporated into iscoms (table I), in this review, we will mainly address data obtained with iscoms containing viral antigens. We will discuss the advantages and disadvantages of using Quil-A or iscoms as adjuvants. Furthermore, we will postulate a working hypothesis with respect to the mechanisms by which the iscom exerts its specific adjuvant activity.

The preparation procedure and structure of the iscom have been extensively described earlier (Höglund *et al.*, 1989). Recent studies have generated detailed information on the structure of the iscom. The typical cage-like structure is formed by Quil-A/cholesterol micelles which are held together by hydrophobic forces (Özel *et al.*, 1989). This structure is very stable. Iscoms can resist repeated freeze-thawing and the structure remains intact upon lyophilization. The minimal requirements for the formation of iscoms have been extensively described (Lövgren and Morein, 1988). Quil A is a necessary constituent of the iscom structure. The size and mor-

Table I. Characteristics of immune-stimulating complexes containing different antigens.

Antigen nature	Name	Carrier	Route	Dose	Isotypes/ biol. activity	T cells	Species	Protection	Reference
Protein	BSA		sc	0.1-1 μg		NI	Mouse	NI	Morein *et al.*, 1990
	OVA		or	10-100 μg		DTH,CTL	Mouse	NI	Mowatt *et al.*, 1991a/b
			sc	0.1-50 μg		DTH,CTL			
Peptide	LHRH, FMDV 144-159	Influenza virus proteins	sc	1 and 3 μg	NI	NI	Mouse	NI	Lövgren *et al.*, 1987
Virus									
Herpesviridae	HSV1		sc	1-15 μg	NI	NI	Mouse	+ +	Erturk *et al.*, 1989
	BHV1		im	50 μg	VN	NI	Rabbit, calf	+ +	Trudel *et al.*, 1987, 1988
	CMV		id	15 μg	G	LST	Rhesus monkey		Wahren *et al.*, 1987
	Pseudorabies virus			170 μg	VN	NI	Mouse, pig	+ +	Tsuda *et al.*, 1991
	EBV		sc	2-5 μg	VN	LST	Tamarin	+ +	Morgan *et al.*, 1988
Hepadnaviridae	HBV		ip	1-5 μg		NI	Mouse	NI	Howard *et al.*, 1987
Togaviridae	Rubella V		im	100 μg	VN	NI	Rabbit	NI	Trudel *et al.*, 1988
Flaviviridae	BVDV		sc/im	50 μg	VN	NI	Sheep	+ +	Carlson *et al.*, 1991
Paramyxoviridae	MV		im	2-20 μg	VN/FI/HLI	DTH	Mouse, monkey	+ +	De Vries *et al.*, 1988a
	CDV		im				Dog	+ +	De Vries *et al.*, 1988b
			im		VN		Seal	+ +	Visser *et al.*, 1989
	BRSV		im	100 μg		NI	Guinea pig	NI	Trudel *et al.*, 1989
	HRSV		im/in	3.5 μg	G	IL2/CTL	Mouse	NI	Trudel *et al.*, 1992
Rhabdoviridae	Rabies virus		sc/im	0.3 μg	NI	NI	Mouse/dog	+ +	Fedaku *et al.*, 1992
Orthomyxoviridae	Influenza virus		sc/in	1-10 μg	G, A, M		Mouse	+ +	Lövgren *et al.*, 1988, 1990
			in	5 μg	G, A, M	NK/CTL	Mouse	NI	Jones *et al.*, 1988
Retroviridae	FeLV		im	3 μg	NI	NI	Cat	+ +	Osterhaus *et al.*, 1989
	BLV		sc	0.3-50 μg		NI	Mouse/calf	NI	Merza *et al.*, 1991
	SIV		im				Rhesus monkey	+ +	Osterhaus *et al.*, 1992
	HIV-1		sc	1 μg	VN	CTL	Mouse/rhesus	NI	Takehashi *et al.*, 1990
						NI	Mouse	NI	Pyle *et al.*, 1989
Bacteria	*E. coli*, pili		sc/im	5-40 μg	NI	NI	Rabbit, pig	NI	Nagy *et al.*, 1990
	B. abortus, porin-OPS		sc	1-5 μg		NI	Mouse	+ +	Winter *et al.*, 1988
Parasites	*Toxoplasma gondii*		sc	0.1-10 μg	NI	NI	Mouse		Overness *et al.*, 1991
	Plasmodium falciparum		im	40 μg	NI	NI	Rabbit	NI	Sjölander *et al.*, 1991
Cell	Erythrocyte membrane antigen		sc/ivag ip	10-50 μg	G/A		Mouse		Thapar *et al.*, 1991
Other	Biotin	Influenza virus proteins	sc	1-10 μg			Mouse		Lövgren *et al.*, 1987

NI = not investigated; LHRH = luteinizing-hormone-releasing hormone; FMDV 144-159 = peptide containing foot-and-mouth virus amino acid sequence 144-159; abbreviations for viruses are as indicated in the text; im = intramuscular, in = intranasal, sc = subcutaneous, or = oral, id = intradermal, ip = intraperitoneal, ivag = intravaginal; G = IgG, A = IgA, M = IgM; VN = virus-neutralizing antibodies, FI = fusion-inhibiting antibodies, HLI = haemolysis-inhibiting antibodies; DTH = delayed-type hypersensitivity; CTL = cytotoxic T lymphocytes; LST = lymphocyte stimulation test; IL2 = *in vitro* antigen-specific interleukin 2 production; NK = natural killer cells; + + = positive protection at the indicated doses.

Table II. A schematic outline representing the procedures leading to iscom production.

1.	Solubilization of (membrane) proteins by adding detergent *e.g.* TX-100, MEGA-10, octylglucoside.
2.	Separation of solubilized and non-solubilized proteins.
3.	Addition of Quil-A, cholesterol and phospholipid.
4a.	Removal of detergent by dialysis.
4b.	Removal of detergent by centrifugation on a Quil-A-containing sucrose gradient.
5.	Removal of free Quil-A by centrifugation through a 10 % sucrose cushion.
6.	Analysis of iscom formation and quality by: negative contrast electron microscopy, SDS-PAGE, immunoblotting and antigen-specific ELISA.

phology of an iscom is independent of the protein incorporated. In fact, "empty" iscoms can be made which solely contain Quil A and cholesterol. This cholesterol iscom or iscom matrix is a very rigid and uniform structure. The incorporation of amphipatic oligo- or polypeptides requires the addition of phospholipids like phosphatidyl ethanolamine or phosphatidyl choline. It is speculated that the incorporation of phospholipids provides the flexibility necessary for the incorporation of proteins in the structure (Lövgren and Morein, 1988).

Table II represents a schematic outline of the principles used for the formation of iscoms. They are formed spontaneously when detergent is removed in a controlled fashion from a mixture of cholesterol, protein, phospholipid, detergent and Quil-A. Detergents are generally needed to obtain and keep (membrane) proteins in a monomeric form. The best results with many viral membrane proteins were obtained when octylglucoside or MEGA-10 (decanoyl-N-methylglucamide) were used as detergents. The detergent can be removed in several ways, but dialysis and centrifugation on a Quil-A-containing sucrose gradient have been used most frequently. The centrifugation method may be used when a non-dialysable detergent like Triton-X100 is used. Iscom formation is confirmed by negative contrast electron microscopy (fig. 1). Furthermore, iscoms have a well defined sedimentation (19 S) constant in a sucrose gradient.

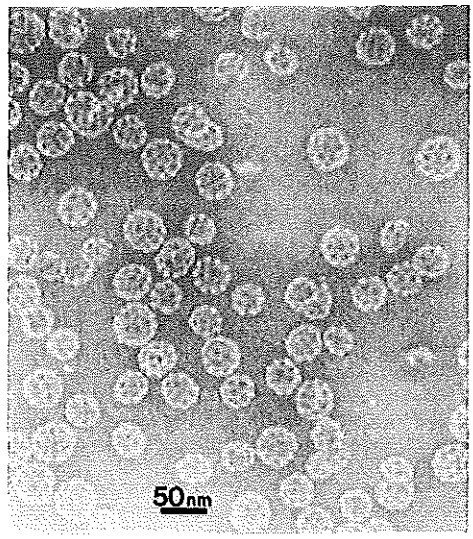

Fig. 1. Electron micrograph × 140,000 after negative contrast staining of iscoms which contain measles virus antigens showing the typical cage-like structure.

One of the interesting features of iscom technology is the fact that a wide range of proteins can be incorporated in the structure. Most viral membrane proteins are spontaneously incorporated upon formation of the iscom structure. For the introduction of proteins which do not possess membrane insertion sequences several methods have been developed. Pretreatment of hydrophilic proteins, *e.g.* BSA, by adjustment at low pH (2.5), reveals hydrophobic regions normally hidden inside the protein structure and allows these regions to interact with the iscom matrix (Morein *et al.*, 1990). Palmitification of proteins, the coupling by overnight incubation with palmitoyloxy-succinimide, prior to iscom formation, is also used to enable the incorporation of non-membrane proteins. The immune response against ovalbumin iscoms prepared in this way proved to be

as expected for iscoms (Mowat *et al.*, 1991a). The acid treatment may be harmful especially to certain conformation-dependent epitopes. The water-soluble nucleoprotein (NP) of influenza virus was chemically linked to bacterial lipopolysaccharide which enabled its subsequent incorporation in the iscom structure (Weiss *et al.*, 1990).

So far only limited data are available on the incorporation of peptides into iscom and the immunogenicity of such iscoms, although many studies are presently being carried out in this area (K. Lövgren, personal communication). It was shown that the response against a peptide based on the foot-and-mouth disease virus amino acid sequence which was coupled to preformed influenza virus iscoms did not induce very high anti-peptide antibody titres (Lövgren *et al.*, 1987). Using recombinant DNA techniques, a *Plasmodium falciparum* antigen sequence was produced as a fusion protein in *Escherichia coli.* When coupled to influenza virus iscoms, this antigen was shown to be immunogenic in rabbits (Sjölander *et al.*, 1991).

Protective immune responses against viruses induced by iscoms

Herpesviridae

Viruses which belong to the *Herpesviridae* family are important pathogens which can establish acute and latent infections in many animal species including man. Both humoral and cellular immunity are considered important for protection against infection with these viruses. Herpes simplex virus 1 (HSV1) and 2 (HSV2) are members of the *Alphaherpesvirinae* subfamily which infect man. Iscoms have been prepared with membrane proteins of these viruses and tested for their immunogenicity and protective potential. Mice generated significant antibody titres after immunization with HSV1 iscoms and were protected against a lethal challenge with HSV1 and HSV2 after a single immunization with 5 μg HSV1 iscom (Erturk *et al.*, 1989).

Pseudorabies virus (PrV), another member of the *Alphaherpesvirinae* subfamily, causes disease and death in piglets. The selective incorporation of viral proteins in iscoms could be useful to discriminate between vaccinated and non-vaccinated animals with subclinical infection. Iscoms containing the gII glycoprotein of PrV induced complement-dependent virus-neutralizing antibodies in mice (Tsuda *et al.*, 1991). A 1-μg dose provided partial protection in mice. Pigs immunized with 170 μg gII iscoms were completely protected against lethal challenge.

Bovine herpes virus type I (BHV1), yet another member of this subfamily, is an important pathogen in cattle which causes infectious rhinotracheitis and maybe responsible for other clinical manifestations. Differentiation between vaccinated and infected cattle on the basis of serology is possible when subunit vaccines are used that selectively incorporate certain proteins. Iscoms containing the envelope glycoproteins of BHV1 were shown to be immunogenic in rabbits (Trudel *et al.*, 1987) and calves (Trudel *et al.*, 1988a; Merza *et al.*, 1991a). Both species developed virus-neutralizing and haemagglutination-inhibiting antibodies. Calves vaccinated with either 50 or 100 μg BHV1 iscoms were protected against challenge with 5×10^6 $TCID_{50}$ BHV1. A control group of animals which had received a commercial inactivated vaccine proved to be unprotected against viraemia in the same experiment (Merza *et al.*, 1991b).

Infection with cytomegaloviruses (CMV), which belong to the subfamily *Betaherpesvirinae,* results in the establishment of latent infections in many species that may eventually lead to serious complications in immunocompromized hosts. Monkeys which were immunized with either CMV-infected monocytes or CMV iscoms developed anti-CMV antibody titres (Wahren *et al.*, 1987). Cellular responses, measured in lymphocyte stimulation tests (LST), were of similar magnitude in both groups. Less antigen (15 μg) was needed when CMV iscoms were administered intradermally. However, 150 μg of free unadjuvanted CMV nucleocapsid invoked poor cellular responses. No protection studies were carried out in this study.

Epstein Barr virus (EBV), belonging to the subfamily *Gammaherpesvirinae,* infects man and is the causative agent of infectious mononucleosis in industrialized countries. In certain developing countries it is associated with the development of undifferentiated nasopharyngeal carcinoma and Burkitt's lymphoma. Cottontop tamarin monkeys *(Saguinus oedipus)* develop tumours after infection with a high dose of EBV. Iscoms containing the EBV glycoprotein gp340 induced virus-neutralizing antibodies. A 5-μg dose of gp340 iscoms given 3 times protected the monkeys completely from tumour development (Morgan *et al.*, 1988). Human T cells from EBV-seropositive donors could be stimulated *in vitro* with gp340 iscoms (Ulaeto *et al.*, 1988).

Hepatitis B virus

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, causes a chronic infection in man which may eventually lead to the development of primary liver cancer. The first generation vaccines consisted of hepatitis B surface antigen (HBsAg) particles (20 nm) purified from plasma of infected donors. Recombinant DNA techniques have made it possible to produce HBsAg particles in yeast cells (McAleer *et al.*, 1984). Alum was required as an ad-

juvant to enhance immunogenicity of these particles. Immunization of mice with iscoms containing HBsAg derived from yeast generated high levels of HBV-specific antibodies (Howard *et al.*, 1987).

Rubella virus

Infection with rubella virus, a member of the family *Togaviridae,* may cause a mild morbilliform rash, occasional fever, and a rather predictable lymphadenopathy in man. Since infection during pregnancy leads to neonatal malformations, vaccination during childhood is widely practiced. A live attenuated rubella virus is used for vaccination worldwide. Iscoms containing the E1 glycoprotein (haemagglutinin) and the nucleoprotein of rubella virus have been prepared. Humoral immune responses in rabbits were as high as those observed with a commercial live-virus vaccine. The presence of virus-neutralizing and haemagglutination-inhibiting antibodies was demonstrated. Small amounts of the viral nucleoprotein were present in these iscoms which may be beneficial for the development of cellular immunity (Trudel *et al.*, 1989b).

Bovine virus diarrhoea virus (BVDV)

BVDV a member of the *Flaviviridae* family, usually causes subclinical infections in sheep and cattle. Vertical transmission of the virus may lead to abortion and teratogenic defects. Lectin affinity-purified BVDV proteins were incorporated in iscoms. Pregnant sheep were given two 50-μg doses of BVDV iscoms. All animals developed anti-BVDV antibodies with neutralizing activity. Significant protection was observed against a challenge of the sheep with BVDV. Some 26 lambs out of 29 were born in the vaccinated group whereas only 5 lambs out of 26 foetuses were born in the non-vaccinated animals (Carlsson *et al.*, 1991).

Paramyxoviridae

The potential of iscoms for the development of subunit vaccines against members of the genus morbillivirus has been explored in our laboratory. Whole measles virus iscoms, which contain the fusion protein (F) and minor amounts of the haemagglutinin protein (H), induced virus-neutralizing, haemagglutination-inhibiting and fusion-inhibiting antibodies. Iscoms containing immuno-affinity-purified F (F-iscom) induced antibodies with haemolysis inhibition (HLI) activity but devoid of VN activity. Both F and H iscom preparations induced protection against intracerebral challenge with a rodent-adapted measles virus strain (De Vries *et al.*, 1988a; Varsanyi *et al.*, 1987). Immunization with F-iscom induced a measles-virus-specific cellular response, delayed type hypersensitivity (DTH) and measles-virus-specific T-cell clones could be generated from these mice (De Vries *et al.*, 1988a). MV iscom also induced humoral (in monkey) and cellular (in mice) immune responses in the presence of passively transferred antibodies, a situation that mimics vaccination in the presence of maternal antibodies (De Vries *et al.*, 1990).

Canine distemper virus (CDV) is a morbillivirus closely related to measles virus that causes a disease in dogs with a pathogenesis similar to measles in man. CDV iscoms were shown to protect dogs against viraemia and clinical signs upon intranasal challenge with a virulent strain of the virus (De Vries *et al.*, 1988b).

CDV iscoms were also used to vaccinate seals against phocoid distemper virus (PDV), a recently discovered morbillivirus that caused mass mortality among seals in the North Sea in 1988 (Osterhaus *et al.*, 1989b; Visser *et al.*, 1989). Vaccination was successful since all animals developed PDV-neutralizing antibodies. Upon challenge with PDV, vaccinated animals were protected whereas two sham-vaccinated animals developed clinical signs and died.

Respiratory syncytial virus (RSV) is another *Paramyxovirus* which causes severe lower respiratory tract infections in man and cows. Guinea pigs immunized with iscoms based on human or bovine RSV developed cross-neutralizing antibody titres. In mice, intramuscular immunization with RSV-iscoms induced virus-neutralizing antibodies, CTL activity and protection against virus challenge (Trudel *et al.*, 1989; Trudel *et al.*, 1992).

Rabies virus

Rabies virus belongs to the *Rhabdoviridae* family and causes fatal disease in warmblooded animals. Most currently used vaccines against rabies virus are inactivated viruses which are used for pre- and post-exposure treatment of rabies infection. Rabies virus iscoms prepared from whole purified virus were among the first iscoms described (Morein *et al.*, 1984). *In vitro* experiments showed that human $CD4^+$ T-cell clones could be stimulated with these iscoms. Moreover, *in vitro* stimulation of human peripheral blood lymphocytes with rabies virus iscoms led to the production of virus-neutralizing antibodies. Immunization of mice with rabies virus iscoms induces a DTH response and neutralizing antibody titres (Höglund *et al.*, 1989). In our laboratory, we have generated data which show that both MHC class-I- and class-II-restricted T-cell responses can be generated after immunization of mice with rabies virus (Claassen *et al.*, submitted). Iscoms which contain lentil-lectin-purified rabies virus glycoprotein

were shown to be immunogenic in both mice and dogs. These iscoms could protect dogs against a lethal challenge with street rabies virus after pre-exposure immunization. Moreover post-exposure protection against street rabies virus challenge could be induced with three 120-ng doses of rabies virus glycoprotein iscom in 90 % of mice but not with three doses of a widely used human diploid cell vaccine (HDCV). When mice were given four doses of HDCV for post-exposure treatment they died of anaphylactic shock. No such phenomenon was observed when the mice had been immunized with the iscom preparation (Fedaku *et al.*, 1992).

Influenza virus

Influenza viruses (family *Orthomyxoviridae*) primarily infect the respiratory tract of many animal species. Therefore, local immune responses in the lung are considered important for protection. The two membrane proteins important for eliciting protective immune responses are haemagglutinin (HA) and neuraminidase (NA). The HA molecule is subject to antigenic drift which interferes with the protection elicited by vaccines based on the relevant strains. Cross-reactive cellular immune responses rapidly reduce virus titres in the lung and are therefore considered important.

Intranasal immunization of mice with two doses of 5-μg HANA iscoms elicited antibody titres (IgM, IgG and IgA) comparable to those elicited by virus infection (Jones *et al.*, 1988 and Lövgren, 1988). Furthermore, virus-specific antibody-secreting cells could be demonstrated in the lungs. In contrast, the same dose of HANA micelles induced lower responses of mainly IgM antibodies and no local B-cell memory. Serum antibody responses could also be demonstrated in guinea pigs and horses (Sundquist *et al.*, 1988). Both iscoms and micelles induced CTL precursors in the lungs of mice but the levels were four-fold higher in iscom-immunized mice. Natural killer cells were found in similar quantities after immunization with either of the antigen preparations (Jones *et al.*, 1988). Complete protection of mice against intranasal challenge with influenza virus could be induced by intranasal and subcutaneous immunizations with HANA iscoms (Sundquist *et al.*, 1988 and Lövgren *et al.*, 1990). Iscoms containing the influenza nucleoprotein induced virus-specific antibody titres but no specific CTL responses were observed. About 50 % of the immunized mice survived challenge with a virulent mouse-adapted strain (Weiss *et al.*, 1990).

Retroviridae

Iscoms containing the gp70/85 envelope protein of feline leukaemia virus (FeLV), a type C virus of the *Retroviridae* family, can induce protection in cats. Antibody responses as determined by ELISA, immunofluorescence, virus neutralization and Western blotting showed that over 80 % of iscom-immunized cats responded compared to only 6 % of a group of cats vaccinated with a commercial vaccine (Åkerblom *et al.*, 1989; Osterhaus *et al.*, 1985 and 1989a).

Iscoms containing gp51 of bovine leukaemia virus (BLV, HTLV/BLV group) induced good humoral immune responses in mice and calves (Merza *et al.*, 1991a). In calves, neutralizing antibodies could be measured after the second inoculation. These results showed the potential of iscoms for retrovirus vaccine development.

Human immunodeficiency virus type 1 (HIV1) (subfamily *Lentivirinae*) is the causative agent of AIDS in man. Until now, no safe and effective vaccines against this disease are available. The development of a suitable vaccine is hampered by the variability of the viral envelope protein gp120 which induces neutralizing antibodies. Multiple injections in rhesus monkeys with iscoms containing gp120 of HIV1 induced ten-fold higher neutralizing titres than comparable amounts of gp120 with an alum adjuvant (Pyle *et al.*, 1989). In mice, a 5-μg dose of these gp120 iscoms induced two-fold higher titres when compared with a 50-μg dose of gp120 in complete Freunds' adjuvant. Sera from rhesus monkeys *(Macaca mulatta)* cross-neutralized RF and MN isolates of HIV. The induction of class-I-restricted precursor CTL in mice was demonstrated using gp160-iscom-immunized mice. Spleen cells obtained from these mice could kill target cells expressing HIV gp160 or pulsed with a known immunodominant CTL peptide (Takahashi *et al.*, 1990).

Several animal models exist to study the immunity and pathogenesis of HIV-related viruses. Simian immunodeficiency virus (SIV) is a closely related lentivirus which can infect rhesus macaques and cause an AIDS-like syndrome. In a recent study from our laboratory, it was demonstrated that a SIV-iscom preparation as well as whole inactivated SIV adjuvanted with muramyl dipeptide (MDP) could protect animals against an intravenous homologous challenge with 10 MID_{50} of cell-free SIV. All animals were protected from developing SIV-specific viraemia for at least a twelve-week period after challenge. Fifty percent of the vaccinated animals in a similar experiment were also protected for at least nine weeks against an intravenous challenge with SIV-infected peripheral blood mononuclear lymphocytes (Osterhaus *et al.*, 1992). In another study, cynomolgus monkeys *(Macaca fascicularis)* were infected with HIV2 after immunization with HIV2-iscoms or inactivated HIV2 with complete Freund's adjuvant (CFA) (Putkonen *et al.*, 1991) HIV2-iscom-immunized monkeys were not protected against infection with HIV2 which was probably

due to the virtual absence of gp125 in the iscom preparation used. These results demonstrate that vaccination against lentivirus infections should not be considered impossible.

General considerations for the use of iscoms as an antigen-presenting moiety

Mode of administration

Iscom preparations have generally been administered intramuscularly or subcutaneously. Only small amounts — 1 to 10 μg — of incorporated antigens are needed to elicit effective immune responses in mice. It was found that the time between first and second immunization is critical for the development of a booster response. An interval between 6 to 8 weeks seems optimal to elicit a booster effect (Lövgren *et al.*, 1990). Short immunization protocols like the NIH efficacy test for rabies vaccines, in which case the interval is only one week, give lower titres and protection levels. One intraperitoneal immunization of mice with 360 ng of rabies virus glycoprotein iscom is sufficient to protect almost 90 % against intracerebral challenge with rabies virus (Fedaku *et al.*, 1992).

Recently the use of iscoms for oral immunization was also investigated. Attempts to investigate the possibility of oral immunization were undertaken with influenza virus iscoms but these were largely unsuccessful, probably because the administered antigen dose was too low, *i.e.* 5 μg (Lövgren, 1988). No systemic antibody response could be measured and it was speculated that iscoms could not resist degradation in the gastrointestinal tract. Intranasal immunization with influenza virus iscoms, however, led to a specific antibody response of all classes and subclasses, including IgA, and also to cellular responses in the lung (Jones *et al.*, 1988). This showed that local immunization with iscoms may lead to the induction of both local and systemic immune responses. Ovalbumin(OVA)-containing iscoms were shown to be immunogenic after systemic but also after oral immunization (Mowat *et al.*, 1991). Oral immunization with OVA iscom induced specific serum antibodies. Cellular responses were measured by DTH response. The required dose for oral immunization was 10 times higher (100 μg) than that needed for parenteral immunization, which is probably due to partial degradation of the immunogen in the gastrointestinal tract before iscoms can reach immunocompetent cells or lymphoid organs in the gut. Repeated feeding with OVA-iscom induced MHC class-I-restricted CTL specific for OVA (Mowat *et al.*, 1991). When soluble OVA was given orally in comparable amounts no responses could be measured.

Secretory immune responses in the vagina of mice could be measured after intraperitoneal, subcutaneous or intravaginal immunization with iscom containing sheep red blood cell (SRBC) membrane antigens. Non-mucosal immunization in the pelvis induced higher systemic and local titres than local application, and local application required 10 times higher amounts of antigen (Thapar *et al.*, 1991).

The finding that iscom can be used for local immunization is important for the development of protection against pathogens which acquire access through mucosal membranes and therefore require musocal immunity. The importance of the mucosal immune system for vaccine development has recently been reviewed (McGhee *et al.*, 1992).

Toxicological aspects

When iscoms are considered as vaccine candidates for humans, toxicological aspects of the incorporated adjuvant Quil-A should be considered. So far, one study has been published on the *in vivo* effects of Quil-A-containing iscoms after intramuscular injection (Speijers *et al.*, 1988). Only a moderate inflammatory reaction was observed in 1 out of 6 rats which had received an iscom dose containing 60 μg Quil-A. It should be noted that the haemolytic activity of Quil-A incorporated in iscom is ten times lower than observed with free Quil-A (Kersten, 1991).

Attempts have been made to separate the individual components of Quil-A in order to segregate the haemolytic and adjuvant activity (Kensil *et al.*, 1991; Kersten, 1991). Kensil described the separation by reverse-phase HPLC of Quil-A into 4 fractions which all retained their adjuvant activity. One fraction (QS-7) showed no direct lethal effect in mice at a dose of 500 μg. Unfortunately this fraction was not tested for its ability to form iscoms. Kersten, using the same approach, reported the separation of Quil-A into 23 fractions. Six of these purified saponins could be used to form iscom-like particles of varying size, but these were consistently larger than iscoms prepared from unpurified Quil-A. Immunogenicity studies were performed using iscom with the gonococcal PI protein incorporated. Iscoms made with the so-called fraction QA-3 were as immunogenic as total Quil-A iscoms. The haemolytic activity of QA-3 was 15 times reduced as compared to total Quil-A. Since the mean size of QA-3-iscoms is between 110 and 145 nm, the influence of particle size on immunogenicity has to be evaluated.

It should be noted that no negative side effects have been observed with the use of an iscom-based commercial equine influenza vaccines in horses (Sundquist *et al.*, 1988), or with any other iscom preparation (Nagy *et al.*, 1990). Moreover, Quil-A is accepted in veterinary vaccine preparations for pigs

and cows at 10 to 100 milligram doses, about 100- to 1,000-fold higher than that used in iscom preparations. Nevertheless, before iscom can be used in vaccines for human application, further toxicological studies are needed. These studies should focus on iscoms and their individual components and should deal with acute and subacute toxicity as well as possible mutagenic effects and sensitization. This may be omitted for iscom administered orally since saponins are accepted food additives.

How do iscoms work?

The quantitative and qualitative effects on the immune responses as found after iscom immunization raise the question as to what determines the immunogenicity of the iscom structure. The higher and, in some cases, longer persisting antibody titres can easily be explained if one assumes that incorporated Quil-A retains its normal adjuvant activity. It has been suggested that the retention time of the immunizing antigen at the injection site and the uptake amount in the lymphoid organs, as observed with influenza iscoms (Watson *et al.*, 1989) and with Quil-A as adjuvant (Scott *et al.*, 1985) may be important for the observed effects. However, when Quil-A was used as adjuvant, the inflammatory reaction and antigen retention could be completely inhibited by the addition of cholesterol without affecting adjuvant activity (Scott *et al.*, 1985). A progressive redistribution of iscom-bound radioactive labelled antigen to the spleen was observed when compared to antigen in micelles (Watson *et al.*, 1989). Furthermore, we have recently observed that iscoms *in vivo* are taken up in mice by a distinct subset of macrophages in the spleen which is not involved in the uptake of free inactivated antigen (Claassen *et al.*, submitted).

Furthermore, the multimeric presentation form of the incorporated proteins and the presence of Quil-A in the same unique structure proved to be essential for the observed effects. Using different biotin/protein ratios, it was shown that at least 3 biotin molecules have to be coupled to a preformed iscom to induce an antibody response 10 times higher than that achieved when only one biotin molecule is coupled (Lövgren *et al.*, 1987). This finding clearly illustrates that the multimeric form is important for the observed adjuvant activity of the iscom structure. Although speculative, this might also be necessary for the induction of MHC class-I-restricted CTL, even when proteins like OVA, which are normally not immunogenic for these T cells are included. It is generally accepted that at least two distinct routes exist for the degradation of foreign antigen and the presentation different subsets of T cells (Yewdell and Bennink, 1990). First, the uptake of antigens into lysosomes, which leads to the degradation of foreign proteins and to the formation of peptides which then associate with MHC class II molecules and are recognized by T cells bearing the CD4 marker. Secondly, the cytosolic processing pathway into which enter proteins which are synthesized in the cytosol, released into the cytosol by lysis of endosomes or by antigens penetrating through the plasma or endosomal membranes. This pathway generates peptides that associate with MHC class I molecules which are recognized by $CD8^+$ T cells. In general, non-replicating antigens do not enter the cytosolic pathway, so that no priming for MHC class-I-restricted CTL occurs. The ability of iscoms to stimulate $CD8^+$ CTL *in vivo* has been demonstrated with different iscom preparations (Jones *et al.*, 1988; Takahashi *et al.*, 1990; Mowat *et al.*, 1991; Trudel *et al.*, 1992), even after oral immunization (Mowat and Donachi, 1991). In our laboratory, data have been generated which show that iscoms can stimulate MHC class-I-specific CTL *in vitro* (van Binnendijk *et al.*, submitted) and *in vivo* (Claassen *et al.*, submitted). The *in vitro* stimulation of measles-virus-specific CTL is dependent on the presence of MHC class-I-matched antigen-presenting cells. It may thus be hypothesized that at least *in vitro* iscoms are able to enter the cytosolic processing pathway which leads to expression of peptides in the context of MHC class I molecules. Iscoms could act as a carrier enabling proteins to penetrate the plasma and endosomal membranes partially or entirely, thus exposing iscom-bound protein to both proteolytic compartments. Perhaps iscoms are not only taken up by APC to enter the lysosomal compartment but they may also pass through or into the membrane of the cells, meanwhile protecting the protein for proteolytic degradation, and thus exposing the protein to cytoplasmic proteases. Intact and partially degraded iscoms could be demonstrated attached to macrophage cell membranes and within phagosomal membranes in close association with phagosomal membranes but not in the cytoplasm (Watson *et al.*, 1989).

The proposed capability of iscom to pass into the membrane may be explained by their hydrophobic structure, and because they contain saponins which can intercalate into cholesterol membranes (Özel *et al.*, 1989). This in fact determines the haemolytic properties of Quil-A. Iscoms are extremely stable structures and thus may be able to pass through cell or lysosomal membranes and so enter the cytosolic processing pathway thereby stimulating $CD8^+$ CTL.

Conclusion

In summary, we conclude that the iscom matrix is a promising moiety for the presentation of antigen to the immune system. It lacks many of the disadvantages of traditional live vaccines as regards

safety. The major points in favour of an iscom approach to developing new vaccines are the following: they generate long-lasting biologically functional antibody responses; they generate immunological responses in the presence of maternal antibodies; they generate functional cell-mediated immune responses; they generate protection in several systems. However, before iscom technology can be applied to the production of vaccines for human use the toxicity of individual iscom preparations will have to be studied and documented in more detail. Since there is an urgent need for new generations of vaccines, especially against viral infections like HIV1, EBV and RSV, the further development of the iscom as an immune-enhancing moiety deserves more attention.

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Comparison of adjuvants for immune potentiating properties and side effects in mice

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Abstract

Four types of adjuvants were evaluated as alternatives to the use of Freund's complete adjuvant in mice. The adjuvants evaluated included a water-in-oil emulsion (Specol), a microorganism (*Lactobacillus*), preformed immune-stimulating complexes (ISCOM) containing rabies virus glycoprotein and a saponin, Quil A. The adjuvants and saline were combined with three weak immunogens (a synthetic peptide, a self antigen and a particulate antigen) and given by three different routes (intraperitoneal, subcutaneous and dorsal in the foot). The evaluation was based on clinical observations, behavioural studies, pathological lesions and capacity to support immunological responses to weak immunogens. Lesions were most severe after injection of antigen combined with Freund's adjuvant or Quil A, mild to moderate with Specol and minimal with *Lactobacillus*, iscom conjugates or saline. Despite pathological changes, no signs of prolonged pain or distress could be demonstrated based on clinical observations and behavioural studies. Minimal immunological responses were found after injection of antigen in combination with saline or *Lactobacillus*. T-cell activation and high antibody responses were found after injection of antigen–iscom conjugates or antigen in Freund's adjuvant emulsions. After Specol/antigen immunisations T-cell activation was demonstrated and high antibody titres were found except for Specol/self antigen immunisations. Presented data suggest that Specol is a possible alternative to Freund's complete adjuvant for the induction of an immune response against weak immunogens except possibly self antigens, for which preformed iscoms seem very suitable.

Keywords: Evaluation; Adjuvants; Pathology; Antibody; T-cell activation

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1. Abbreviations

FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; FA, Freund's adjuvant; iscom, immune-stimulating complex; PBS, phosphate-buffered saline; i.p., intraperitoneal(ly); s.c., subcutaneous(ly); d.f.p., dorsal foot pad; SP, synthetic peptide; MBP, myelin basic protein; PLN, popliteal lymph node; IL-2-PC, interleukin-2 producing cells; IFN-γ-PC, interferon-γ producing cells.

2. Introduction

To elicit effective B- and T-cell responses, Freund's complete adjuvant (FCA) has become the most widely used adjuvant in laboratory animals. However, besides the marked enhancement of the immune response, a broad spectrum of unwanted side effects can also be found (Toth et al., 1989). Pathological changes seen after injection of FCA are known to be very painful in humans (Chapel and August, 1976). Growing concern with respect to the severe side effects caused by FCA is leading to stricter regulations. In Canada (Canadian Council on Animal Care, 1991), the USA (National Institute of Health, 1988) and the Netherlands (Veterinary Public Health Inspectorate, 1993) for example, guidelines have been publicised giving recommendations on the use of adjuvant products in laboratory animals. One of the reasons for continuation of the use of FCA is a lack of information on possible alternatives to FCA, with regard to both side effects and immunological properties. Many products with adjuvant activity are available. Dependent on the type of immune response desired, an adjuvant may be applied. In an earlier study (Leenaars et al., 1994) we evaluated adjuvants for the production of polyclonal antibodies and side effects in rabbits.

For the induction of a specific immune response it is important to know the mode of action of the adjuvant. To elicit immune responses efficiently the production of cytokines is required. Two major cytokines produced by activated helper T cells are IL-2 (Kuziel and Greene, 1990) and IFN-γ (Gustafson and Rhodes, 1992). T-cell activation can also be studied by revealing the 39 kDa membrane protein (gp39) expressed on activated helper T cells, this molecule is essential for the activation of resting B cells into antibody producing cells (Noelle et al., 1992; Van den Eertwegh et al., 1993).

Since mice are generally used in immunological research we conducted a study to compare side effects and immunomodulating effects of alternative adjuvants to FCA in mice. The adjuvants, selected on their mechanism, low toxicity and supposed immunological properties, included a water-in-oil emulsion, *Lactobacillus* strains, immune-stimulating complexes and saponin. To mimic normal laboratory problems in evoking immune responses and to ease discrimination between the adjuvants to be evaluated, the antigens used (a synthetic peptide, a self antigen and a particulate antigen) were weak immunogens.

3. Animals, materials and methods

3.1. Animals

Male and female BALB/c mice were bred specific pathogen free (SPF) at the National Institute of Public Health and Environmental Protection (RIVM) breeding facilities, Bil-

thoven, the Netherlands and were used at 10–14 weeks of age. At the RIVM, animals were housed in groups of five under SPF conditions in polycarbonate cages, environmental temperature of 20–22°C, relative humidity of 50–80% and with a 12 h day/night cycle. Mice were fed a commercial diet (Hope Farms, Woerden, the Netherlands) and provided with bottled water ad libitum.

3.2. Adjuvants

The following adjuvants were used: Freund's adjuvant (water-in-oil emulsion, Freund's incomplete adjuvant (FIA) containing mycobacteria (FCA); Difco Laboratories, Detroit, MI, USA), water-in-oil emulsion (Specol described by Bokhout et al., 1981; Institute for Animal Science and Health (ID-DLO), Lelystad, the Netherlands), non-pathogenic micro-organism, *Lactobacillus* (*Lactobacillus plantarum* (ATCC 8014, 10^9 ml^{-1}), *Lactobacillus casei* (ATCC 393, 10^9 ml^{-1}); Claassen et al., 1995), preformed iscoms containing rabies virus glycoprotein (RV-iscoms; preparation described below; Claassen and Osterhaus, 1992) and purified saponin Quil A ('Spikoside', Iscotec, Luleå, Sweden).

Rabies virus (RV-Pasteur strain) was propagated in Vero-cell monolayer cultures (Van Wezel et al., 1978). Culture supernatant was cleared by filtration and concentrated by Amicon ultrafiltration (cut-off 10^6 Da). Virus was inactivated with β-propiolactone (BPL) and stored at -70°C at a concentration of 900 μg ml^{-1}. This rabies virus antigen was used for the preparation of RV-iscoms as described earlier (Fekadu et al., 1992).

3.3. Antigens

Synthetic peptide (SP215, an analogue of SP29) was synthesised as described by Boersma et al. (1989). SP215 is a sequence derived from the hinge region of human IgG2, comprising 21 amino acids. The self antigen was myelin basic protein from bovine brain (MBP, Van Noort et al., 1993; Sigma, St. Louis, MO, USA), consisting of 173 amino acids (18.5 kDa). The particulate antigen *Mycoplasma pneumoniae* (MAC strain) was a kind gift of Dr. A. Angulo (RIVM, Bilthoven, the Netherlands) and was prepared by culture into Chanock broth, concentrated by centrifugation and washed with phosphate-buffered saline (PBS). Inactivation was done at 57°C during 30 min. Particles of *M. pneumoniae* are 0.1–0.8 μm in diameter.

3.4. Adjuvant/antigen preparation

For the preparation of adjuvant/antigen mixtures, the antigens (amounts given in Table 1) were diluted in sterile physiological saline and mixed with the adjuvant, except when antigens were injected in combination with preformed iscoms. SP215 (2 mg) was coupled covalently to 120 μg RV-iscoms using 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) as described previously (Deen et al., 1990). MBP (2.1 mg) was coupled to 80 μg RV-iscoms using EDC. After coupling for 30 min at room temperature (RT), iscoms were dialysed against PBS overnight to remove uncoupled antigens and EDC. Freund's adjuvant (FCA in primary and FIA in secondary immunisation, abbreviated as FA) and Specol emulsions, containing antigen, were prepared as recommended by the manufacturer of the

Table 1
Injected volumes per type of antigen and per route of administration[a] and dose of antigen applied in this study

Antigen	Dose (μg)	Volume (ml)		
		i.p.	s.c.	d.f.p.
SP215	50	0.1	0.1	2×0.040
MBP	100	0.2	0.2	2×0.040
M. pneumoniae	15	0.2	0.1	2×0.040

[a]i.p., intraperitoneal; s.c., subcutaneous; d.f.p., at the dorsal site of the hind feet.

adjuvant. *Lactobacillus*/antigen mixtures were prepared by adding antigen in sterile physiological saline to *Lactobacillus* (1:1) and mixing well. Quil A dissolved in water was added to the antigen and mixed. Intraperitoneally (i.p.) injected animals received 10 μg Quil A per injection while animals injected subcutaneously (s.c.) or at the dorsal site of the hind foot (d.f.p.) received 20 μg Quil A per injection.

3.5. Experimental design

Adjuvants were evaluated in three experiments. In each experiment one type of antigen was combined with each of the adjuvants, with one exception: in combination with *M. pneumoniae*, preformed iscoms were substituted by Quil A. The size of this particulate antigen makes it unfeasible to couple to preformed iscoms. Per antigen and per injection route, a control group, injected with antigen in sterile physiological saline was included. Injected volume per route of administration and per type of antigen are given in Table 1. Five mice were used per group. Animals were given a primary injection with the adjuvant/antigen or saline/antigen preparations on Day 0. Identical secondary injections were given on Day 42, except that FCA was replaced by FIA and the secondary injections of MBP–iscom conjugate were given on Day 91 owing to unavailability of the conjugated antigen.

Blood samples were taken from the tail vein on Day −1 to determine preimmune titres, 1 week after primary immunisation and at 7 day intervals thereafter. Sera were pooled per group except for sera collected at necropsy. These sera were collected and titrated individually. Sera were stored at −20°C. One week after secondary immunisation mice were bled after being anaesthetised by intramuscular inoculation of 0.1 ml KRA (mixture of Ketamine (50 mg ml^{-1}), Rompun (20 mg ml^{-1}) and Atropine (1 mg ml^{-1}) in a 7:3:1 ratio). Animals were examined for gross lesions and relevant tissue samples were collected for histopathology.

3.6. Clinical observation and body weight

Specific changes in common clinical signs to indicate pain, distress or discomfort in experimental animals were evaluated as described by Morton and Griffiths (1985). General condition was evaluated daily and mice were weighed twice a week, starting on Day −1. Injection sites were palpated for signs of pain and swelling at least once a week.

3.7. Behavioural and physiological state

The behavioural changes and physiological state of the mice were studied in the primary observation test (POT), a systematic quantitative procedure described by Irwin (1968) and modified by Olivier (Solvay-Duphar, Weesp, the Netherlands).

The POT procedure involved an initial phase of undisturbed observation and a later manipulative phase during which the animal was subjected to different stimuli. Animal behaviour was studied before immunisation, 3 days after primary immunisation and weekly thereafter.

The procedure started by placing a group of mice in a viewing jar followed by an adaptation period of 1 h. The observation study began by observing the animal's undisturbed behaviour within the viewing jar, i.e. dispersion in the cage, apathy, startle-reaction, restlessness, watchfulness and respiration. Thereafter, the animals were individually transferred onto the viewing arena for testing: arousal response to transfer, spatial locomotion, gait, slip resistance, righting reflex, exophthalmos and pilo-erection. Throughout individual handling, touch-reflex, provoked-freezing, provoked biting, skin colour, body tone, pupil size, urination–defecation and vocalisation were studied. After handling, mice were again placed in the viewing jar and wash activity was observed. Behavioural changes and physiological state of each mouse were scored.

3.8. Pathology

Necropsy consisted of complete dissection and examination of injection sites and major organs. Tissue of lesions and organs showing macroscopic abnormalities were preserved in 4% buffered formaldehyde. A sample of the omentum was preserved of all i.p. injected animals. The size of the popliteal lymph node (PLN) of d.f.p. injected animals was scored. The left PLN was fixed in 4% buffered formaldehyde, while the right PLN was immediately frozen in liquid nitrogen for immunohistochemistry for demonstration of cytokine-producing cells and gp39 expression. Frozen tissues were stored at −70°C in air-tight aluminium containers containing a small amount of ice to prevent dehydration on prolonged storage. For histopathological evaluation, a selection of fixed tissue samples was embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin. In the i.p. injected animals the extent of abdominal lesions was scored based on the relative amount of white plaques in the visceral and diaphragmatic peritoneum. The severity of peritonitis in these groups was scored based on gross detection of adhesions and omentum retrahens and on microscopic evaluation of (peri)pancreatitis, fat necrosis and micro abscesses. In animals s.c. injected lesions were scored separately for primary and secondary injection site. The score was based on diameter of lesions and on microscopic evaluation of infiltrates and the presence of necrosis. In the d.f.p. injected animals swelling was scored grossly and severity of lesions was scored microscopically by involvement of deep layers and the presence of ulceration. Gross and histopathologic lesions were scored as follows: −, minimal; +, mild; + +, moderate; + + +, marked.

3.9. Immunoassays

Serum antibodies to MBP, SP215 and *M. pneumoniae* were determined using a direct enzyme-linked immunosorbent assay (ELISA). For animals immunised with SP215 and

MBP, ELISA procedures were performed essentially as described by Zegers et al. (1991). Sera were diluted 1:100, 1:300 and 1:900.

For *M. pneumoniae* antibodies, ELISA was performed as described in detail by Leenaars et al. (1994). The sera were diluted using three-fold dilutions from 1:270 to 1:65610.

3.10. Immunohistochemistry

Detection of cytokine producing cells and $gp39^+$ cells in PLN were performed essentially as described by Van den Eertwegh et al. (1993). Cryostat sections ($-20°C$, 8 μm) were fixed for 10 min in fresh acetone containing 0.02% H_2O_2. The murine monoclonal antibody (mAb) DB-1 (specific for rat IFN-γ and cross-reacting with murine IFN-γ) and the rat mAb S4B6 (specific for murine IL-2) both conjugated to alkaline phosphatase (AP) were used as cytokine-specific antibody conjugates. After washing the slides with PBS, AP activity (blue) was demonstrated by naphthol-substrate as described by Claassen et al. (1986). Immunohistochemical demonstration of gp39 was performed with MR-1 (a hamster mAb specific for murine gp39) horizontally overnight under high humidity at 4°C. Slides were washed three times with PBS followed by the murine mAb specific for rat/hamster-Igκ (RG-7) chain conjugated to peroxidase (HRP) horizontally for 1 h under high humidity at RT. RG-7-HRP slides were washed three times with PBS and HRP activity (red) was demonstrated by AEC-substrate as described by Claassen et al. (1986).

For both substrate types, the reaction was stopped by transferring slides to PBS. Sections were counterstained with haematoxylin, rinsed with tap water and embedded in glycerin–gelatin. Cytokine-producing cells (IL-2 producing cells (IL-2-PC) and IFN-γ producing cells (IFN-γ-PC)) and $gp39^+$ cells were counted per cryostat section.

3.11. Statistical evaluation

For statistical evaluation change in body weight was calculated as body weight on test day minus body weight on last measured day before. Differences between adjuvant and no-adjuvant groups were evaluated for significance using analysis of variance. Serum antibody responses at necropsy were analysed by the two-sample Student's *t*-test for comparison of two empirical means in a normally distributed population. A probability of 0.05 or less was considered significant.

4. Results

4.1. Clinical findings

After 7 days, the body weights of immunised animals were equal to or higher than body weights of control mice for the remainder of the post-immunisation period. Pilo-erection was observed two days after primary and secondary immunisation, mainly in FA groups and when *M. pneumoniae* preparations were injected. Abnormalities (swelling, signs of pain etc.) after immunisation are described below per injection route.

4.1.1. i.p. injection

Body weights decreased significantly in groups of mice ($n=5$) i.p. immunised with FCA/antigen (3 out of 3 groups), Specol/antigen (2/3), *Lactobacillus*/antigen (1/3) or antigen–iscom conjugates (2/2).

After primary immunisation, i.p. injected animals showed no abnormalities at the injection site and palpation of the abdomen did not lead to any sign of pain such as vocalising or struggling. Within 1 h after secondary immunisation (i.p.) with FIA/SP215 emulsion animals died. Within 2–3 days after secondary injection (i.p.) of iscom conjugates some animals died (combined with SP215, 2/5 and combined with MBP, 5/5).

4.1.2. s.c. injection

When mice were immunised subcutaneously, body weights significantly decreased after primary injection of FCA/antigen (2 out of 3 groups), Specol/antigen (2/3), *Lactobacillus*/antigen (1/3) or antigen–iscom conjugates (1/2). At the s.c. injection site of FA/antigen emulsions, palpable nodules, varying in size between 1 and 5 mm, were found. One hour after secondary immunisation (s.c.) with FIA/SP215 emulsion, one mouse died. In some animals s.c. injected with *M. pneumoniae* combined with Specol or *Lactobacillus* a yellow spot was found at the site of injection. Secondary injection of Quil A/*M. pneumoniae* preparations (s.c.) resulted in nodules of 2–5 mm.

4.1.3. d.f.p. injection

In animals injected with FCA, body weights were significantly decreased 2 days after immunisation. An ulcerative lesion was found at the injection site in two animals injected with Quil A/*M. pneumoniae.*

4.2. Behavioural changes and physiological state

Pilo-erection was observed in the first days after primary immunisation (i.p.) with the FCA/SP215 or FCA/MBP emulsions. No significant changes in behaviour or physiological state of the mice were observed in other groups after primary immunisation. After secondary injections, pilo-erection was observed in all groups i.p. or s.c. injected with *M. pneumoniae* preparations. Secondary injections resulted in mortality in some groups (specified above).

4.3. Pathological findings at necropsy

4.3.1. i.p. injection

Table 2 shows gross and histopathologic lesions at necropsy. The most prevalent abdominal abnormality was white smooth shiny thickening of the peritoneum covering liver, diaphragm and spleen. Microscopically these 'plaques' are granulomatous peritonitis. This was present in all FA and Specol treated animals. In animals that died immediately after FIA/SP215 injection (i.p.) the same plaques were found. Moreover, they showed cyanosis of extremities and congestion of intestinal serosa. Animals that died after secondary injection (i.p.) of antigen–iscom conjugate showed a red nose and congestion of intestinal serosa and liver while no abdominal granulomatous lesions were found. Histology of the kidneys of three animals showed acute degeneration of convoluted tubules. Omentum retrahens was

Table 2
Score of gross and histopathologic lesions at necropsy (7 weeks after primary and 1 week after secondary injection), from mice injected with various adjuvant/antigen or saline/antigen mixtures via three injection routes[a]

Adjuvant	Antigen	Pathological findings[b]					
		i.p.		s.c.		d.f.p.	
		Extent[c]	Severity[c]	Primary[c]	Secondary[c]	Gross[c]	Histo[c]
FA	SP215	[d]		+ +	[e]	+ +	+
	MBP	+ +	+ + +	+ + +	+	+ +	+
	M. pneumoniae	+ + +	+ + +	+	+ +	+ +	+ +
Specol	SP215	+	–	–	+	+	–
	MBP	+	–	–	–	+ +	–
	M. pneumoniae	+ +	+	+ +	+	+ +	+
Lactob.[f]	SP215	–	–	–	–	–	–
	MBP	–	–	–	–	–	–
	M. pneumoniae	+	+ + +	–	+ +	+	–
Iscoms	SP215	–[g]	–	–	–	–	–
	MBP[h]	[i]		–	+	–	–
Quil A	*M. pneumoniae*	+	+ + +	–	+ +	+ + +	+ + +
Saline	SP125	–	–	–	–	–	–
	MBP	–	–	–	–	–	–
	M. pneumoniae	–	–	–	–	–	–

[a]i.p., intraperitoneal; s.c., subcutaneous; d.f.p., at the dorsal site of the hind feet.
[b]Comparison of data is possible intraroute and not interroute because of anatomical differences.
[c]–, minimal; +, mild; + +, moderate; + + +, marked.
[d]No data comparable since animals died within 1 h after secondary injection (see Section 4).
[e]Primary and secondary injection on the same site.
[f]*Lactobacillus* spp.
[g]In one of three animals lesions were found (see Section 4.3.1.).
[h]14 weeks after primary injection.
[i]No data comparable since animals died within 48 h after secondary injection (see Section 4).

present in all groups treated with *M. pneumoniae*–adjuvant preparations. Intestinal adhesions were only found in FA/MBP (3/5 animals) and in SP215–iscom conjugate (1/3) injected animals. This last animal showed arteritis in the pancreas.

4.3.2. s.c. injection

FA injection sites most consistently showed lesions both 1 week after FIA and 7 weeks after FCA application. FCA injection resulted in white subcutaneous nodules measuring 1–9 mm, microscopically designated as fibrosing granulomas. FIA induced lesions grossly showed white fluid diffusely in the s.c. fat. Microscopy revealed oil spaces and fat necrosis surrounded by active fibrous tissue rich in granulocytes. At the primary injection site of Specol/*M. pneumoniae*, in 3/5 mice thin-walled cysts (2–3 mm) with yellow fluid were present, microscopically diffuse pyogranulomatous panniculitis. Secondary injection of Specol/*M. pneumoniae* induced subcutaneous white lesions which microscopically are granulomas around large oil spaces. A soft yellow mass was found after secondary injection of *Lactobacillus*/*M. pneumoniae* and Quil A/*M. pneumoniae*, which microscopically appeared to be exudative panniculitis and diffuse necrotising panniculitis, respectively.

Table 3
Relation between T-cell activation, enlargement of popliteal lymph nodes (PLN) and humoral response of animals d.f.p. injected with various adjuvant–antigen preparations

Antigen	Adjuvant	IL-2[a]	IFN-γ	gp39	PLN[b]	Antibody response[c]
SP215	FA	+	+	+	+ +	1.68 ± 0.4
	Specol	+	+	+	+ +	1.78 ± 0.2
	Lactob.[d]	–	–	+	–	0.19 ± 0.2
	Iscoms	+	+	+	–	1.97 ± 0.05
	Saline	–	–	–	–	0.04 ± 0.5
MBP	FA	+	+	+	+ +	0.15 ± 0.2
	Specol	+	+	+	+ +	0.07 ± 0.03
	Lactob.	–	–	–	–	–
	Iscoms	+	+	+	+ +	1.83 ± 0.5
	Saline	–	–	–	–	–
M. pneumoniae	FA	+	+	+	+ +	≥ 6
	Specol	+	+	+	+ +	≥ 6
	Lactob.	+	+	+	+	5
	Quil A	+	+	+	+	≥ 6
	Saline	+	+	+	–	4

[a]IL-2-PC, IFN-γ-PC, gp39$^+$ cells: –, no staining observed; +, staining observed.
[b]PLN: –, not enlarged; +, twice enlarged; + +, three times or more enlarged.
[c]Serum antibody responses 1 week after secondary injection; SP215 and MBP results are expressed as absorbance at 405 nm for diluted (1:300) sera. Values represent the mean ± SD of antibody responses of five mice. *Mycoplasma pneumoniae* results are shown as ^{3}log serial dilutions with a serum dilution from 1:270 (1) to 1:65610 (6).
[d]*Lactobacillus* spp.

4.3.3. d.f.p. injection

Injection of FA/antigen or Specol/antigen preparations resulted in diffuse swelling of the metatarsal region and local swellings at the dorsal side just proximal to the metatarsophalangeal joints and of the tarsal region. Histologically granulomatous inflammation in the subcutis was present. In the FA treated animals lesions also involved muscles, tendon sheaths and periost. Lesions were most severe after d.f.p. injection of Quil A/*M. pneumoniae*. Feet were swollen and hyperaemic and two animals showed an ulcerative lesion. Microscopically these lesions were extremely exudative and also involved the deep layers. The relative enlargement of the PLN after d.f.p. injection is shown in Table 3.

4.4. Antibody response

High serum anti-SP215 responses were found after injection of SP215 in combination with FA, Specol or preformed iscoms (Fig. 1). The d.f.p. injection resulted in antibody responses comparable to s.c. injection. Primary injection of SP215 emulsified in FCA or Specol resulted in high antibody responses which reached a plateau at Week 5 while secondary injection had limited effect on antibody responses. The secondary injection of SP215–iscom conjugate had enhancing effect on the immune response. When SP215–iscom conjugates were injected d.f.p., antibody responses were low until secondary immunisation.

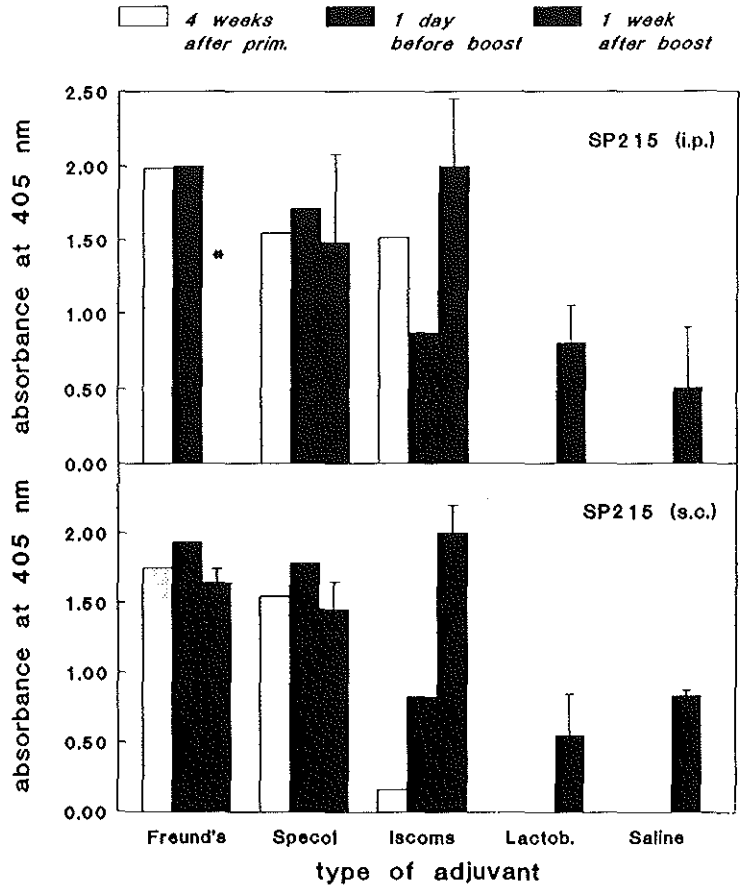

Fig. 1. Serum anti-SP215 antibody responses in a direct ELISA. Mice were primary immunised and boosted (i.p. or s.c.) with different adjuvant/SP215 preparations. The results are expressed as absorbance at 405 nm for diluted (1:300) sera, collected 4 weeks after primary immunisation, 1 day before secondary injection and 1 week after secondary injection. *, no data available. Data from 1 week after secondary injection represent mean ± SD of antibody responses of five mice.

A secondary injection (d.f.p.) of SP215–iscom conjugate enhanced the antibody response to a level comparable to FA/SP215 or Specol/SP215 emulsions.

The i.p. injection of FA/MBP emulsions resulted in high antibody titres (Fig. 2). After MBP–iscom conjugate injection (i.p.) serum antibody responses were still rising at the moment of secondary immunisation. When MBP was s.c. injected (Fig. 2) antibody titres were similar to those produced after d.f.p. injection. Low anti-MBP antibody responses were found after injection of FA/MBP emulsions via s.c. or d.f.p. route also after a secondary injection. After primary injection of MBP–iscom conjugate (s.c. or d.f.p.) anti-MBP antibodies increased till secondary immunisation (Week 13). Secondary injection (s.c. or d.f.p.) of MBP–iscom conjugate enhanced the immune response significantly.

After primary injection of adjuvant/*M. pneumoniae* preparations minimal differences in antibody responses were observed between the adjuvants (data not shown). A secondary

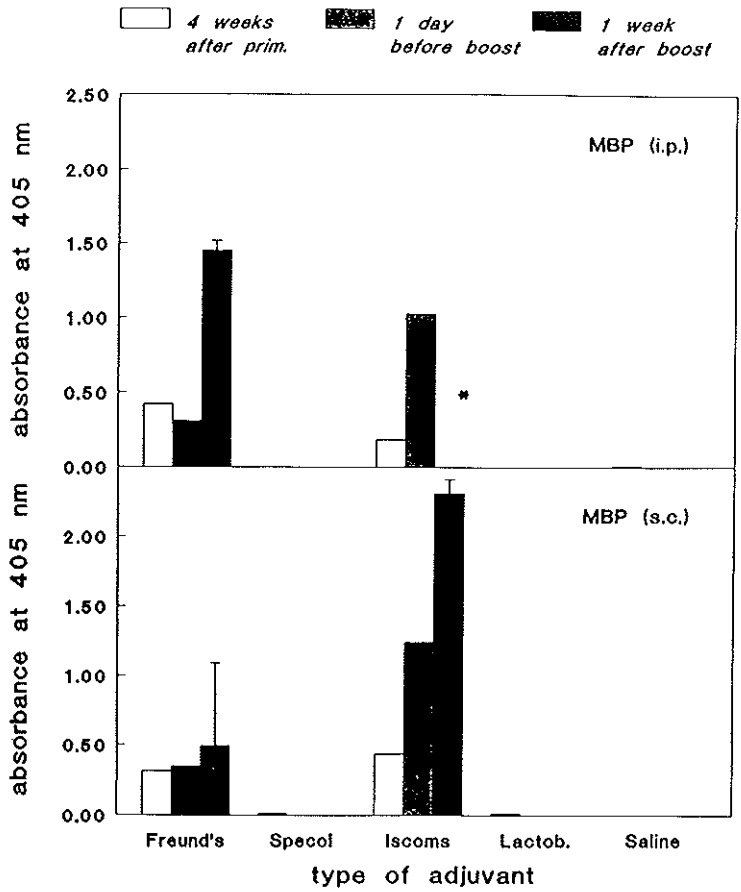

Fig. 2. Serum anti-MBP antibody responses in a direct ELISA. Mice were immunised and boosted (i.p. or s.c.) with different adjuvant/MBP preparations. The results are expressed as absorbance at 405 nm for diluted (1:300) sera, collected 4 weeks after primary immunisation, 1 day before secondary injection and 1 week after secondary injection. *, no data available. Data from 1 week after secondary injection represent mean ± SD of antibody responses of five mice.

injection resulted in enhanced antibody responses in all groups. The effect of secondary injection of adjuvant/*M. pneumoniae* preparations on antibody responses was higher than in the saline/*M. pneumoniae* injected animals.

4.5. T-cell activation

Cytokine production (IL-2, IFN-γ) and gp39 expression were demonstrated by immunohistochemistry (Table 3). IL-2-PC and IFN-γ-PC cells were observed in PLN of animals injected with FA/SP215 (Fig. 3(a)) or Specol/SP215 emulsions or with SP215–iscom conjugate. $Gp39^+$ cells were demonstrated in PLN of all animals injected with different adjuvant/SP215 preparations. No gp39 expression or cytokine production were demonstrated in PLN of saline/SP215 injected animals.

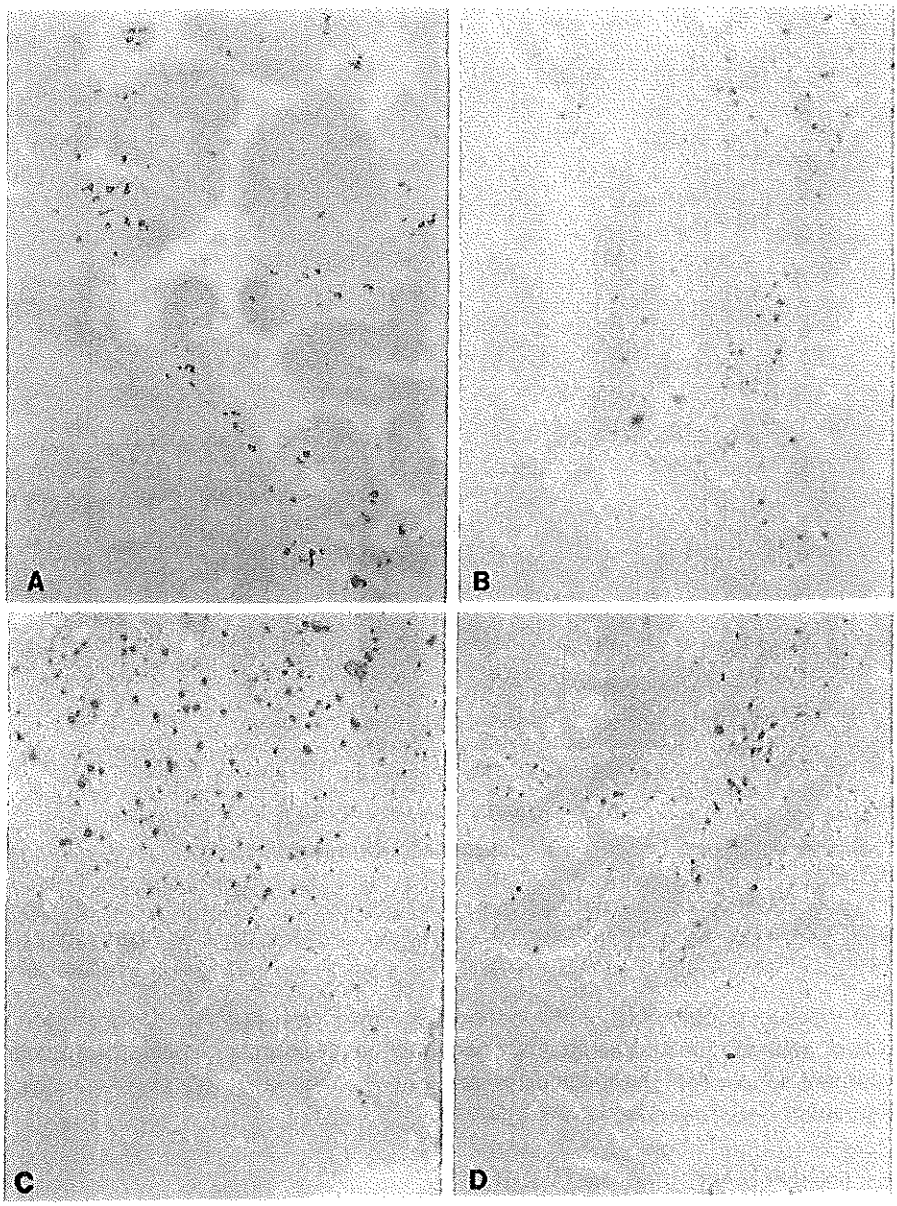

Fig. 3. Immunohistochemical visualisation of IL-2 producing cells and gp39$^+$ cells in the PLN after secondary immunisation with antigen/adjuvant mixtures. Cryostat sections of murine lymphoid tissue were incubated with specific immuno-conjugates, followed by the appropriate substrates, as described in Section 3. IL-2-PC in a cryostat section of the PLN of a mouse, 1 week after secondary immunization with FIA combined with SP215 (A). gp39$^+$ cells in a cryostat sections of the PLN of a mouse, 1 week after secondary immunization with Specol/ *M. pneumoniae* (B), *Lactobacillus*/*M. pneumoniae* (C) or Quil A/*M. pneumoniae* (D).

In PLN of animals injected with FA/MBP, Specol/MBP or MBP–iscom conjugate IL-2-PC, IFN-γ-PC and gp39$^+$ cells were demonstrated. No cytokine-producing cells or gp39$^+$ cells could be demonstrated in animals injected with *Lactobacillus*/MBP or saline/MBP mixtures. IL-2-PC, IFN-γ-PC and gp39$^+$ cells (Figs. 3(b)–3(d)) were observed in PLN of all animals injected with preparations containing *M. pneumoniae* as antigen.

5. Discussion

In a comparative study in mice we here show that preformed iscoms can be used as an effective adjuvant for the production of specific antibodies to self antigen and small peptides while inducing minimal side effects when injected subcutaneously. In a previous study in rabbits (Leenaars et al., 1994) we concluded that Specol might be an acceptable alternative to FCA/FIA. Based on data of this study with three antigens, we suggest that this conclusion can be extended to the mouse. An exception was self antigen MBP, where only a very small amount of anti-MBP antibodies could be detected in serum of Specol/MBP injected animals. Cytokine-producing cells and gp39$^+$ cells were demonstrated in PLN of animals d.f.p. injected with all three antigen/Specol emulsions while PLN were enlarged and many plasma cells were present (data not shown). The enlargement of the PLN can be explained by the aspecific immune-stimulating effects of Specol as described by Boersma et al. (1992). The low level of antibodies might be explained by the minimal presence of specific B-cells for self antigen (MBP). Furthermore, we were unable to induce Experimental Allergic Encephalomyelitis (EAE) in SJL/J mice with Specol/MBP as opposed to FCA/MBP (data not shown), indicating yet again that T-cells were not antigen specifically activated by Specol/MBP. High antibody responses found after immunisation with Specol/SP215 confirm the data of Boersma et al. (1989) who found 25 μg of SP29 emulsified in Specol to be sufficient in mice to elicit an anti-peptide response. Specol can be a possible alternative to FCA; however, more research is needed to confirm that it is an alternative to FCA for a wide range of antigens. Pathological changes after i.p. injection of Specol emulsions were similar to those observed after injection of Freund's adjuvant emulsions, but the area affected was less extensive.

Only the injection of MBP–iscom conjugate induced high antibody responses to MBP after s.c. and d.f.p. injection. The responses exceeded those induced by Freund's adjuvant. The secondary injection of MBP–iscom conjugate was given in Week 13 owing to the availability of the conjugated antigen. At Week 7, anti-MBP antibody levels in the MBP–iscom group were already higher than those found in the FA/MBP group which received a secondary injection at Week 6. Moreover, levels still increased in the period until the secondary immunisation (at Week 13) and had not reached a plateau at that time.

For the coupling of antigen to preformed iscoms, the amounts of antigen given in Table 1 were used per animal. However, possibly not all antigen was coupled to preformed iscoms and therefore the amount of antigen injected in combination with preformed iscoms may be lower than given in Table 1. It has been shown that when antigen is coupled to preformed iscoms immunisation can be performed at reduced antigen concentrations (Morein et al., 1987). Coupling of haptens and peptides to preformed iscoms is a laborious process and

not all types of antigen can be coupled. These are disadvantages if preformed iscoms are to be used as a ready-to-use product that serves as alternative to FCA.

Besides the adjuvant, type of antigen and route of injection influenced the severity of the lesions found after immunisation with the adjuvant/antigen preparations. When different antigens were compared most severe lesions were found after injection of *M. pneumoniae* preparations. The nature of the antigen injected may be involved in the severity of induced lesions. When different injection routes were compared, i.p. injection resulted in relatively severe lesions (including intestinal adhesions, (peri) pancreatitis, abscesses) compared with s.c. injection and in some groups animals died after secondary i.p. immunisation. Within 1 h after secondary i.p. injection of FIA/SP215 emulsions, animals most probably died as a result of anaphylactic shock as suggested by time course and observed circulatory disturbances (peripheral cyanosis and abdominal congestion). After the secondary i.p. injection of SP215–iscom conjugate, two animals died after 2–3 days (not suitable for microscopy). In one survivor arteritis was observed which suggests the possibility of a type III hypersensitivity reaction. Secondary i.p. injection of MBP–iscom conjugate resulted in death of the whole group within 48 h. No evidence of acute immune complex disease was found (i.e. no endothelial proliferation or polymorphonuclear infiltration in vessels or glomeruli) in these five mice. The presence of free Quil A in iscom preparations is not considered the cause of death. Quil A is known to have intrinsic haemolytic activity in vitro. When Quil A is incorporated in iscom the haemolytic activity is ten times lower than observed with free Quil A (Kersten, 1990). Macroscopically no signs of haemolysis were found and microscopically no haemoglobin was present in renal tubular epithelium. Morein et al. (1987) and Speijers et al. (1988) observed minimal pathological effects after injection of iscoms. Speijers et al. (1988) injected 0.25 ml measles virus iscoms containing 90 μg Quil A intramuscularly in rats and observed minimal local and no haematological effects. Other immunopathologic events may have caused the death of these animals since these animals died within 48 h after secondary injection while circulatory disturbances were found as may be observed in shock state. Besides these immunopathologic events after i.p. injection, minimal pathological lesions were observed after injection of preformed iscoms.

At necropsy severe pathological changes were found after injection of FA. Despite the severe lesions we could not demonstrate signs of prolonged pain or distress based on clinical observations, weight gain or several behavioural studies. This is in correspondence with results of Toth et al. (1989) who did not find prolonged signs of pain in mice after i.p. injection of 0.5 ml FA/saline (1:1). Van den Broek et al. (1994) however, suggest that i.p. injection of 0.1 ml FCA may lead to severe distress. The reasons for the fact that we could not demonstrate prolonged pain or distress while severe lesions were found could be that these lesions do not induce pain and distress in mice or the methods we used were not sufficient to detect prolonged animal distress. In order to control possible pain and distress, the induction of severe pathological changes need to be kept to a minimum. Amyx (1987) suggested that most of the undesirable side effects of the use of Freund's complete adjuvant can be eliminated by careful control of injection quantity and site selection.

In conclusion, when alternative adjuvants are compared, the effect of the nature of antigen on immune responses and side effects, should be taken into account. We suggest that Specol is a possible alternative to FCA for the production of specific antibodies to small peptide and particulate antigen. We demonstrated that s.c. injection of preformed iscoms can be

used for the production of specific antibodies to self antigen or small peptide and cause minimal side effects.

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I.4 Aims of the present thesis

The aim of this thesis is to generate data that could help in the rational design of novel generations of safe and effective subunit vaccines. Therefore, the studies described in this thesis address the evaluation of strategies that could be helpful in developing viral and bacterial candidate vaccines using the iscom and OMV antigen presentation systems.

Rabies virus (RV) infection of mice was chosen as a viral model system in which the potential of the iscom presentation system could be evaluated. To this end a non adjuvated rabies vaccine was used as a basis to generate RV iscoms. The vaccine itself served as a control preparation. One basic question to be addressed with this system would be whether differences exist in the routing and persistence of the respective antigen preparations at the level of tissues and cell types involved in antigen presentation and immune responses. Since macrophages are known to play a major role in the presentation of particle antigens, it was considered important to specifically study the role of macrophages and other phagocytosing cells in this context. Methods, involving clodronate containing liposomes, were available to eliminate these cells *in vivo,* which would allow studies concerning the differences in immune responses between depleted and control animals.

It is known that in protection against certain viral diseases CTL play an important role. Given the published data which have shown that unlike most inactivated antigens inactivated rabies vaccines may induce CTL responses, we were also interested to see whether RV-iscom could induce a functional CTL response more efficiently. It was considered that the availability syngeneic target cells persistently infected with RV in a mouse model, would allow studies to compare the different antigen preparations in this respect.

Since cytokines play an important role in the induction of immune responses and at least some of the effector mechanisms of adjuvants are dependent on the release of certain cytokines, we designed experiments in which the response against RV could be modulated when rabies vaccine was directly adjuvated by the addition of different cytokines that are known to be involved in immuno-modulation.

Neisseria meningitidis infection of mice was chosen as a bacterial model system in which the potential of iscom and OMV, being two different antigen presentation systems with built- in adjuvant activity, could be evaluated and compared. To this end it was decided to test different candidate vaccine formulations containing meningococcal class 1 OMP -purified OMP, OMV and iscom- with regard to their ability to generate an anamnestic and bactericidal antibody response. The responses were characterized with regard to IgG subclasses and involved epitopes.

With the data generated in both systems the potential and limitations of the respective antigen presentation systems for the development of future candidate subunit vaccines against viral and bacterial infections, are discussed in the final chapter of this thesis.

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II. Iscom and other adjuvants in viral system: rabies as a model

Chapter II.1

Antigen detection *in vivo* after immunization with different presentation forms of rabies virus antigen:Involvement of marginal metallophilic macrophages in the uptake of immune stimulating complexes.

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Antigen detection *in vivo* after immunization with different presentation forms of rabies virus antigen: involvement of marginal metallophilic macrophages in the uptake of immune-stimulating complexes

Several mechanisms have been postulated to explain the relatively high immunogenicity of antigens presented in immune-stimulating complexes (iscom). Their potency can in part be explained by the specific targeting of these structures to cells presenting antigens to the immune system. However, until now no method for the subcellular detection of iscom *in situ* was available. In the present study, a novel, fast and simple method for the detection of iscoms *in situ* is demonstrated. By making use of the lipophilic fluorescent carbocyanine dyes, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO), rabies virus antigen and iscom prepared with this antigen were visualized with fluorescence microscopy. The labeled antigen and iscoms were observed in macrophages of spleen and liver of mice within 1–2 h after intravenous administration. When administered intramuscularly or in the footpad, uptake in macrophages of draining lymph nodes could be demonstrated. In the spleen, labeled inactivated virus antigen localized preferentially in the marginal zone macrophages and to a lesser extent in the red pulp macrophages. In contrast, antigen presented in iscom was taken up mainly by the marginal metallophilic macrophages and to a much lesser extend by marginal zone macrophages or follicular-dendritic and -B cells. This method enables the detection of iscom and membrane viruses and allows the analysis of their relation to antigen-presenting cells *in situ*. Here, we demonstrate that iscom containing rabies virus antigen are taken up by a subset of macrophages in the spleen distinct from those that take up inactivated rabies virus antigen not presented in iscom, thereby possibly explaining the observed difference in immunogenicity of these antigen preparations. Furthermore, we show a lower efficiency on the induction of humoral and cellular responses after intravenous immunization for both types of antigen when compared with subcutaneous immunization.

1 Introduction

Since immune-stimulating complexes (iscom) were initially described by Morein et al. [1], numerous studies have demonstrated their potential as a vaccine with built-in adjuvant properties. Bacterial, parasitic, viral and other proteins and peptides have been incorporated into iscom to enhance the immune response against these antigens [2]. Immunization with iscom generally induces both humoral and cell-mediated immune responses [3]. The antibody responses against antigens incorporated in iscom are generally higher and longer-lasting than those induced by the same amount of antigen not incorporated in this matrix. Furthermore, unlike most inactivated antigens, iscom can induce an MHC class-I-restricted cytotoxic T cell response specific for the immunizing antigen [4–6]. Little is known about the early events after immunization with iscom, *e.g.* which antigen-presenting cells are involved or in which anatomical location (organ or compartment) the response is elicited. Watson et al. [7] described the inflammatory response and organ distribution of antigen after intraperitoneal immunization with influenza virus glycoprotein in iscom or micelles using radioactively labeled antigens, and showed that the spleen is important in the uptake and retention of iscom. However, no detailed information on the involvement of splenic antigen-presenting cells (*e.g.* macrophages, dendritic cells, B cells) in the uptake, processing and presentation of iscom or inactivated virus is available. Such knowledge would allow the study of distinct subsets of antigen-presenting cells in the differential immunogenicity of viral antigens presented in different ways. Here, we present a simple post-formation method for labeling virus and iscom by means of lipophilic fluorescent dyes. This method allows the study of virus and iscom distribution in the lymphoid organs at the cellular level.

[I 13939]

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Abbreviations: **DiI:** 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate **DiO:** 3,3'-Dioctadecyloxacarbocyanine perchlorate **iscom:** Immune-stimulating complex **MZM:** Marginal zone macrophages **MMM:** Marginal metallophilic macrophages **NSE:** Nonspecific esterase **RPM:** Red pulp macrophages **RV:** Rabies virus **RV-BPL:** β-Propiolacton-inactivated rabies virus

Key words: Immune-stimulating complex / Marginal zone / Macrophages / Fluorescence

2 Materials and methods

2.1 Animals and chemicals

Female BALB/c mice aged 12–16 weeks, were kept in Macrolon cages under an 11-h dark/13-h light regimen at 20 °C and were given acidified water (pH 3) and pelleted mouse food (Hope Farms, Woerden, The Netherlands) *ad libitum*. DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; D-282), and DiO (3,3'-dioctadecyloxacarbocyanine perchlorate; D-275) were obtained from Molecular Probes (Eugene, OR). Monoclonal antibodies ERTR-9, specific for marginal zone macrophages, and MOMA-1, specific for marginal metallophillic macrophages, were a kind gift of Prof. Dr. G. Kraal, Free University, Amsterdam, The Netherlands.

2.2 Antigens

Rabies virus (RV-Pasteur strain) was propagated in Vero cell monolayer cultures [8]. Culture supernatant was cleared by filtration and concentrated by Amicon ultrafiltration (cut-off 10^6 Dalton). Virus was inactivated with β-propiolactone (BPL) and stored at −70 °C at a concentration of 900 µg/ml. This rabies virus antigen was used for the preparation of RV-iscom as described [9]. Briefly, RV proteins were solubilized with 10 % MEGA-10 (decanoyl-N-methylglucamide) for 2 h at room temperature. The solubilized virus was layered on top of a 10 % sucrose cushion and centrifuged for 2 h at 200 000 × g. Quil-A (Iscotec AB, Luleå, Sweden), cholesterol and phosphatidylcholine (Sigma, St. Louis, MO) were added to the supernatant and the mixture dialyzed overnight against PBS. Free Quil-A and unincorporated proteins were removed by centrifugation over a 20 % sucrose cushion for 48 h at 150 000 × g. Incorporation of RV proteins was analyzed by SDS-PAGE and a glycoprotein-specific ELISA [10].

2.3 Immunological responses and protection

Immunological responses were measured 4 weeks after i.v. or s.c. immunization. RV-specific neutralizing antibody responses were measured by rapid fluorescence focus inhibition test (RFFIT) and T cell responses were measured as described [11, 12]. Briefly, spleen cells were isolated and stimulated with different concentrations of RV-BPL. After 5 days, cells were pulsed with [^{3}H] thymidine for 18 h. Cells were harvested on glass filters and incorporated radioactivity was measured in a Betaplate scintillation counter (LKB, Uppsala, Sweden). For protection studies, BALB/c mice were challenged intracerebrally with 30 LD_{50} rabies challenge virus standard CVS-26 according to standard NIH potency testing of vaccines [13].

2.4 Fluorochrome labeling of RV antigen and RV-iscom

Stock solutions of DiI and DiO were made in pure ethanol or DMSO (2.5 mg/ml). RV-BPL (100 µg), RV-iscom or empty iscom in 1 ml PBS were vigourously mixed with 8 µl (20 µg) DiI stock solution and incubated for 1 h at 37 °C. Any unincorporated DiI formed crystals during this incubation period. After labeling, the samples were filtered using 0.45-µm filters to separate crystalline DiI and labeled antigens [14]. Labeled material was stored in the dark at 4 °C for up to 4 weeks.

Total amounts of carbocyanine label incorporated in virus and iscoms were determined with a Beckman DU-40 UV/Vis spectrophotometer. Wavelength scans were made from 380 to 600 nm with peaks at 552 nm for DiI and 492 nm for DiO. Association of carbocyanine dye with the iscom structure was demonstrated by purification of DiO-labeled iscom by density centrifugation on a 10–60 % sucrose gradient for 18 h at 40 000 × g [15].

2.5 Fluorescence microscopy

Frozen 8-µm sections of organs were examined and photographed directly after cryo-sectioning with an Olympus (Tokyo) VANOX fluorescence microscope. DiI fluorescence was observed both as red with green light (excitation filter BP 545) and rhodamine/TRITC optics (emission: EO 570) and as golden-yellow with blue light (BP 490 + EY 455) and FITC optics (EO 515). DiO fluorescence was observed only with FITC optics as green (excitation: BP 490 + EY 455, emission: EO 515).

2.6 Enzyme- and immunocytochemistry

Organ sections used for localization studies based on fluorescence microscopy were air-dried and fixed in 1 % paraformaldehyde at 37 °C for 15 min for simultaneous fluorescence and immunochemical detection (acetone dissolves lipid membranes, causing a significant decrease in fluorescence of labeled iscom/virus). Sections for enzyme staining (nonspecific esterase; NSE) were fixed in acetone after fluorescence observation and photomicrography. Monoclonal antibody staining on fixed sections for marginal zone metallophilic macrophages (MOMA-1) was performed as described [16]. Endogenous acid phosphatase staining for the identification of all splenic and liver phagocytes and NSE staining for the detection of marginal metallophilic macrophages (MMM) was performed as described [16]. Localization of the respective subsets of splenic macrophages has also been described [17].

3 Results

3.1 Incorporation of dyes in virus and iscom

The efficiency of the labeling of viral antigen and iscom is shown in Fig. 1. At the optimal dye concentration used (200 µg dye/mg iscom), up to 80 % was integrated in the RV-iscom. Under the same conditions, RV-BPL took up approximately 50 % of the dye. The carbocyanine dye associates specifically with the iscom structure, as shown by sucrose gradient density centrifugation of DiO-labeled iscom and in a recent report dealing exclusively with mucosal localization of iscom [15]. To determine whether the incorporation of these dyes influences the structure and immunological properties of iscom or viral antigens, labeled antigens were analyzed morphologically by electron microscopy as well as for their immunogenicity in mice.

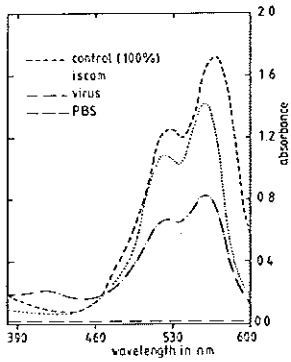

Figure 1. Incorporation of carbocyanine DiI in virus and iscom as determined by spectrophotometric measurements (wavelength scans from 390–600 nm) after 2 h incubation (see Sect. 2.4). Control 100% contains the same amount of label as was added to the antigen preparations without filtration. Iscom, RV-BPL and PBS were all filtered to remove dye which was not take up in the lipid phase after incubation.

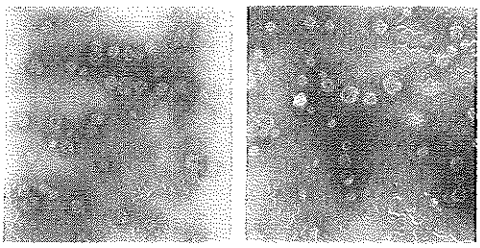

Figure 2. Electron micrographs (× 106000) of unlabeled (left panel) and DiI-labeled (right panel) iscom matrix after 0.2-μm filtration: note no differences in typical cage-like iscom structure.

Fig. 2 shows representative electron micrographs of iscom matrix before (left panel) and after (right panel) DiI incorporation. No changes in the structure were observed after DiI uptake, and all samples were devoid of any particulate material other than the iscom. Results obtained with RV-BPL (data not shown) were essentially the same as for RV-iscom. Labeled virus and iscoms were shown to retain their normal immunogenic properties (data not shown) as the same results were obtained as presented in Fig. 7 for non-labeled material.

3.2 Localization of antigen and iscom in the spleen after i.p. or i.v. injection

After i.p. and i.v. injection, both antigens could be demonstrated in the Kupffer cells of the liver within 15 min, shown, for iscom only, in Fig. 3a. No differences in liver localization pattern were observed between RV-BPL or RV-iscom.

When DiI-labeled antigens were injected i.p. or i.v., uptake in splenic macrophages could be observed as early as 20 min after administration. Labeled RV-BPL preferentially localized in marginal zone macrophages (MZM) (Fig. 3b). When large amounts of RV-BPL were administered, antigen was also taken up by red pulp macrophages (RPM). Viral antigen was detected in roughly equal concentration ratios in MZM and RPM after 1–7 days irrespective of the dosage given.

Labeled iscom were taken up by MZM as shown for other antigens. However, iscoms preferentially localized within MMM. As shown in Fig. 4, a section showing the uptake of DiI-labeled iscoms in the spleen was photographed (Fig. 4a) and subsequently fixed in acetone. Following fixation, the section was incubated with a substrate for NSE, an MMM endogenous enzyme marker (Fig. 4b). It is clear from these pictures that similar patterns are obtained for DiI-labeled iscoms and NSE activity. Although iscoms were not found exclusively in MMM, but also in MZM, almost all NSE-containing macrophages can be seen to have ingested iscoms, whereas RV-BPL could not be observed in these cells.

The involvement of MMM in the uptake of iscom was also demonstrated by a double-fluorescence study. First, DiI-labeled iscom were injected in mice, and after 2 h the spleen was removed, frozen, cryo-sectioned and paraformaldehyde-fixed. After fixation, sections were incubated with MOMA-1 antibody and goat anti-rat conjugated to FITC. Using FITC optics, DiI iscoms were observed as golden yellow and MOMA-1-staining cells as green. FITC did not fluoresce when using green light for excitation, showing iscom in red. Most MOMA-1 – positive cells were positive for DiI fluorescence, albeit with differences in intensity, and hence for iscom uptake (data not shown).

Follicular localization (either on follicular-dendritic or -B cells) of iscom was also observed [green dots in F (follicle) Fig. 5] in a similar fashion as that shown for TNP-Ficoll [18] and for liposomes [19].

3.3 Double-fluorescence labeling studies

To confirm the differences in localization pattern of RV-BPL and RV-iscom, the following experiment was performed. RV-BPL was labeled with DiI and RV iscom with DiO. When observed using FITC optics, these labels fluoresce golden yellow for RV-BPL and green yellow for iscom, respectively. Mixtures of these antigens were injected intravenously, and after 2 h, the spleens were removed. Observation of sections revealed that RV-BPL and iscom co-localized in MZM, but a small ring of cells on the border between marginal zone and periarteriolar lymphocyte sheath (PALS) contained only iscom (green yellow) and no labeled virus material (Fig. 5).

3.4 Localization after intramuscular or subcutaneous injection

When RV-BPL or RV-iscom were injected s.c. or i.m., both antigens were rapidly found in the draining lymph nodes.

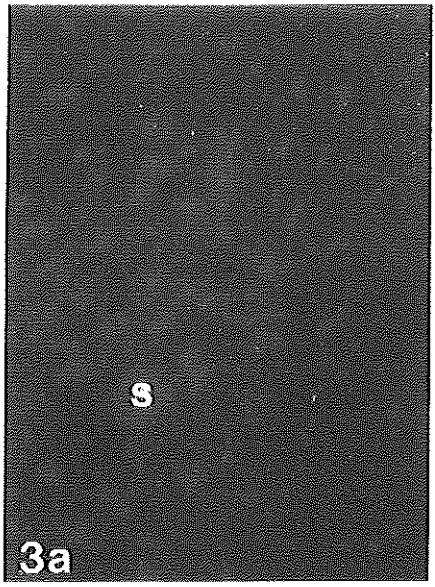

Figure 3. (a) Fluorescence microscopic image of an 8-μm liver cryo-section showing DiI-iscom localization in Kuppfer cells 2 h after intraperitoneal injection. S = sinus. (b) Fluorescence microscopic image of an 8-μm spleen cryo-section showing localization of RV in a typical ring of MZM, 2 h after i.v. injection.

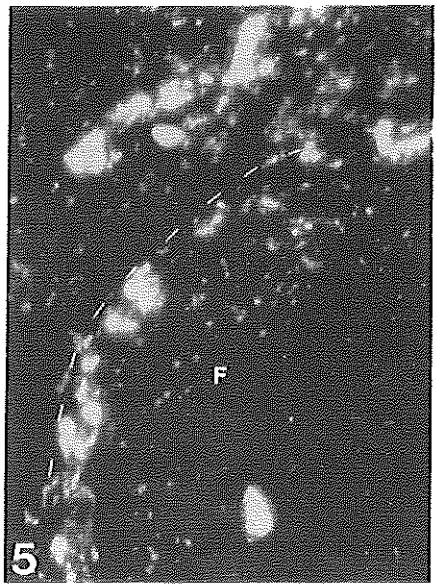

Figure 5. This shows the different localization of DiI-labeled rabies virus (golden yellow) in MZM and DiO-labeled iscoms (green yellow) after i.v. injection. Two follicles (F) are segregated by the marginal zone. MMM are located on the border. The dashed line indicates the border between MMM and MZM. Also, follicular localization of the DiO-labeled iscoms (green spots) in the follicle can be seen.

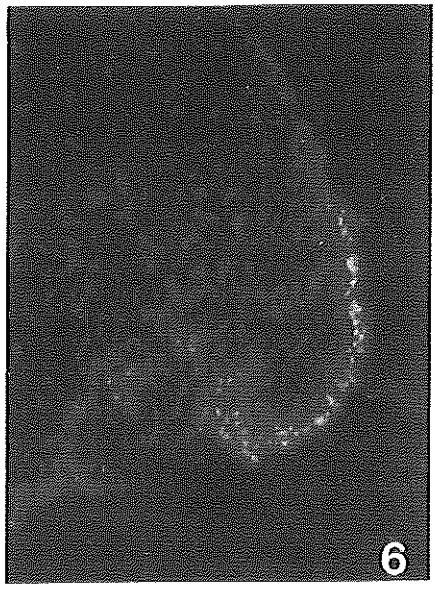

Figure 6. Fluorescence microscopic image of an 8-μm cryo-section showing DiI-iscom localization in the popliteal lymph node 2 h after s.c. injection in the footpad.

Intramuscular injection in the hind leg resulted in the uptake of both antigens in popliteal and inguinal lymph nodes. Footpad-injected antigens were found in the popliteal lymph node only. In lymph nodes, labeled antigens localized initially in the subcapsular macrophages, and after a few hours in the medullary macrophages. Localization is shown for s.c.-injected iscom only in Fig. 6. No differences between RV-BPL or RV-iscom localization patterns or intensity were observed.

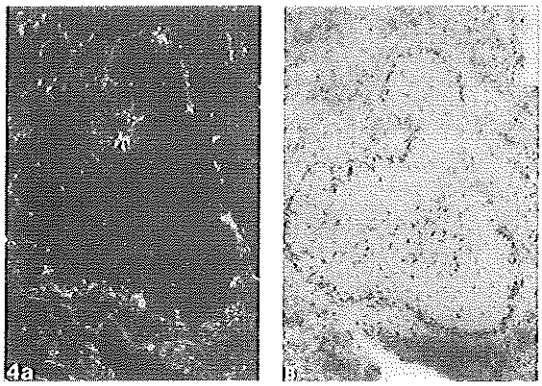

Figure 4. (a) Fluorescence microscopic image of an 8-µm spleen cryo-section showing DiI-iscom localization 2 h after i.v. injection. (b) Shows the same section after acetone fixation and incubation with NSE substrate, specifically enzymatically staining the MMM.

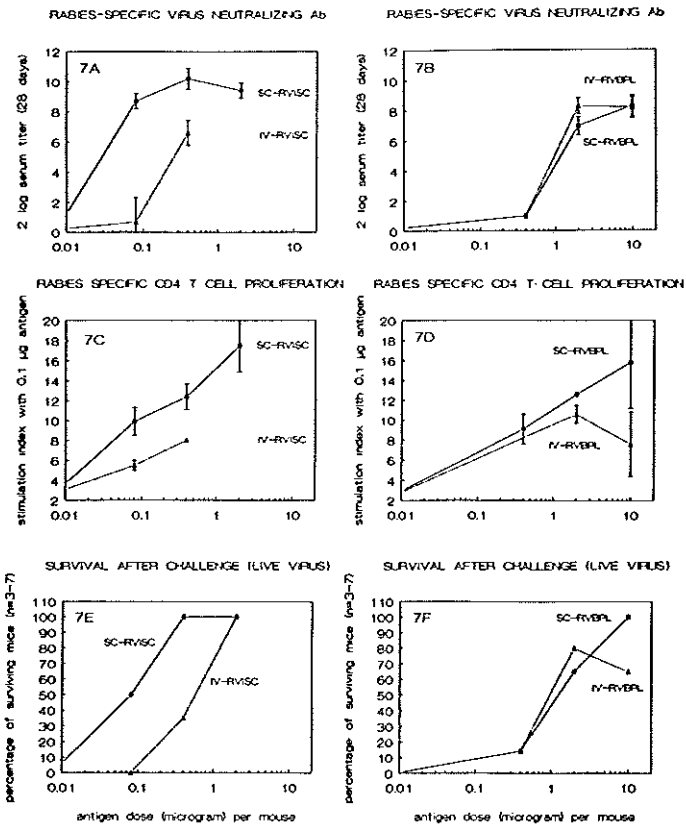

Figure 7. (A) and (B) show the serum titer of RV-specific neutralizing antibodies 28 days after a single immunization with different antigens. (C) and (D) show RV-specific T cell responses of immune spleen cells when stimulated with 0.1 µg inactivated rabies virus *in vitro*. (E) and (F) show the percentage of survival following challenge with 30 LD_{50} live CVS-21 after a single immunization with different antigens. Virus neutralizing titers and T cell stimulation indices are mean values for at least three animals in a representative experiment. Survival data after challenge were determined in separate experiments. SC-RVBPL: BPL-inactivated RV given subcutaneously, SC-RVISC: RV iscom given s.c., SHAM: no immunization, IV-RVBPL: BPL-inactivated RV given i.v., IV-RVISC: RV iscom given i.v.

3.5 Immunological responses after immunization with RV-BPL and RV-iscom

In view of the observed differences in localization patterns of RV-BPL and RV-iscom after i.v. and i.p. injection, mice were immunized to study the induction of B and T cell responses and protection against challenge with virulent RV. The results, of which a representative experiment is shown in Fig. 7, showed that in mice, RV-iscom are about 20–30-fold more potent than RV-BPL when injected subcutanously. RV-iscom and RV-BPL both induced good virus-neutralizing antibodies via both routes (Fig. 7A,B). Via the s.c. route, 0.08 μg RV-iscom induced RV-neutralizing antibody titers as high as those found with 10 μg RV-BPL, demonstrating the higher immunogenicity of the RV-iscom. When administered i.v., comparable amounts of RV-iscom induced significantly lower titers. T cell proliferation *in vitro* after stimulation with RV-BPL was used as a measure of T cell priming *in vivo*. Fig. 7C,D show that RV-iscom prime T cells more efficiently and at lower doses than RV-BPL. Immunization doses as low as 0.08 μg RV-iscom still elicited T cell responses *in vitro*, and these data corresponded with the induction of neutralizing antibody titers.

After intracerebral challenge with 30 LD_{50} CVS-26, all sham-treated animals ($n = 7$) died (data not shown). Full protection was obtained with iscom with a dose as low as 0.4 μg ($n = 6$) when given via the subcutaneous route (Fig. 7E). RV-BPL was fully protective at a dose of 10 μg s.c. (Fig. 7F). When given intravenously, RV iscom were two- to fivefold less effective, but full protection could still be obtained with 2 μg RV-iscom i.v. ($n = 3$). Taken together, these data confirm the stronger immunogenicity of proteins incorporated into iscom, and also show a lower efficiency of the i.v. route of immunization over the s.c. route, especially for RV-iscom.

4 Discussion

RV-iscom, in contrast to inactivated RV antigen, are preferentially taken up by MMM of the murine spleen after i.v. or i.p. administration. This is an interesting finding since, until now, no involvement of this particular subset of macrophages in antigen uptake has been demonstrated. RV-iscom were more immunogenic than RV-BPL in terms of induction of virus-neutralizing antibodies, T cell responses, and protection against disease. The subcutaneous immunization route, which primarily activates the draining lymph nodes, proved more efficient than immunization via the i.v. route. Furthermore, it was shown that carbocyanine dyes can be used effectively to label membrane viruses and iscom, permitting localization pattern studies of these antigens *in situ*.

DiI and DiO were taken up within a few seconds in viral membranes and iscom. The iscom matrix is highly hydrophobic and membrane proteins are easily incorporated during its formation. Due to this property and the lipophilic character of DiI and DiO, these dyes are easily incorporated in the iscom structure. It should be stressed that these carbocyanine dyes are taken up exclusively in lipophilic structures, *i.e.* membranes, and, therefore, do not bind to or interfere with function or immunogenicity of surface proteins [14]. The use of antigens labeled in this way has enabled us to obtain data on the early processes of antigen handling *in vivo*.

Recently, this method was used to study the kinetics of liposome uptake in which MZM and RPM play an important role [14]. In this study, it was surprising to find that there are indeed differences in the cell populations which are involved in uptake of the antigens when they enter the spleen. MZM are known to play an important role in the uptake of particulate antigens (*e.g.* liposomes, bacteria) since they are among the first cells encountered upon entry in the spleen.

Labeled RV and RV-iscom could be found in MZM within 2 h after i.v. or i.p. injection, as would be expected. However, only iscom were found in MMM, a cell population which is located at the border of the marginal zone, characterized by slender processes protruding into the outer PALS, which is the main site of antibody production in the spleen. The role of these cells in the handling of antigens is unclear [20] but their involvement in the processing of antigens has been suggested by Kraal et al. [16].

It should be noted that studies recently showed that selective uptake and retention of antigens by the MZM *in vivo* functions as a way of rapid antigen removal and elimination, thereby obscuring it from the immune system and hampering a normal response [21]. This may explain the relative inefficiency of RV-BPL taken up by MZM to induce immune responses at low doses, in contrast to results found with iscom, which are taken up by MMM. Similarly, the fact that significant amounts of both antigens given by the i.v. or i.p. route are taken up by the MZM could also explain the relative inefficiency of this route.

The importance of the microenvironment for antigen processing and presentation *in vivo* has often been stressed [20, 22]. From numerous studies, it is clear that extensive traffic and intimate contact of the cells in the spleen is necessary for the generation of the immune response. MZM degrade the entering antigens which are then transferred to secondary cells. It is not clear whether MMM behave differently from MZM in this respect. The strategic localization of MMM at the border of the marginal zone and close to the PALS (Fig. 4b) makes them ideal candidates for the transfer of antigens to antigen presenting B cells or perhaps interdigitating dendritic cells in the inner PALS. However, it is not clear which factor determines the specific uptake of iscom by MMM while other antigens, *i.e.* proteins, erythrocytes, bacteria or RV-BPL, are not found in this cell population. RV-iscom and empty iscom matrix localized in the same way. This finding demonstrates that the presence or absence of proteins in the iscom matrix does not influence localization behavior or the involvement of a particular subset of macrophages. Since low amounts of liposomes do not preferentially localize in MMM, a direct influence of cholesterol in this phenomenon can also be excluded, leaving Quil-A or the uncommon structure of the iscom as possible factors.

The labeled iscom we detected in the splenic follicles within hours after administration could be indicative for complement-mediated uptake [18] and a possible role for follicu-

lar-dendritic cells in antigen presentation. As reviewed by Kosco [23] follicular dendritic cells have the capacity to pass antigen to B cells which in turn are capable of processing and presenting it to T cells, leading to germinal center formation [24]. On the other hand, macrophages can also efficiently present antigen to T cells, thereby functionally implicating subsets such as the MMM until proven otherwise, as it the case for the suppressive MZM. Notwithstanding the follicular localization of the iscoms, a role for follicular-dendritic or -B cells in the presentation and processing of iscom, which is also partly major histocompatibility complex class I-dependent, remains to be proven.

Until now, only one study has described the early events in the immune response following i.p. injections of iscoms compared to micelles. In this study, it was shown that there was a significant increase in the inflammatory response and an increase in the uptake of radiolabeled antigen in the spleen following immunization with iscom [7]. Even more interesting was the observation that a larger amount of antigen in iscom is retained in the spleen when compared to antigens in micelles. In the present study, fluorescence in spleen and lymph nodes could still be demonstrated after 1 week, but not differences were observed between RV-BPL or iscom.

In conclusion, we have shown that macrophages from the marginal zone, and in particular the MMM, are involved in uptake and, therefore, probably also in processing and presentation of iscom-associated antigens. No evidence was found for direct interactions between iscom and T cells (iscom were not found in the main T cell areas). However, the follicular localization of iscom indicates direct activation of B cells, a not-unlikely mechanism in view of the fact that no differential macrophage localization was observed in the lymph nodes after s.c. or i.m. immunizations. Further studies, involving splenic macrophage elimination [25] to establish the role of MMM and MZM in the immune response against iscoms and viral antigens, are now underway.

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Chapter II.2

Fluorescent labelling of virus, bacteriae and iscoms: *In vivo* systemic and mucosal localisation patterns.
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FLUORESCENT LABELLING OF VIRUS, BACTERIA AND ISCOMS: *IN VIVO* SYSTEMIC AND MUCOSAL LOCALISATION PATTERNS

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INTRODUCTION

For gut associated immune responses several ways of antigen uptake have been described: non-specific uptake between epithelial cells common non-specific uptake via epithelial cells: specific uptake via epithelial cells and specialised uptake via M cells, the latter of which seems to be most efficient.[1] Immunity against a variety of organisms which infect the body via mucosal surfaces is probably most effective when antibodies and immunocompetent cells are present at the site of infection. Mucosal antibody production, especially secretory IgA, is generally induced very poorly after intramuscular or subcutaneous immunization. Oral administration of antigens can be used to target the specialized lymphoid organs associated with the gut termed Peyer's patches, which are embedded in the small intestine. This immunization route works well with live (recombinant) vaccines in both viral and bacterial systems. With non replicative vaccines, however, a major disadvantage is the degradation of the antigens in the stomach and in the gut.

Due to degradation, extremely large doses of the immunizing antigen are required to induce satisfactory immunity. The use of slow release particles (Challacombe *et al.*, these proceedings) or immune stimulating complexes (iscoms) for the presentation of antigens may circumvent this problem. Iscoms are cage-like antigen carriers (30-40 nm) with built-in adjuvant (Quil-A) activity (2, for review see 3). Iscoms containing antigens of different origin were shown to be immunogenic by parenteral and local immunization routes. Recently the use of iscoms for local mucosal immunization was also investigated. Intranasal immunization with influenza virus iscoms leads to a specific antibody response of all immunoglobulin classes and subclasses, including IgA, and also to cellular immune responses in the lung.[4] This showed that local mucosal immunization with iscoms may lead to the induction of both local and systemic immune responses. Ovalbumin (OVA) containing iscoms were shown to be immunogenic after systemic but also after oral immunization.[5] Oral immunization with OVA iscoms induced specific serum antibodies. Cellular responses were measured by DTH. The required dose for oral immunization was ten times higher (100 µg) than that needed for parenteral immunization. This is probably due to partial degradation of the immunogen in the gastrointestinal tract before the iscoms can reach immunocompetent cells or lymphoid organs in the gut. This dose is 100 times

less than that needed to induce a response by OVA alone. In addition to this repeated feeding with OVA, iscoms induced MHC Class I restricted CTL specific for OVA.[6] In this study we describe the labelling of different types of antigens and an *in vitro* method to study the uptake and routing of different antigens in the gut.

MATERIALS AND METHODS

Labelling of Antigens

Iscoms, rabies virus antigen and *Lactobacilli* were labelled with DiO (3,3'-dioctadecyloxacarbocanine perchlorate; D275) using standard procedures[7] for one hour at 37°C. The efficiency of labelling of virus and iscoms was determined with a UV/Vis spectrophotometer. Wavelength scans were made from 380 to 600 nm with a peak at 492 nm for DiO. Association of DiO with the iscom structure was demonstrated by purification of DiO labelled iscoms by density centrifugation. Latex microspheres were 0.5 µm Covaspheres MX-FITC, Covalent Technology. Ann Arbor, MI.

In vitro Incubation of Isolated Gut Loops

BALB/c mice were euthanized and the small intestine containing the Peyer's patches was rinsed with RPMI. Small segments of the ileum containing at least one Peyer's patch were filled with 200 µl of an antigen dilution in RPMI and incubated in RPMI medium for 2 hr at 37°C. After incubation gut loops were washed with PBS, frozen in liquid nitrogen, and 8 µm sections were cut and analyzed by fluorescence microscopy.

RESULTS

DiO labelling of particulate antigens is very efficient due to the lipophilic character of carbocyanines.[8] Using our methodology with optimal doses, up to 80 % of the dye is incorporated in the lipid phase of the antigens.[9] To demonstrate stable association of the dye with the iscoms structure, labelled iscoms matrix was purified on a 10-60 % sucrose gradient. As shown in Fig. 1B, a distinct band at approximately 30 % sucrose could be seen after centrifugation. This yellow band contained virtually all labelled material (1A). Furthermore, iscoms could only be visualized in fractions 7-10 by electron microscopy (data not shown).

Attempts to demonstrate labelled antigens in lymphoid organs in the gut after oral administration were unsuccessfull. Subsequently, the method previously described by Pappo[10] to study *in vitro* uptake of antigens in isolated gut loops was adapted for use. Considerable autofluorescence in the Peyer's patch was observed (2A) after sham incubation. Incubation of gut loops with labelled *Lactobacilli* showed that within 2 hr the bacteria were taken up specifically in the M cells in the Peyer's patches. Iscoms were taken up very rapidly and efficiently in the M cells. Free rabies virus did not localize in the Peyer's patch but was demonstrated bound to the surface of epithelial cells (not shown). Uptake of FITC-labelled latex microparticles could not be demonstrated in any mucosal cell type in the gut.

DISCUSSION

Recently we introduced a new method to study *in vivo* localisation patterns of intact particulate antigens.[11] In these studies particulate antigens were labeled with DiI. In this study we extended the use of carbocyanin dyes for labelling and *in situ* detection of iscoms. Iscoms were labeled with DiO because of the significant golden yellow autofluorescence of

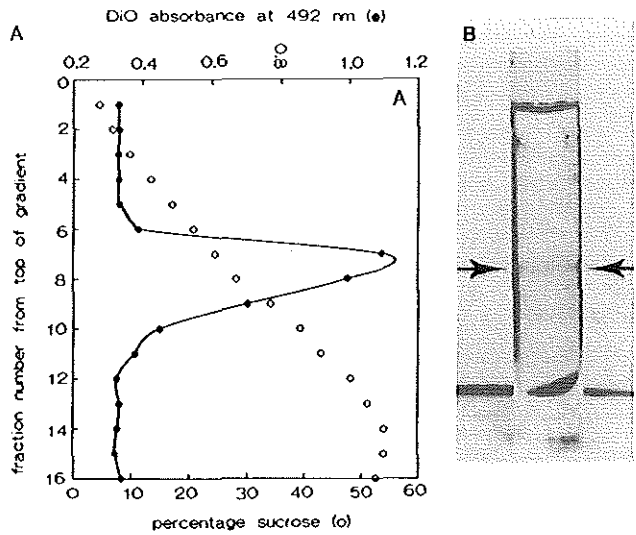

Figure 1A shows the absorbance at 492 nm and the sucrose concentration of fractions obtained from a sucrose density gradient after centrifugation. Fractions 7-10 contain iscoms. 1B shows one distinct band present after density centrifugation which contains all bound DiO.

macrophage-like cells in the Peyers patches. DiO has a bright green fluorescence (DiI = yellow) and can therefore easily be distinguished from the autofluorescent background.

In this paper we present novel data on the uptake of different types of intact particulate antigens in isolated gut loops. After labelling with DiO, antigens can be easily visualized using standard fluorescence microscopy. The present approach shows that iscoms can be labelled efficiently post formation, which is an advantageous characteristic of the method.

After parenteral administration iscoms localize distinctly from rabies virus antigen. Following i.v. administration iscoms were taken up by a distinct subset of splenic macrophages, namely the marginal metallophilic macrophages. In contrast to this, rabies virus antigen was found mainly in marginal zone and red pulp macrophages.[9] The finding that iscoms can be used for local mucosal, especially oral, immunization is important for the development of protection against pathogens which acquire access through mucosal membranes and therefore requires mucosal immunity. The importance of the mucosal immune system for vaccine development has recently been reviewed.[12] Orally administered antigens are only immunogenic if they can reach lymphoid cells or organs in the gut. The observed differences in uptake and localisation of antigens after parenteral immunization may be responsible for observed differences in immunogenicity. In view of the differential localisation pattern observed after i.v. or i.p. administration, it was decided to investigate whether a similar difference in localisation pattern between virus and iscoms was also found in the GALT.

Iscoms and *Lactobacilli* were taken up specifically by M cells in the Peyer's patch. Again rabies virus antigen localized distinctly and was mainly found in close association with epithelial cell surface and not with Peyer's patch M cells. This variation in localisation might explain the observed differences in efficacy (RV-iscoms vs RV free antigen). Using isolated gut loops we could not demonstrate any uptake of 0.5 μm latex particles in the Peyer's patches. This finding is an extension of the experiments of Eldridge[13] who showed uptake of polystyrene and polylactate particles, but not of cellulose-acetate or ethyl-cellulose

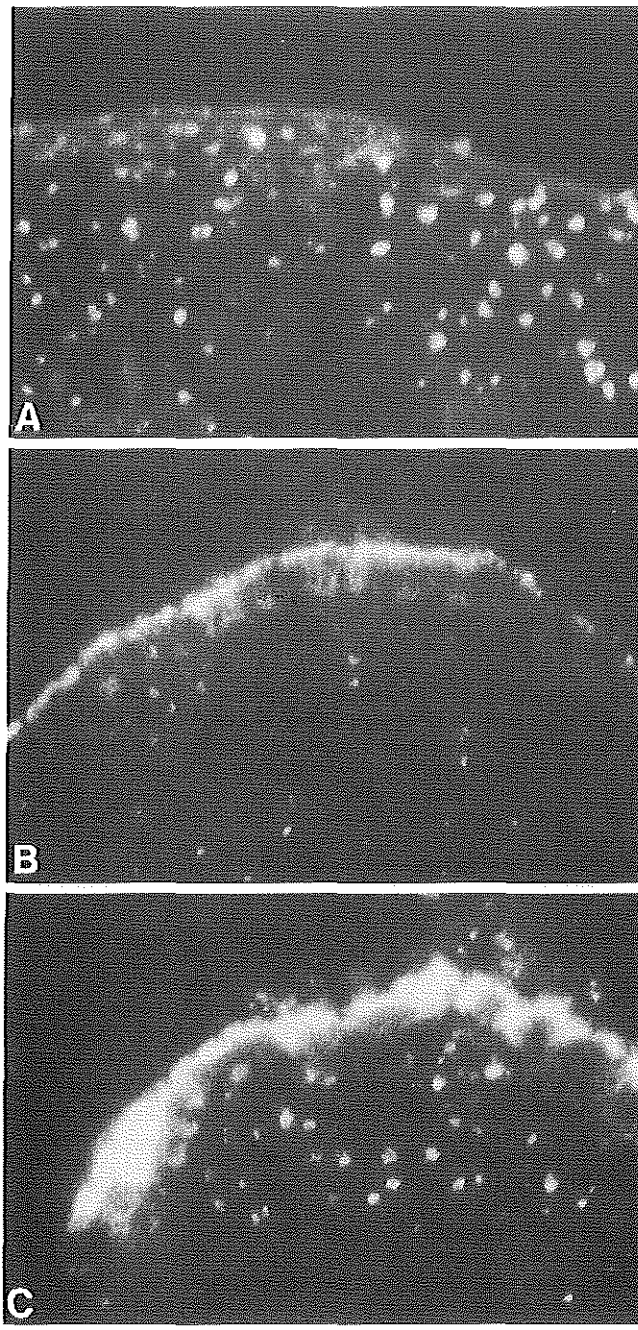

Figure 2. Fluorescence micrographs of 8 µm sections of Peyer's patches after incubation with RPMI-medium (2A), RPMI containing labeled *Lactobaccilli* (2B) and RPMI containing labeled iscoms (2C).

microparticles, after oral administration. Elridge showed that particles of small size and high hydrophobicity were well absorbed in Peyer's patches. The high uptake of iscoms in Peyer's patch may well be explained by the similar hydrophobicity of the structure. However, relatively high doses of iscoms, when compared to parenteral immunization, are required for oral immunization to induce responses, probably due to degradation. Nevertheless, this study demonstrates the potential of iscoms as antigen carriers to target antigens to specialised antigen presenting cells in lymphoid organs of the gut.

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Chapter II.3

A new method for the removal of mononuclear phagocytes from heterogeneous cell populations in vitro, using the liposome mediated macrophage 'suicide' technique
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A new method for removal of mononuclear phagocytes from heterogeneous cell populations in vitro, using the liposome-mediated macrophage 'suicide' technique

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In this study we present a new method for the elimination of mononuclear phagocytic cells from cell suspensions. By making use of liposome-encapsulated dichloromethylene diphosphonate we were able to effectively remove macrophages from spleen cell suspensions. This effect was not observed when using the free drug or control (PBS) liposomes. The use of this procedure has no effect on other cell types, as measured by growth, protein production, antigen presentation and antigen specific T cell proliferation, though PBS liposomes in very high doses were able to inhibit antigen presentation. The finding that lymphocytes are not affected by the liposome encapsulated drug suggests that the observed loss of lymphocytes in vivo, after intravenous dichloromethylene diphosphonate liposome treatment, may be due to damage inflicted by lysosomal enzymes released from dying macrophages. This method permits the removal of both macrophages and monocytes from heterogeneous cell populations (i.e., blood, lymphoid tissue suspension) in vitro with a very high rate of reliability. With the concentrations and incubation time used, no negative effects on other cell types were observed.

Key words: Mononuclear phagocyte; Liposome encapsulated dichloromethylene diphosphonate; Heterogeneous cell population; Removal of mononuclear phagocytes

Introduction

In order to selectively remove monocytes and macrophages from heterogeneous cell populations (e.g., blood, spleen suspensions) several approaches have been proposed. Macrophages can be eliminated with silica, asbestos (cf, Kagan and Hartmann, 1984) or 1-carrageenan (cf., Ishizaka et al., 1989) or they may be removed by the use of carbonyl iron (cf., Wong and Varesio, 1984). Sephadex G-10 has been proposed as a column matrix for the removal of macrophages from suspensions of immunologically reactive cells (cf., Mishell and Mishell, 1984).

Recently we developed a new method for the selective elimination of phagocytic cells in vivo, using liposome-encapsulated dichloromethylene disphosphonate (Cl_2MDP, reviewed in Van Rooijen and Claassen, 1988; Van Rooijen, 1989). Unlike free Cl_2MDP, liposome-entrapped Cl_2MDP is taken up only by phagocytes that have easy access to the liposomes (e.g., in the liver, spleen and circulation) after intravenous administration. Once taken up, the liposomal membranes are digested by phospholipases in the lysosomes and the drug is released inside the cell. Macrophages can

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be effectively killed in this way, as we demonstrated at the light-microscopical level (Van Rooijen and Van Nieuwmegen, 1984), by enzyme-histochemical methods (Claassen et al., 1987a), functional tests (Claassen et al., 1986) and at the ultrastructural level (Van Rooijen et al., 1985). In order to study the role of macrophages in vitro under the same conditions as we used in our in vivo elimination studies, an in vitro equivalent of this macrophage elimination technique was required. Moreover the cause of the observed effects of Cl_2MDP-liposomes on lymphocytes in vivo (cf., Van Rooijen et al., 1985; Claassen et al., 1986), due either to free Cl_2MDP, Cl_2MDP-liposomes or enzymes released by dying macrophages was a subject requiring further investigation.

In the present study we show that the approach used for the elimination of macrophages in vivo is also effective for heterogeneous cell populations in vitro.

Materials and methods

Experimental design

Cells, from spleen suspensions (cf., Claassen and Van Rooijen, 1985), or cell lines (UytdeHaag et al., 1987), were washed and resuspended at a concentration of 10^5 cells/ml in DMEM (Gibco) tissue culture medium. After incubation with liposomes the suspension was mixed with an equal volume of Lymphoprep (density 1,077 g/ml, Nyegaard, Oslo Norway) and centrifuged for 15 min at 1600 rpm ($400 \times g$). The supernatant with free liposomes was removed and the pellet was resuspended and washed again in lymphoprep/DMEM (1 : 1). Cells were counted and used for various assays/culture systems. Data were analyzed with the two-sample Student's t test for comparison of two empirical means in a normally distributed population (Sachs, 1984), P values are given in relation to untreated and PBS-liposome treated controls.

Preparations of liposomes

Multilamellar liposomes were prepared (sterile) as described earlier (Van Rooijen and Van Nieuwmegen, 1984). To summarize the procedure briefly, 86 mg phosphatidylcholine and 8 mg cholesterol were dissolved in chloroform in a roundbottom flask. By vacuum rotary evaporation at 37°C a thin film formed on the wall of the flask. Cl_2MDP (1.89 g dissolved in 10 ml PBS) was enclosed by gently shaking for 10 min. The suspension was kept for 2 h at room temperature and sonicated for 3 min at 20°C. After an additional 2 h at room temperature free Cl_2MDP was removed by centrifugation ($100{,}000 \times g$, 30 min). Liposomes were resuspended in 4 ml PBS. The amount of liposome-encapsulated Cl_2MDP was determined according to Claassen and Van Rooijen (1986).

Immunocytochemistry

Cytospin preparations of cell suspensions (and 8 μm cryostat sections) of spleen tissue were fixed for 10 min in acetone containing 0.02% H_2O_2 (to minimize endogenous peroxidase activity) freshly prepared.

Endogenous acid phosphatase activity of splenic macrophages was demonstrated by incubation with naphthol AS-BI phosphate and pararosaniline for 30–45 min at 37°C (Claassen et al., 1987a). Monoclonal antibody staining was performed essentially as described previously (Claassen et al., 1988). Briefly, cryostat sections and cytospin preparations were air-dried, fixed and incubated for 1 h at 4°C with the appropriate dilution of the monoclonal antibody supernatant, rinsed thrice in PBS and incubated with rabbit anti-rat Ig coupled to horseradish peroxidase (Dako, Copenhagen) and 1% normal mouse serum for 1 h at room temperature. After rinsing three times in PBS, HRP activity was demonstrated by incubation for 10 min with 3,3-diaminobenzidine (0.5 mg/ml in 0.05 M Tris-HCl, pH 7.6, containing 0.01% H_2O_2). The reaction was stopped by transferring the slides to PBS. The monoclonal antibodies used included: MOMA-2 for the detection of all splenic macrophages (Kraal et al., 1987), ERTR-9 for marginal zone macrophages (Van Vliet et al., 1985), MOMA-1 for marginal metallophilic macrophages (Kraal and Janse, 1986), Thy-1 for T lymphocytes (cf., Claassen et al., 1988), MT-4 for T-helper lymphocytes (Pierres et al., 1984), Lyt-2 for T cytotoxic/suppressor lymphocytes (Ledbetter and Herzenberg, 1979), anti-IgM for B lymphocytes (Braun and Unanue, 1980), and were a kind gift

from Dr. Georg Kraal (VUA, Amsterdam) and Dr. Willem Van Ewijk (EUR, Rotterdam). Slides (either counterstained or not with haematoxylin for 15 s) were mounted in glycerine gelatin or in malinol after dehydration.

Hybridoma growth and antibody production

A mouse monoclonal hybridoma cell line ($SH_{4.1c9}$), producing anti-TNP antibodies of IgG1 isotype, was used to study growth and antibody production. The use of these antibodies (as reference sera) and the anti-TNP ELISA has been described in detail before (Claassen et al., 1988). Cells were incubated with different doses of liposomes (Fig. 2) in DMEM medium (10^5 cells/ml). After incubation for 1 h at 37°C and 7% CO_2, cells were separated from liposomes and 1.7×10^4 cells were seeded per well (24 well plate, Costar). After 3 and 5 days supernatants were removed for ELISA and the cells harvested and counted.

Antigen presentation and proliferation by B and T cells

IL-2-dependent, MHC class-II-restricted, T cell clones and Epstein-Barr virus-transformed B cells (EBV-BC) were cultured as described earlier (UytdeHaag et al., 1987). In lymphocyte transformation assays cloned T cells (10^4/well) were cultured in the presence of autologous irradiated (3000 rads) EBV-BC (10^4/well) as antigen presenting cells and stimulating antigen (autologous immunoglobulin) in RPMI 1640 (Gibco) containing 10% (v/v) pooled human AB serum. After 4 days the cells were pulsed for 18 h with 0.5 μCi [6-^{3}H]thymidine (Amersham)/well. Cells were harvested and DNA-incorporated [^{3}H]thymidine was measured with a LKB liquid scintillation counter (1205, Betaplate).

To study the effect of Cl_2MDP, 10^5 T cells or 10^5 EBV-BC were incubated in the presence of different doses (see results section) of free or liposome encapsulated Cl_2MDP (in a final volume of 1 ml) for 2 h at 37°C. After incubation 0.5 ml Lymphoprep was added and the cells separated from the liposomes by centrifugation (15 min, $400 \times g$). The cells were then washed thrice in RPMI 1640 containing 2% (v/v) human AB serum and were tested as described above.

Results

Spleen suspensions from Cl_2MDP-treated animals

Spleen sections prepared from frozen spleens, of Cl_2MDP-liposome treated and control animals, were stained for the presence of endogenous acid phosphatase and the macrophage markers MOMA-2, MOMA-1 and ERTR-9 (see materials and methods section). Cl_2MDP-liposome treated animals (3 days after intravenous injection) contained no macrophages. In cytospin preparations of cell suspensions made from these spleens (non-frozen part) we could not find any macrophages (this study) or monocytes (Huitinga et al., manuscript in preparation). Furthermore, these suspensions contained normal numbers of T cells. However decreased amounts (from 42% ± 6% to 33% ± 8%) of B cells were found.

Cl_2MDP liposome-treated spleen cell suspensions

Elimination of acid phosphatase positive cells could be achieved with doses from 50 to 200 μl/ml suspension in a dose and time related manner (Fig. 1*a*). Disappearance of all macrophages (as detected by endogenous acid phosphatase activity) correlated fully with disappearance as determined by monoclonal antibody staining for: all macrophages (MOMA-2); (Fig. 1*b*), marginal zone macrophages (ERTR-9, not shown) and marginal metallophils (MOMA-1, not shown). Because, damage and eventually elimination could be detected 1–2 h earlier with the enzyme staining, the latter was used in subsequent experiments.

Fig. 1*a* shows complete elimination of cells containing acid phosphatase after incubation of spleen cell-suspensions with 50–200 μl/ml Cl_2MDP liposomes for 4 h at 37°C.

No differences in the number of the following cells were observed after Cl_2MDP or Cl_2MDP liposome treatment as compared to the normal (untreated) or PBS liposome-treated mice: T lymphocytes (Thy-1)(Fig. 1*b*), T helper lymphocytes (MT-4), T cytotoxic/suppressor lymphocytes (Lyt-2), B lymphocytes (anti-mouse immunoglobulin-M)(fig. 1*b*).

Effects of Cl_2MDP on growth and proliferation

Fig. 2*a* shows no significant effect ($P < 0.09$)

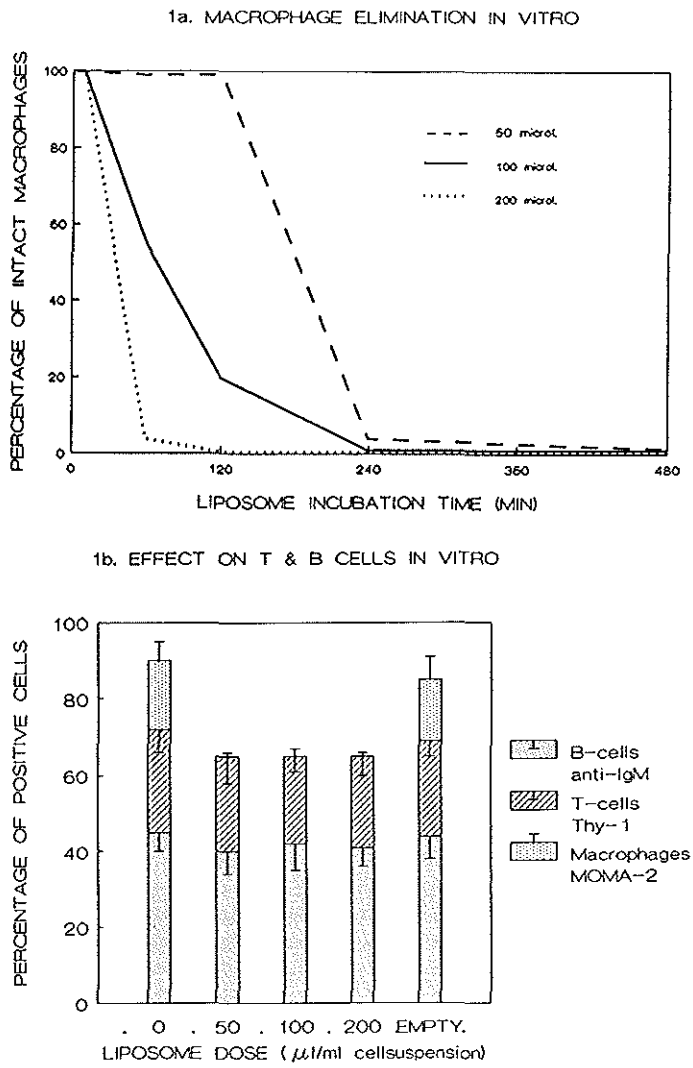

Fig. 1. Elimination of macrophages, with different doses of Cl_2MDP-liposomes, from 1 ml spleen suspensions of BALB/c mice. *a*: data expressed as a percentage of endogenous acid phosphatase positive cells relative to controls (PBS-liposomes or untreated). *b*: decrease of macrophages and no effect on T and B cells as shown by immunostaining (see materials and methods section).

of Cl_2MDP-liposomes in concentrations ranging from 10 to 200 µl/ml on the increase in number (19–33 fold) of hybridoma cells. The same was found for Cl_2MDP liposome-treated T or B cells, used in the antigen presentation assay. However, a decrease in proliferation was found upon incuba-

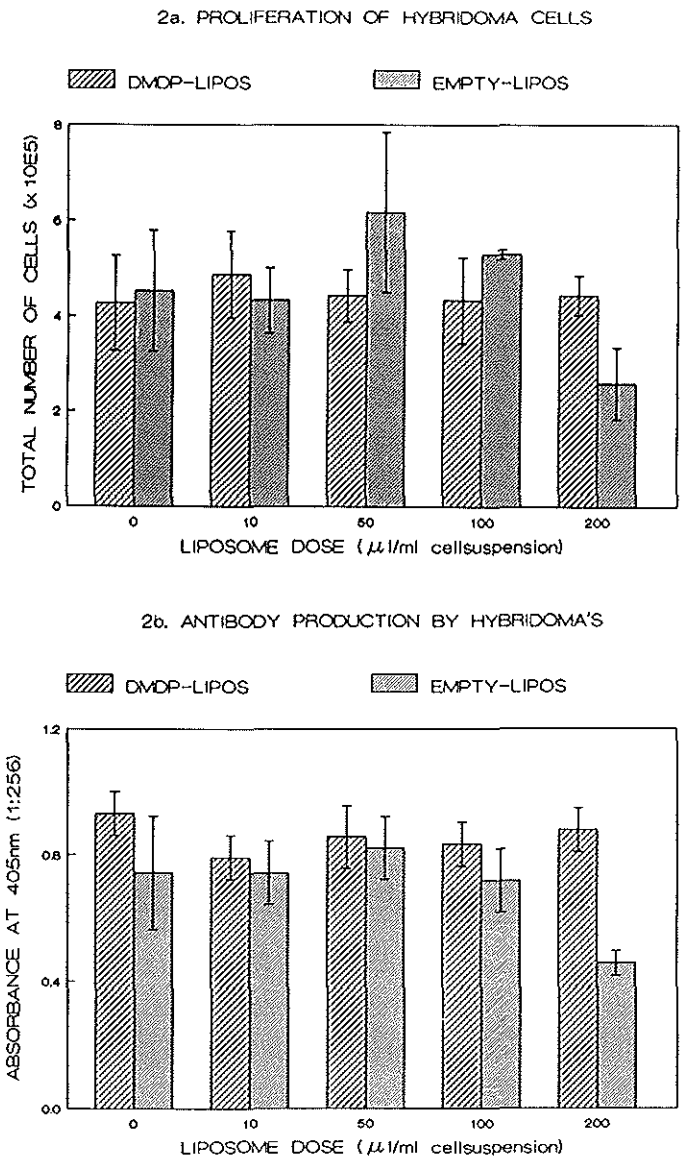

Fig. 2. Effects of PBS and Cl_2MDP liposomes on antibody producing hybridoma cells (treated for 1 h with liposomes), measured after 5 days incubation (at 37°C, 7% CO_2). *a*: number of cells/ml obtained from seeding 0.17×10^5/well at day 0. *b*: anti-TNP antibody production measured in a TNP-ELISA using a 1 : 256 dilution of the culture supernatant.

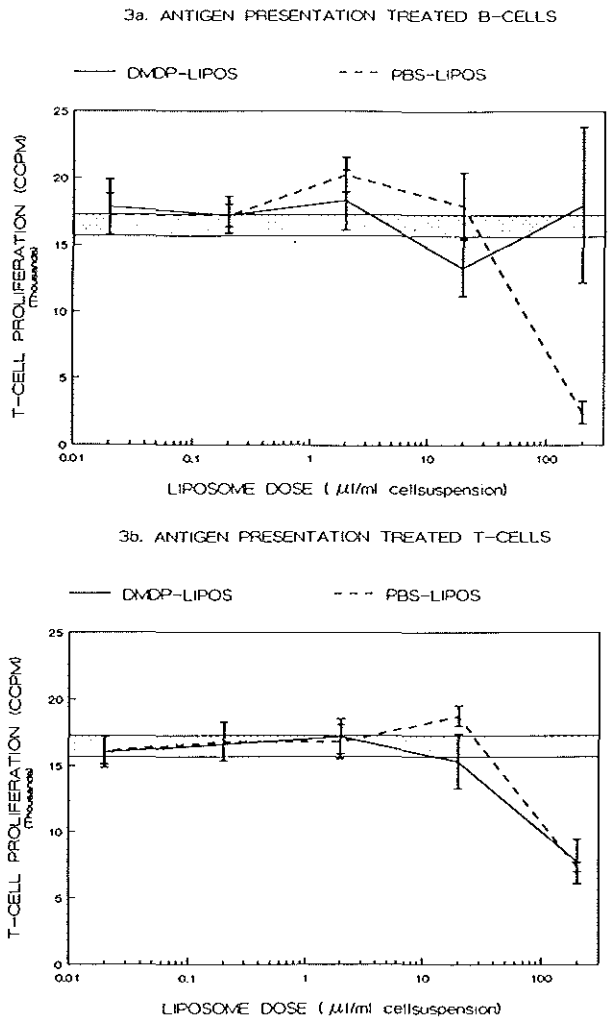

Fig. 3. Effects of PBS and Cl_2MDP containing liposomes on [^{3}H]thymidine incorporation by T cells 4 days after treatment and antigen stimulation. *a*: antigen presentation by treated EBV-BC to normal T cells; *b*: treated T cells with antigen presentation by normal EBV-BC. Data are presented as mean ± standard deviation of triplicate cultures from two experiments ($n = 6$). The dotted zone indicates upper and lower response limits of untreated B and T cells.

tion of hybridoma cells with PBS-liposomes at 200 µl/ml ($P < 0.05$).

Effects of Cl_2MDP on antibody production by hybridoma cells

As Fig. 2*b* shows, production of anti-TNP antibodies by SH4.1C9 hybridoma cells followed essentially the same profile as the hybridoma growth curve. Using Cl_2MDP liposomes no significant ($P < 0.09$) effects were observed. Again, PBS-liposomes exerted a significant effect at 200 µl/ml ($P < 0.001$).

Effects of Cl_2MDP on antigen presentation

Fig. 3 shows T cell proliferation as a function of the dose of liposomes used for pre-treatment. The dotted zone gives the absolute upper and lower response limit of untreated or Cl_2MDP-treated cells. No significant effects of Cl_2MDP liposomes were found on the antigen presenting capacities of B cells (Fig. 3*a*). However, a marked inhibition was observed after treatment with 200 μl/ml PBS-liposomes ($P < 0.005$). Fig. 3*b* shows essentially the same for treated T cells. In addition 200 μl/ml Cl_2MDP liposomes also inhibited proliferation. Incubation with Cl_2MDP (either free or in liposomes) or PBS liposomes had no effect on the viability of the cells, as measured by [^{3}H]thymidine incorporation of un-irradiated cells (data not shown).

Discussion

In this study we have demonstrated the elimination of mononuclear phagocytes from heterogeneous cell suspensions by means of liposome entrapped Cl_2MDP. Free Cl_2MDP, PBS or PBS liposomes in similar concentrations were unable to eliminate these phagocytes.

Using doses as low as 50 μl/ml cell suspension, complete elimination of phagocytes could be obtained. In the mouse a dose of 50–100 μl/ml blood is necessary for complete elimination of all macrophages. After exposure of cells for 30 min to 1 h all phagocytes were killed so that in vitro and in vivo dose effects were in agreement. Only the time interval that elapsed before acid phosphatase activity could no longer be observed was increased by decreasing doses of liposomes; thus short (e.g., 30 min) incubation with the liposomal suspension, and subsequent washings to remove the liposomes was sufficient to remove phagocytic cells The sensitivity of the cells was dependent on the ambient temperature and agitation of the suspension (pilot studies, not shown) and therefore the optima should be determined for specific applications. Initially, this study was aimed at the generation of macrophage-free suspensions using spleens and lymph nodes of Cl_2MDP liposome-treated animals. Although in vivo elimination of phagocytes with Cl_2MDP liposomes proved very effective, B cell numbers were reduced and hence these conditions would not be applicable where cells other than phagocytes must remain unaltered.

In vitro, in contrast, the present studies clearly showed that, within the dose range 50–100 μl/ml, no effects on B or T cell growth, protein synthesis or antigen presentation were observed. Thus, unlike the use of silica or asbestos (cf., Wirth et al., 1980; Elliot and Hartmann 1984), this treatment does not influence lymphoid cells in vitro. Furthermore, this method appears to eliminate both macrophages and monocytes (Huitinga et al., manuscript in preparation), independently of the adherence properties of these cells. Adherence properties are frequently used to remove macrophages (Mishell and Mishell, 1984; Wong and Varesio, 1984).

In the present study no toxic effects of free Cl_2MDP (cf., Labat et al., 1984) were found on the cells investigated, indicating that within the dose range used (0.1–10 mg/ml) the effects were due to liposome-mediated uptake. Furthermore, leakage of Cl_2MDP from the liposomes or dying macrophages during incubation of the samples did not affect the lymphoid cells, since the maximum dose released (by complete disruption of all liposomes) was 2–4 mg for the 50–100 μl/ml solutions used. This observation also has implications for in vivo studies in which we found a decrease of marginal zone B cells after treatment with Cl_2MDP liposomes, as studied by both immunocytochemistry (Claassen et al., 1986) and electronmicroscopy (Van Rooijen et al., 1985). In view of the present findings this loss of B cells could not be explained by the toxic effects of Cl_2MDP liposomes or free Cl_2MDP, since the concentrations of these substances in vivo would be much too low for such an effect. Consequently we feel that the loss of marginal zone B cells was due to enzymes released by the dying macrophages, as previously suggested (Van Rooijen et al., 1985).

In earlier in vivo studies we demonstrated that charges in marginal zone macrophages, which specifically take up but do not process thymus independent type-2 (TI-2) antigens such as TNP-Ficoll are not responsible for either a decrease in the specific immune response against these antigens after splenectomy (Claassen et al., 1989) or

for macrophage elimination by Cl_2MDP-liposomes (Claassen et al., 1986a). To exclude the possibility that the B cells present in the marginal zone were in any way hampered by the liposome treatment, we performed a number of functional studies with B cell lines in this study. It is clear that these cells are not damaged in terms of growth, proliferation, antigen presentation or antibody production. From the data demonstrating antigen presentation by B cells and the fact that this process is not hampered by Cl_2MDP liposomes we can conclude that B cells may be involved in antigen presentation of TI-2 antigens after macrophage elimination (cf., Claassen et al., 1989).

PBS liposomes are supposedly nontoxic carriers (cf., Gregoriadis, 1985), and in our in vivo studies we have never been able to demonstrate toxic effects of phosphatidylcholine-cholesterol liposomes (cf., Claassen et al., 1988), though a small number of studies have reported unwanted side effects of liposomes, with different compositions, in vitro (Chen and Keenan, 1977; Ng et al., 1978; Nuzzo et al., 1985; Grover and Sundharadas, 1986; Mayhew et al., 1987). This study again shows effects of PBS liposomes on lymphoid cells. However these effects were only observed at very high doses and never in vivo (Claassen et al., 1988). In view of the fact that the numbers of T or B cells were not affected but only their ability to proliferate (cell division) we feel that this 'toxicity' can be ascribed to changes in the phospholipid membrane, e.g., by uptake from cholesterol by the cells from the liposome (cf., also Mayhew et al., 1987).

In conclusion we feel that this novel method for the elimination of mononuclear phagocytic cells from heterogeneous cell suspensions has definite advantages over existing methods for macrophage removal. In contrast to other methods, liposome encapsulation of the active drug selectively targets it to macrophages, thus preventing it from affecting non-phagocytic cells such as lymphocytes.

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Chapter II.4

Antigen detection *in vivo* after immunization with different presentation forms of rabies virus antigen, II. Cellular, but not humoral, systemic immune responses against rabies virus immune-stimulating complexes are macrophage dependent.

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Antigen detection *in vivo* after immunization with different presentation forms of rabies virus antigen, II. Cellular, but not humoral, systemic immune responses against rabies virus immune-stimulating complexes are macrophage dependent

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SUMMARY

In this paper we describe the effect of depletion of splenic macrophages on the uptake, and immune response against, different formulations of rabies virus antigen. Splenic macrophages were removed by intravenous injection with clodronate liposomes. β-propiolacton inactivated rabies virus (RV-BPL) and immune-stimulating complexes (iscom) containing these antigens were given to macrophage-depleted and control mice. In the absence of phagocytic cells in the spleen, antigen is still trapped in the red pulp and to a lesser extent in the peri-arteriolar lymphocyte sheaths (PALS) for both antigen formulations. The localization pattern in the main area of immune response induction, namely the follicles, was unaltered after macrophage depletion. Functionally, the depletion of splenic and liver macrophages had no influence on the induction of specific antibody responses in both RV-BPL or RV-iscom immunized mice, even though the latter presentation form was clearly associated with specific localization in the marginal metallophillic macrophages. In RV-BPL immunized mice, macrophage depletion had no influence on proliferative T-cell responses. However, macrophage-depleted mice that were immunized with RV-iscom showed a significant decrease in proliferative T-cell responses. These results confirm existing ideas on the spleen as a physical filter rather than an induction site for humoral responses and shed new light on the efficient role of iscoms as antigen-presenting moieties in relation to their specific *in vivo* localization patterns and partial macrophage dependency.

INTRODUCTION

Several mechanisms have been postulated to explain the relatively high immunogenicity of antigens presented in immune-stimulating complexes (iscoms).[1,2] Their potency can in part be explained by the specific targeting of these structures to antigen-presenting cells (APC) of the immune system. Recently we developed a novel method for the *in situ* detection of iscoms.[3] Employing this technique we demonstrated that iscoms containing rabies virus antigen were taken up by a different subset of macrophages in the spleen than inactivated rabies virus antigen, thereby possibly explaining the difference in immunogenicity. Furthermore, we found a surprising preference for iscom, rather than intact virus, in the follicular trapping/uptake of these antigens. These results led to a biphasic presentation model for iscom with a specific localization pattern in marginal metallophilic macrophages (MMM), away from antigen-degrading macrophages, in the first phase and direct presentation to follicular cells in the second phase. This model implies an entire or at least partial dependency of the iscom-induced immune response on this particular macrophage subset. From several studies it has now become evident that the spleen processes antigen in two possible ways, depending on different types of non-lymphoid cells. Firstly, there seems to be a macrophage-dependent pathway for particulate-and thymus-independent type I (mitogenic polysaccharides) antigens.[4] Second, there is an important role for the follicular (dendritic and B) cells of the spleen in trapping and presenting soluble protein and TI-2 antigens to the immune system.[5] Because both virus (RV-BPL) and iscom can qualify as particulate antigens it remains to be established whether these antigens also require macrophage (pre)-processing before they can evoke an effective immune response. In view of our

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Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; iscom, immune stimulating complex; MZM, marginal zone macrophages; MMM, marginal metallophilic macrophages; NSE, non-specific esterase; RPM, red pulp macrophages; RV rabies virus; RV-BPL, β-propiolacton inactivated rabies virus.

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earlier study on the difference in splenic localization of these two formulations of rabies antigen and based on the available literature suggesting a suppressive rather that immune-stimulating role of splenic macrophages we decided to study he contribution of splenic macrophages in these responses. By making use of a well-established technique for splenic nacrophage elimination, employing clodronate liposomes, the role of macrophages, as opposed to the function of follicular cells (which are not affected), can be determined.[5] By making use of the discrete differences in repopulation kinetics of different splenic macrophage subsets[6] their role in immune response induction could be studied. Furthermore, this elimination model can be combined with the above mentioned labelling technique, thereby enabling the study of *in situ* antigen localization under macrophage-free conditions.

MATERIALS AND METHODS

Animals and chemicals

Female BALB/c mice aged 12–16 weeks, were kept in macrolon cages under an 11 hr dark/13 hr light regimen at 20° and were given acidified water (pH 3) and pelleted mouse food (Hope Farms, Woerden, The Netherlands) *ad libitum*. DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; D-282) was obtained from Molecular Probes, Eugene, OR. Clodronate liposomes were prepared as described earlier.[7]

Antigens

Rabies virus (RV-Pasteur strain) was propagated in Vero-cell monolayer cultures.[8] Culture supernatant was cleared by filtration and concentrated by Amicon ultrafiltration (cutoff 10^6 MW). Virus was inactivated with β-propiolacton (BPL) and stored at −70° at a concentration of 900 μg/ml. This rabies virus antigen was used for the preparation of RV-iscoms, as described earlier.[9] Briefly RV-proteins were solubilized with 10% MEGA-10 (decanoyl-*N*-methylglucamide) for 2 hr. at room temperature. The solubilized virus was layered on top of a 10% sucrose cushion and centrifuged for 2 hr at 200 000 *g*. Quil-A (Iscotec AB, Luleå, Sweden), cholesterol and phosphatidylcholine (Sigma-Aldrich, Zwijndrecht, the Netherlands) were added to the supernatant and the mixture was dialysed overnight against phosphate-buffered saline (PBS). Free Quil-A and unincorporated proteins were removed by centrifugation over a 20% sucrose cushion for 48 hr at 150 000 *g*. Incorporation of RV proteins was verified by sodium dodecyl sulphate-polyacrylamide gel eletrophoresis (SDS-PAGE) analysis and a glycoprotein specific enzyme-linked immunosorbent assay (ELISA).[10]

Immunological responses

Clodronate liposomes were prepared as described in detail before,[7] briefly: 75 mg phosphatidylcholine and 11 mg cholesterol were dissolved in chloroform in a 500 ml round-bottomed flask. The thin film that formed on the interior of the flask after low vacuum rotary evaporation at 37° was dispersed by gentle rotation for 15 min with 10 ml PBS (0·15 M NaCl/10 mM phosphate buffer pH 7·4) containing 1·89 g dichloromethylene diphosphonate (a kind gift of Boehringer Mannheim, Germany). The milky white suspension was kept for 2 hr at room temperature and sonicated for 3 min at 20° in a Sonicor waterbath sonicator (50 Hz, Farmingdale, NY). After an additional 2 hr at room temperature, the liposome suspension was diluted to 100 ml in PBS and centrifuged (100 000 *g*, 30 min) to remove free clodronate. Liposomes were resuspended in 4 ml PBS and microscopically checked for formation and size homogeneity. Three animals per group were injected i.v. with 200 μl Clodronate-liposomes or PBS intravenously. 48 hr after treatment animals were immunized with 1 μg RV-iscom or with 10 μg RV-BPL. At day 40 animals were again treated with either Clodronate liposomes or with PBS and animals were booster immunized at day 42. At day 49 animals were killed and the spleen was removed for T-cell proliferation studies. Immunological responses were measured 1 week after the last i.v. immunization at day 42. Rabies virus specific antibody responses were measured by antigen binding ELISA as described earlier. and T-cell responses were measured as described earlier.[11] Briefly, spleen cells were isolated and stimulated with varying concentrations of RV-BPL. After 5 days cells were pulsed with [^{3}H]thymidine for 18 hr. Cells were harvested on glass filters and incorporated radioactivity was measured in a Betaplate scintillation counter (LKB, Uppsala, Sweden). Sera were taken at weekly intervals and were analysed in a rabies-virus-specific ELISA for the induction of specific antibodies.

Fluorochrome labelling of rabies virus antigen and RV-iscoms

Stock solution of DiI and was made in pure ethanol (2·5 mg/ml). 100 μg RV-BPL, RV-iscoms or empty iscoms were mixed with 20 mg DiI in PBS and incubated for one hour at 37°. After labelling the samples were filtered using 0·45 mm filters to separate crystalline DiI and labelled antigens.[12] Labelled material was stored in the dark at 4° for up to 4 weeks. Administration was performed i.v. for several doses ranging from 0·1 to 10 μg per mouse.

Fluorescence microscopy

Frozen sections (8 μm) of organs under study were observed and photographed directly after cryo-sectioning with an Olympus (Tokyo) Vanox fluorescent microscope. DiI fluorescence was observed both as red with green light (excitation filter BP 545) and 'rhodamine/TRITC' optics (emission: EO 570).

Enzyme cytochemistry

Organ sections used for localization studies based on fluorescence microscopy were air-dried and fixed in acetone for enzyme staining. Endogenous acid phosphatase staining for the identification of all splenic and liver phagocytes was performed as described in detail earlier.[13]

RESULTS

Localization of virus and iscom in the spleen after macrophage depletion.

Already at 30 min after i.v. administration of DiI labeled RV-BPL preferential uptake by marginal zone macrophages (MZM) could be observed in control mice (PBS at −48 hr) with some antigen also being taken up by red pulp macrophages. This image was identical to the localization patterns we described in a recent study for normal untreated mice.[3] The preferential uptake by MZM was maintained when observing sections at 60 or 120 min (Fig. 1a) and proved

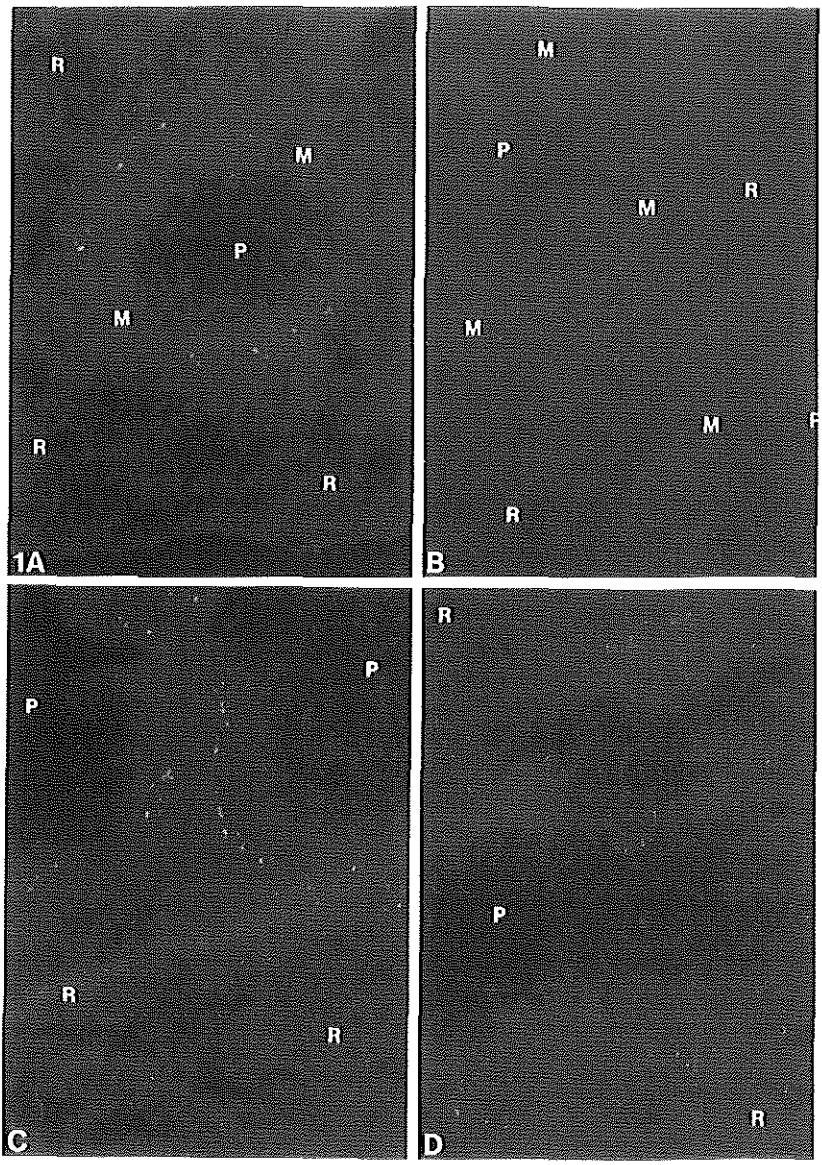

Figure 1. In this figure the uptake of DiI-labelled antigens is shown in the spleen 2 hr after i.v. injection. (a) Uptake of RV-BPL in normal untreated animals. (b) Uptake of RV-BPL in clodronate treated animals. (c) Uptake of RV-iscom in untreated animals. (d) Uptake of RV-iscom in clodronate treated animals, R, red pulp, M, marginal zone, P, peri-arteriolar lymphocyte sheath.

completely in accordance with other localization studies on particulate thymus dependent antigens.[14,15]

Splenic macrophages were depleted by i.v. administration of clodronate liposomes,[16] depletion was complete as confirmed with enzyme histochemistry for acid phosphatase of alternating sections from those shown in Fig. 1 (data not shown). Irrespective of this complete absence of macrophages from the spleen, a massive trapping and retention of DiI-labelled RV-BPL could be observed (irrespective of the dose given). Contrary to the discrete localization pattern found in

normal or PBS treated mice (in MZM and red pulp), RV-BPL seemed to be evenly distributed over all splenic compartments (Fig. 1b).

As described before,[3] iscom was taken up preferentially by MMM (bright yellow dots in Fig. 1c) and to a lesser extent by MZM and on cells in the follicles. In macrophage-depleted animals iscom was trapped and retained almost exclusively in the marginal zone and red pulp of the spleen (irrespective of the dose given). Contrary to RV-BPL almost no iscom localization could be observed in the PALS area of depleted animals (Fig. 1d).

Immunological responses after depletion of spleen macrophages

Based on dose-response curves generated before[3] we chose for similarity of eventual titre rather than dose of immunization (antigen). In this scenario 1-μg of RV-iscom is equivalent to 10 μg of RV-BPL, both resulting in virus neutralising antibodies, specific T cells and 100% survival after challenge (i.e. full protection).[3]

Rabies-virus-specific antibody responses in serum were measured weekly in antigen-specific ELISA. Serum samples were analysed weekly after depletion of splenic macrophages and subsequent immunization to observe possible effects caused by macrophage repopulation of the spleen. It was established that at day 7 no repopulation of the spleen with macrophages has yet occured, marginal metallophils reappear between 14 and 21 days and at day 28 the normal situation is almost completely restored ([4,6,17] and data not shown). As is shown in Fig. 2(a) (represenative experiment from three performed) no difference in the kinetics of the antibody response could be observed when splenic macrophages were removed in RV-iscom immunized animals as compared with untreated animals. It should be noted that a trend towards higher titers in depleted RV-BPL animals could be observed (not significant).

The clodronate treatment was repeated at day 40 and animals were booster immunized with RV-iscom at day 42. Both treated and untreated animals showed increased serum titres at 7 days after boost immunization as compared to primary responses. Again, no effect of the clodronate liposome treatment was observed. Identically, humoral responses after immunization with RV-BPL were unaffected after clodronate liposome treatment.

Proliferative splenocyte responses in RV-BPL immunized animals were unaffected after treatment with clodronate liposomes (Fig. 2c). However, when splenic macrophages were removed prior to immunization with RV-iscom a significant decrease in rabies-virus-specific spleen-cell responses could be observed (Fig. 2d). *In vitro* restimulation of T cells with either inactivated antigen or antigen as provided by paraformaldehyde (PFA)-fixed persistently ERA (Evelyn–Rokitnicky–Abelseth)-infected P815 (H2d, haplotype) cells was also significantly decreased in clodronate liposome-treated animals.

DISCUSSION

In this study we show that the T-cell mediated immune response against rabies virus iscom is partly dependent on splenic macrophages. Depletion of macrophages resulted in almost identical *in situ* localization patterns for rabies virus antigen presented as inactivated virus or in iscom. The elimination of splenic macrophages has no effect on the humoral response against rabies virus for both antigen formulations.

When given in sufficiently high doses, 'empty' liposomes can efficiently block and suppress the macrophage system.[18,19] Similarly, macrophages can be suppressed or depleted by using substances such as carrageenan, silica, carbonyl iron or antibodies.[20] We have developed and applied a liposome-suicide technique for the actual elimination of macrophages.[7,16] The resulting modulation studies have firmly established the role of macrophages in innate (antigen removal) rather than in adaptive (antigen presentation) immunity.[4,5,15] Emphasizing the role of what is now called professional antigen presenting cells, such as dendritic and B cells, in the generation of antibodies and cellular immunity.[5] Not very surprising it could be established in a similar fashion that the macrophage is the principal antigen-presenting cell for liposome-encapsulated antigens[21] as well as for other particulate antigens.[15,22] Furthermore, CTL responses induced by membranous vesicles but not those induced by syngeneic antigen pulsed splenocytes proved to be macrophage dependent[23]. Iscom and inactivated virus can be regarded as hydrophobic particulate antigens. In this study no differences in humoral response were observed in macrophage-depleted cells as compared with untreated animals, at the antigen dose and immunization regime studied. In view of the finding that trapping of both antigens occurs on follicular cells in depleted animals it can be concluded that follicular dendritic or follicular B cells are involved in antigen presentation of both RV-BPL and iscom. This observation, in combination with the observed decrease in T-cell responses against iscom in depleted animals, suggests a role for both follicular cells and MMM in the presentation of iscom. And no or very limited role for MMM in the presentation of RV-BPL.

To induce an efficient MHC class I restricted immune response, involving the production of antigen specific cytotoxic T-cells (CTL), it seems necessary to target the antigen to the cytoplasm[29] or induce processing by dedicated macrophages[22–24]. It was shown that both anionic and cationic[32] pH-sensitive liposomes could enter the class-I restricted (endoplasmatic reticulum/Golgi) pathway.[22,24,25] The addition of Quil A to liposomes has been shown to efficiently enhance their capacity to induce $CD8^+$ CTL.[26] Quil-A is an adjuvant and toxic by itself, and a crucial component of iscom which belong to the most potent adjuvants, for hydrophobic proteins, known.[1–3] Both rabies virus iscom and RV-BPL can induce antigen-specific, class-I restricted CTL. Quil A containing liposomes proved to be an effective vehicle to shuttle hydrophillic proteins into the major histocompatibility complex (MHC) class I pathway, resulting in induction of $CD8^+$ cytotoxic T lymphocytes (CTL). These and other[4,27] data point to a conflicting role for macrophages in direct stimulation of T cells in some systems and suppression of T cells in others.

We have shown previously that the T-cell responses are not directly influenced by treatment with clodronate liposomes.[28] Consequently the observed decrease in T-cell proliferation in RV-iscom immunized animals is likely a result from the absence of splenic macrophages. Because no decrease was observed in the CD4 T-cell dependent humoral response the observed decrease can be explained by in the class-I restricted

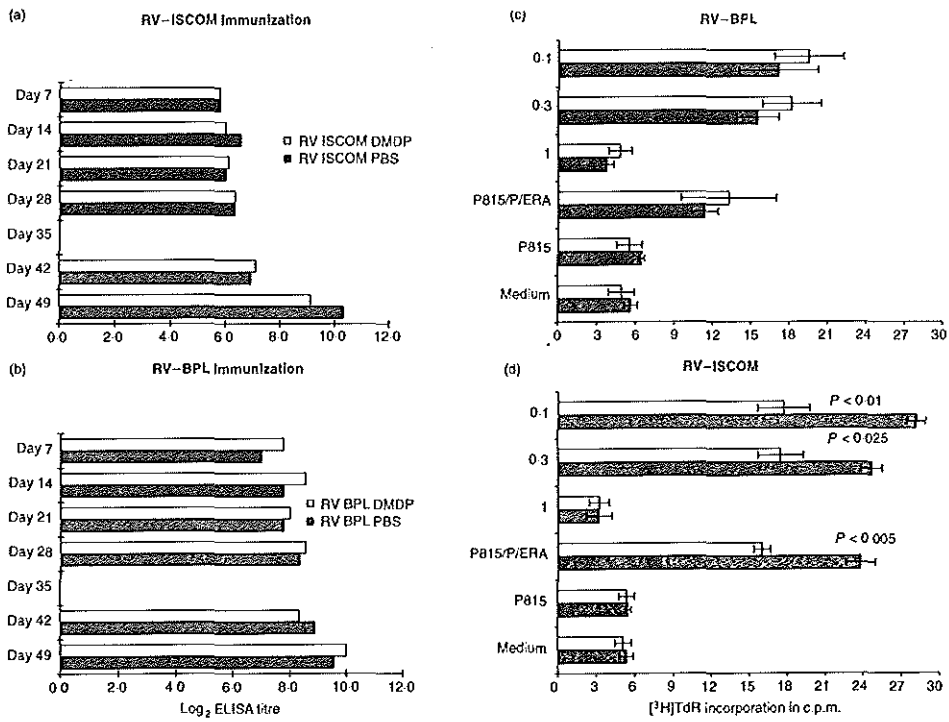

Figure 2. In this figure the rabies virus specific immunological responses are shown before and after treatment with clodronate liposomes. Rabies virus specific ELISA serum titres in RV-BPL (a) and RV-iscom (b) immunized animals immunization. Proliferative spleen cell responses in RV-BPL (c) and RV-iscom (d) immunized animals.

CTL response which is dependent on the presence of macrophages. Such a decrease has been described for the generation of primary ovalbumin (OVA)-specific CTL after splenic macrophage elimination.[29] Why this decrease does not occur in RV-BPL immunized animals, which normally generate CTL activity as well, remains unclear but, in combination with the localization data, it suggests a role for the MMM subset in the (pre)processing of antigen in iscom and not in other unactivated antigen forms.

Removal of splenic and hepatic macrophages with clodronate liposomes is very efficient and actually eliminates phagocytic cells from the tissues altogether.[16] As described before in studies using radioactively labelled liposomes[30] this removal does not result in persistently increased serum levels of the antigen nor in a decrease of splenic uptake or a change of antigen localization patterns. The present results show the localization patterns of this nonphagocytic trapping by the spleen. As observed for a soluble thymus-independent antigen[5] it is evident that antigen is still trapped by follicular (dendritic or B) cells of the spleen after macrophage depletion. In view of the crucial role of dendritic cells[29] and follicular dendritic cells in the presentation of antigens to the immune system[3,5] this is an important observation. Furthermore, this is in accordance with the primary function of the spleen, namely as a filter of the blood.[4,5] In a large number of studies it has now been shown that removal of marginal zone macrophages leads to a relative increase of humoral immune responses against particulate antigens[17] or antigens in oil emulsions.[31] This trend is also visible in this study (Fig. 2b). The preferential localization of iscom in MMM certainly leads to relative protection of the antigen for the degradable action of marginal zone macrophages and provides an explanation for the relative effectiveness (10–25-fold efficiency gain in terms of dose[3]) of iscom in inducing immune responses. Studies involving development or optimization of iscom or other antigen presenting moieties should therefore include similar *in vivo* localization and modulation studies to better predict eventual efficacy. This in turn will lead to vaccines with higher efficacy at much lower antigen concentrations because much less antigen is lost by immunologically non-specific filtering.

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Chapter II.5

Incorporation of inactivated rabies antigen into iscom enhances its capacity to induce specific T-cell mediated immunity and protection in mice.

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submitted for publication

Summary
It is well established that incorporation of rabiesvirus (RV) antigen into iscom enhances its potency to induce virus neutralizing antibodies and protection in mice. Here we show that intramuscular immunization of BALB/c mice with inactivated RV antigen, induces RV specific proliferative and cytotoxic T lymphocyte (CTL) responses more efficiently when the antigen is incorporated into iscom. Analysis of the phenotype of the RV specific CTL by depletion studies showed that Lyt-2 positive cells were involved in the RV specific CTL responses. These data add to our previous findings, illustrating the possibility to reduce the antigen concentration in rabies vaccine by presenting it in an iscom structure.

Rabies continues to be the cause of more than 50.000 cases of fatal encephalitis in developing countries annually (WHO report). Although safe and effective cell culture vaccines for human use are available one of the limiting factors in their availability is the limited production capacity for RV antigen. Previously we and others have shown that incorporation of RV antigen into iscom not only enhanced its potency to evoke neutralizing antibodies but also to induce protection in a pre- and post- exposure setting in mice (Fekadu et al., 1992; Claassen et al., 1995). Virus neutralizing antibodies have been shown to be protective against infection with RV. There are however several indications that RV specific T lymphocytes may play an additional role in the protective immunity against rabies. These include, the demonstration of cellular immunity after infection or immunization (Wiktor et al., 1977a and 1977b), the protective capacities of a rabies virus specific cytotoxic T lymphocyte (CTL) clone against challenge in mice (Kawano et al., 1990), and the induction of protective immunity against RV infection after immunization with nucleoprotein (N), a protein that does not induce any neutralizing antibodies (Dietschold et al., 1987; Tollis et al., 1991). Recently it was demonstrated that protection against RV can be induced by immunizing with a plasmid expressing the full length RV-GP (Xiang et al., 1995). However the slow development of the immune response to genetic immunization makes these vaccines unsuitable for postexposure treatment of RV infection (Ertl and Xiang, 1996).

Besides specific T helper cells, CTL may play an important role in the RV specific immunity: MHC class I restricted $CD8^+$ CTL have been shown to be important for the elimination of several virus infections. For the induction of this type of virus specific CTL, it is generally considered necessary that *de novo* synthesis of viral antigen takes place. Therefore most inactivated vaccines fail to induce these responses. However, some antigenic presentation forms of inactivated vaccines, like the iscom presentation form have been shown to induce these CTL (for review see Claassen and Osterhaus 1992). Interestingly, in the past some reports have indicated that the current inactivated rabies vaccines do induce MHC class I restricted $CD8^+$ CTL responses (Sugumata et al., 1990; Fujii et al., 1994; Kawano et al., 1990). Therefore, we have studied here the

potential of beta propiolacton inactivated rabies vaccine, produced in cell culture, to induce RV specific CTL in its non-adjuvated form and after incorporation into iscom.

Rabies virus is a negative stranded RNA virus and its genome encodes five proteins: the large protein (L) , the glycoprotein (G), the nucleoprotein (N), the phosphoprotein (NS) and the matrix protein (M). Resistance to RV infection is mediated by both humoral and cellular responses. Protection against rabies is mainly dependent on virus neutralizing antibodies (VNA) directed against the G-protein of the virus. Cell mediated immunity (CMI) especially cytotoxic T lymphocytes (CTL) with the G-protein as target have been described but their role in protection is not clear. The N-protein cannot induce VNA but the N-protein and synthetic peptides mimicking parts of the N-protein can induce protection in mice in the absence of VNA at the time of challenge. Although the mechanism of this protection is still unclear it clearly demonstrates a role in protection of either N-protein specific T-helper cells or CTL.
It is generally assumed that CTL recognising infected target cells in the context of the correct MHC class I haplotype play an important role in the elimination of viral infections. For the induction of MHC class I restricted CTL *de novo* synthesis of proteins is regarded essential. However exceptions have been described. Interestingly Wiktor et al. initially showed that inactivated RV can induce CTL responses (Wiktor et al., 1977a).
Most data on RV specific CTL have been obtained after immunization with inactivated or attenuated vaccines. CTL responses have been described after immunization with live attenuated virus (Sugamata et al., 1990; Wiktor et al., 1977b) and with inactivated RV (Wiktor et al., 1977a and 1977b; Morgeaux et al., 1989). CD4 T- cell responses against RV have been described in both mouse and man (Bunschoten et al.,1989,1990; Celis et al., 1988a, 1988b). Alternatively, iscom containing antigen have been shown to induce class I restricted CTL responses (Takahashi et al., 1990; Mowat et al., 1991). Iscoms are small (40-80nm) cage like structures that contain multiple antigen molecules and the adjuvant Quil-A (Morein et al., 1984). The iscom structure is a matrix with high adjuvant activity for the induction of anti viral immune responses in general (for review see Claassen and Osterhaus, 1992). We have described that RV-iscom require a lower antigen dose to induce high VNA titers, proliferative T-cell responses and protection as compared with RV-BPL. Here we have analyzed whether a qualitative difference exists between RV-BPL with RV-iscom for their priming capacity of CTL *in vivo*.
To this end we have constructed RV-iscom, using BPL inactivated rabies virus as described earlier (Claassen et al., 1995). We extended our observations in previous experiments with regard to the induction of rabies virus specific T-cell proliferation and protection with a wider range of antigen concentrations (figure 1). Indeed RV-iscom proved to be more efficient in the induction of protection and proliferative T-cell responses confirming superior immunogenicity of the RV-iscom over RV-BPL.

Similarly CTL responses induced by either of both antigen preparations were measured.
In figure 1 the induction of protection against intracerebral challenge is also shown. Groups of 5 BALB/c mice were immunized intramuscularly with either RV-iscoms (0.001 - 1 mg), BPL inactivated rabies virus (0.01 - 30 mg) intramuscularly (i.m.), or live Flury Low Egg Passage virus (Flury-LEP) intraperitoneally (i.p). As a control mice were immunized with iscom matrix, a cage like structure similar to the iscom structure but not containing any antigen. I.m. immunized mice were booster immunized at day 14 and all mice were intracerebrally (i.c.) challenged with 30LD50 CVS-26 at day 28. Mice immunized with 1 μg RV-iscom were completely protected against i.c. challenge with RV. Groups immunized with 10μg and 30μg RV-BPL (not shown) were also completely protected. 1μg RV-BPL is not sufficient to completely protect against challenge (figure 1A). This confirms data presented earlier by our group that even at doses as low as 0.4μg RV-iscom after subcutaneous immunization all animals were protected (Claassen et al., 1995). Mice immunized with iscom matrix were not protected at all (figure 1D).
To study priming of T-cells for their capacity to respond to rabies virus antigens *in vitro,* spleens of immunized mice were removed at day 21 and mono cell suspensions were prepared. Cells were cultured in Iscoves Modified Dulbeccos Medium (IMDM) supplemented with 10% normal BALB/c serum and conditioned supernatant from rat spleen cells. Paraformaldehyde (PFA) fixed P815 ($H2^d$ haplotype) cells and P815 cells persistently infected with Evelyn-Rokitnicky-Abelseth (ERA) rabies virus (P815-ERA-P) (a kind gift from dr. Lodmell) were added to the spleen cells to specifically stimulate *in vivo* primed CTL. Infection of P815 cells was assayed by immunofluorescence. RV-BPL was added to the wells at concentrations of 0.03, 0.1 and 0.3μg per well to specifically stimulate L3T4 T-cells. 3H thymidine was added after 5 days and incorporation of radioactive label was measured after 18 hours. Stimulation indexes were calculated using the following formula

$$S.I. = \frac{\text{incorporated CPM antigen}}{\text{incorporated CPM medium}}$$

Results of the mean of two independent experiments (3 mice per group) are given in figure 1, left panels. From this figure it can be concluded that RV-iscom more efficiently induce specific T-cell proliferation (S.I.>4) in a range of 0.01-1μg. Immunization with 10 or 30μg RV-BPL induces T-cell responses equally high as those observed after immunization with 1μg RV-iscom (data not shown).
To demonstrate RV specific CTL responses, one week after isolation and stimulation of immune spleen cells with PFA fixed P815-ERA-P cells, the blast cell population was isolated using a percoll gradient centrifugation and the cells were tested in a chromium release assay for their ability to kill infected target cells at different effector/target ratios . Uninfected P815 cells were used as control. P815 and P815-

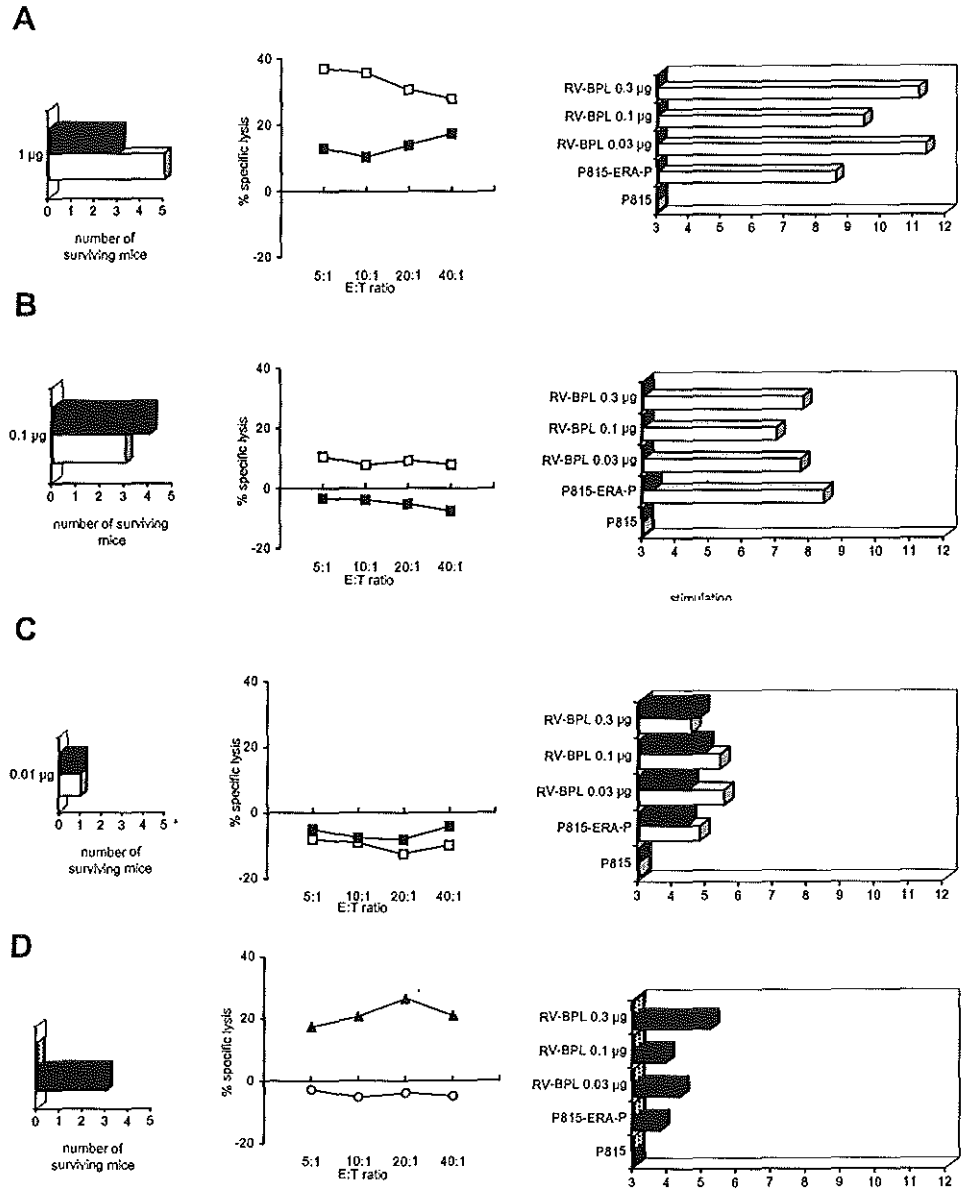

figure 1:Panels from left to right show survival after i.c. challenge in groups of 5 mice, CTL responses and S.I of proliferative responses of spleen cells. 1A shows responses after immunization with 1µg RV-iscom □ or RV-BPL ■ , 1B: immunization with 0.1µg, 1C: immunization with 0.01µg. 1D shows responses after immunization with iscom matrix □,○ , or Flury-HEP ■, ▲ .

ERA-P cells were labelled with ^{51}Cr for one hour and washed with IMDM. Effector cells were added in different ratios to a constant amount of labelled target cells (5 x 10^3 /well). After one hour supernates were collected and counted in a Packard gamma counter. The percent specific cytotoxicity was determined using the following formula:

$$\frac{\text{CPM(experimental)-CPM(spontaneous)}}{\text{CPM(maximum)-CPM(spontaneous)}} \times 100$$

In figure 1 (middle panel) CTL responses one week after *in vitro* stimulation are shown. Again RV-iscom (figure 3A) require about ten-fold lower dose (1µg) to induce specific CTL activity (app.30%) comparable to that after immunization with 10µg RV-BPL (app 40%) (data not shown). In contrast, immunization with 1µg RV-BPL induced only background activity (app 10%). Immunization with iscom matrix did not induce detectable CTL activity (figure 1D).

To demonstrate that in all cases MHC Class I restricted T-cells are involved in the killing of P815-ERA-P target cells L3T4 and Lyt-2 positive T-cells were depleted from the in vitro stimulated T-cell population. T-cell subsets were removed by incubation of the cell populations with monoclonal antibodies specific for the L3T4 or Lyt-2 subset coupled to magnetic Dynabeads®. This treatment resulted in almost complete depletion of one subset as is shown after double labelling with anti L3T4-PE and anti Lyt-2-FITC (Beckton and Dickinson, Mountain View, California) in FACS analysis. Figure 2 shows typical results of FACS analysis after depletion of T-cell subsets. 7 days after *in vitro* stimulation the spleen cells contain both L3T4 cells (upper left quadrant) and Lyt-2 positive T-cells (lower right quadrant) and a population of double negative cells (figure 2A). After depletion with Lyt-2 coated magnetic Dynabeads the lower right quadrant is empty (figure 2B). The upper left quadrant of the panel 2C no longer contains cells after treatment with L3T4 coated Dynabeads.

When the remaining T-cells were tested in a CTL assay only T-cells bearing the Lyt-2 phenotype were able to kill ERA infected P815 cells (figure 2C) as efficient as the unsorted population (figure 2A) . L3T4 T-cells were unable to lyse infected target cells (figure 2B). These data demonstrate that the CTL responses measured after immunisation with either RV-BPL or RV-iscom or live Flury-LEP all restricted by class I MHC molecules. In addition to the above described experiment it should be noted that P815 lacks expression of H-2 molecules belonging to the class II MHC complex.

In the present paper we have described that RV-iscom can prime *in vivo* for CTL activity in vitro. The dose necessary for the induction of CTL was only 1µg protein in the RV-iscom preparation as compared with at least 10µg with RV-BPL.

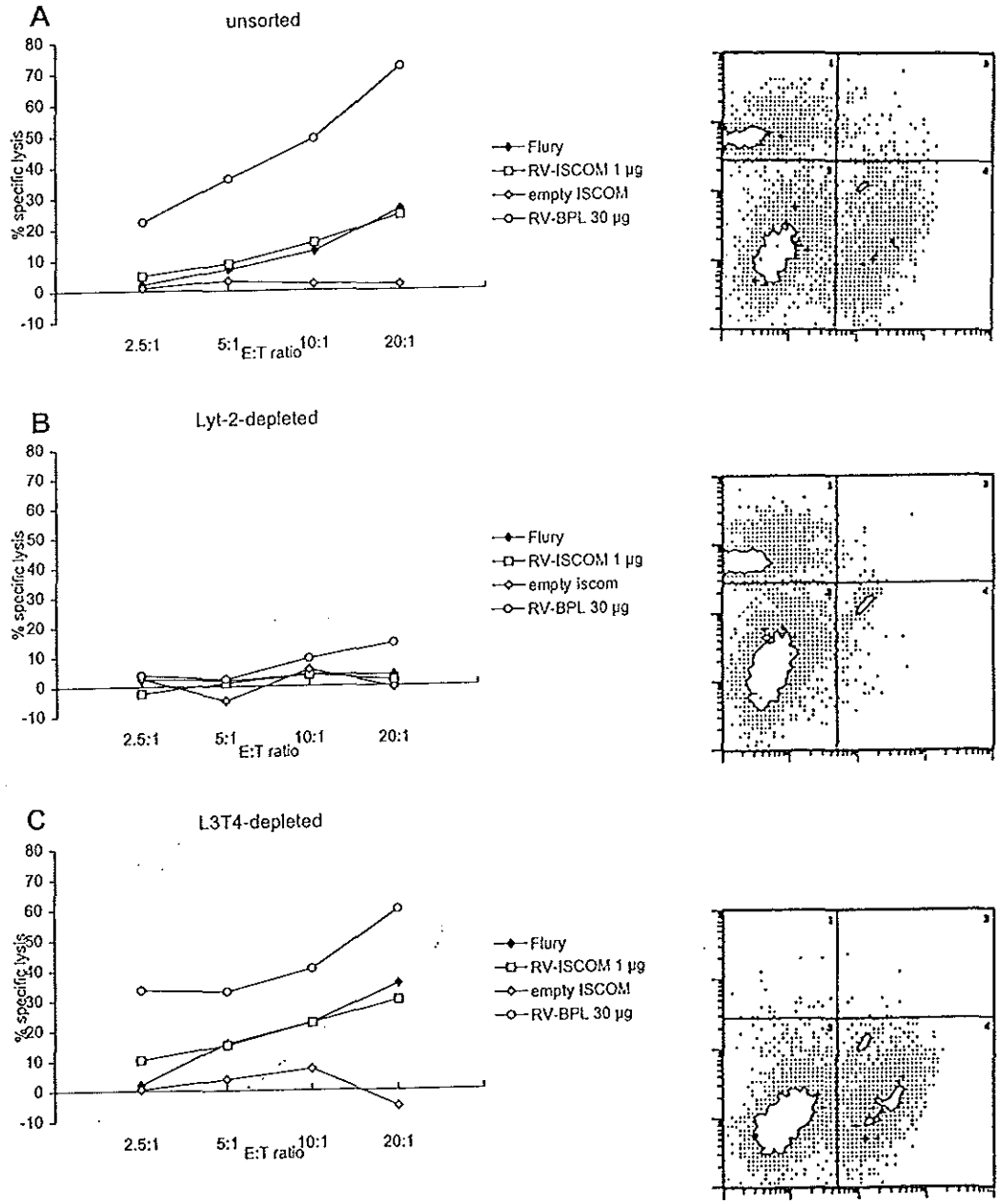

figure 2: shows that CD8+ T-cells are responsible for CTL activity 2A: CTL responses after immunization with Flury, 1µg RV-iscom, empty iscom or 30µg RV-BPL of unsorted spleen cell population, 2B: CTL responses after depletion of L3T4 cells and 2C: CTL responses after depletion of Lyt-2 cells. FACS analysis of unsorted and Lyt-2 depleted or L3T4 depleted spleen cell populations (from top to bottom).

Apparently both iscom and inactivated virus can enter the class I antigen processing - pathway to elicit CTL. For iscom this was demonstrated *in vitro* were measles virus iscom could specifically stimulate measles virus specific $CD8^+$ CTL (van Binnendijk et al., 1992; Morgeaux et al., 1989) demonstrated that purified GP or NP could no longer prime for class I restricted CTL in hybrid $H2^{kd}$ mice. This suggests that at least the way in which the antigen is presented to the immune system is of importance for the induction of the right immune response. Morgeaux described also high killing of target cells due to the induction of natural killer (NK) cells after immunisation with inactivated RV, but not in control immunized mice. Our T-cell depletion studies demonstrate that NK cells cannot be held responsible for the killing we observed in our experiments.
Another possible advantage of iscom for immunization against RV has been published. Iscom which contain lentil-lectin purified RV glycoprotein were shown to be immunogenic in both mice and dogs. These iscoms could protect dogs against a lethal challenge with street RV after pre-exposure immunization. Moreover post-exposure protection against street RV challenge could be induced with three 120 ng doses of RV glycoprotein iscom in 90 % of mice but not with three doses of a presently widely used human diploid cell vaccine (HDCV). When mice were given four doses of HDCV for post-exposure treatment they died of anaphylactic shock. No such phenomenon was observed when the mice had been immunized with the iscom preparation (Fekadu et al., 1992).
The contribution of MHC class I restricted CTL in protection against RV is not clear. The induction of CTL after vaccination with inactivated virus antigen correlated with resistance to intracerebral challenge with RV in one study (Wiktor et al., 1977b) but in vivo depletion of the CD8 subset did not influence resistance to intraperitoneal SRV challenge (Perry et al., 1991). In the same study the depletion of CD4 T-cells resulted in the loss of resistance SRV challenge. In contrast with this finding it has been shown that transfer of GP specific CTL clones induced protection of mice against lethal challenge with RV (Kawano et al., 1990).
Recently it was published that immunisation with inactivated vaccines leads to the generation of GP specific CTL activity only, whereas after immunisation with live virus CTL responses against other structural proteins of the virus (N, NS and M) could also be demonstrated (Fujii et al., 1994). The role of CTL specific for other proteins in protection against RV is unclear.
Collectively our data show that incorporation of RV antigen in iscom, enhances its capacity to induce T-cell mediated immunity and protection in mice.

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Chapter II.6

Modulation of antiviral immune responses by exogenous cytokines: effects of tumour necrosis factor-α, interleukin-1α, interleukin-2 and interferon-γ on the immunogenicity of an inactivated rabies vaccine.

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Modulation of antiviral immune responses by exogenous cytokines: effects of tumour necrosis factor-α, interleukin-1α, interleukin-2 and interferon-γ on the immunogenicity of an inactivated rabies vaccine

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In vivo administration of exogenous cytokines may influence elicited immune responses, and hence may change the efficacy of a vaccine. We investigated the effects of tumour necrosis factor-α (TNF-α), interleukin-1α (IL-1α), interleukin-2 (IL-2) and interferon-γ (IFN-γ) on the immune response elicited by inactivated rabies virus vaccine in a mouse model. Each of the cytokines increased virus-specific IgG responses after primary and after secondary immunization. A single dose of 1·3 ng TNF-α or IL-1α, when injected shortly before vaccination, only marginally stimulated resistance to challenge infection (four- and seven-fold, respectively) without enhancing virus neutralizing antibody (VNAb) responses. In contrast, a single injection of 10^3 units of IFN-γ or five daily injections of 1·6 μg IL-2 increased vaccine dilutions protecting 50% of mice (PD_{50} values) 77- to 50-fold, respectively, with a concomitant enhancement of VNAb. At a 1:10000 dilution of a standard inactivated rabies vaccine preparation both IFN-γ and IL-2 increased protective immunity without enhancing VNAb responses; in non-vaccinated animals this treatment had no effect on resistance to challenge. Combined administration of IFN-γ and IL-2 synergistically enhanced VNAb responses. In contrast to the other cytokines tested, IFN-γ preferentially stimulated virus-specific IgG2a production. It also augmented the vaccine-induced priming of rabies virus-specific splenocyte proliferation. These results document that certain cytokines alone or in combination are potent immunological adjuvants which may direct and modulate immunization-induced antiviral immune responses.

Introduction

Antiviral protection by immunization is established after a cascade of cellular interactions in which soluble mediators collectively termed cytokines are involved. Certain macrophage-derived cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor (TNF) and interferon (IFN) play a critical role in the early activation of accessory cells and in the subsequent co-stimulation of helper and effector lymphoid cells (Unanue & Allen, 1987; Weaver & Unanue, 1990). The secreted cytokine profile of antigen-stimulated T helper cells largely determines the nature of the immune response (Janeway *et al.*, 1988; Mosmann & Coffman, 1989). The factors influencing the distinct lymphokine gene expression in antigen-specific T helper cells are less well understood but appear to depend upon the type and dose of antigen (Parish, 1972; Carding *et al.*, 1989), the lymphoid microenvironment where helper cell activation occurs (Daynes *et al.*, 1990) and the local cytokine profile at the onset of antigen recognition (Seder *et al.*, 1992; Locksley *et al.*, 1991). The latter may follow initial non-specific immune responses including IFN production, activation of natural killer cells or triggering of mast cells and basophils (Romagnani, 1992). Administration of exogenous cytokines may therefore represent a means of improving the magnitude and nature of immune responses elicited by vaccines.

Improvement of the immunogenicity of a vaccine or model antigen has been demonstrated in several systems when exogenous cytokines were administered during the immunization period, for example IL-1 (Staruch & Wood, 1983), TNF (Ghiara *et al.*, 1987), IL-2 (Nunberg *et al.*, 1989), IFN-γ (Playfair & De Souza, 1987; Finkelman *et al.*, 1988; Heath *et al.*, 1991) and IL-6 (Takatsuki *et al.*, 1988). Moreover, the action of

traditional adjuvants like bacterial lipopolysaccharide, Freund's complete adjuvant, $Al(OH)_3$ (Mannhalter *et al.*, 1985) and muramyl dipeptides (Bahr *et al.*, 1987; Oppenheim *et al.*, 1980) is at least partly based on the induction of cytokine secretion (Tomai & Johnson, 1982; Odean *et al.*, 1990). Thus, particular cytokines either administered or induced, may selectively stimulate or inhibit immune reactions. These phenomena may have direct implications for immunization strategies, because pathogens may be eliminated by one effector mechanism but may escape or cause exacerbated reactions during others (Mosmann & Coffman, 1989). Furthermore, studies in this field may contribute to the understanding of the *in vivo* immunoregulatory activities of cytokines.

In this study we investigated the effects of putative stimulatory cytokines, including TNF-α, IL-1α, IL-2 and IFN-γ and of a combination of IL-2 and IFN-γ on the immunogenicity of an inactivated rabies vaccine. For potency assessment we used an *in vivo* protection test, recommended by the WHO for the assessment of human rabies vaccines. Induction of protective immunity by rabies vaccines has largely been attributed to virus neutralizing antibody (VNAb) (Kaplan *et al.*, 1975; Turner, 1976; Wunderli *et al.*, 1991). However, Dietzschold and coworkers (1992) have suggested that it is not solely correlated with neutralizing activity of antibodies but also with their ability to prevent virus spread between cells and to inhibit transcription of viral RNA. Other investigators have demonstrated the induction of rabies virus-specific cytotoxic T lymphocytes (CTLs) by this type of vaccine (Wiktor *et al.*, 1977; Morgeaux *et al.*, 1989; Sugamata *et al.*, 1990), which are not usually primed by non-replicating vaccines. Here we demonstrate that each of the cytokines tested enhance the antiviral immunity induced by an inactivated rabies vaccine to various degrees. IFN-γ, which was shown to enhance the production of antiviral IgG2a, proved to be the most potent and suitable for practical use.

Methods

Mice. Outbred NIH (RIVM:NIH) mice and inbred C57BL/6 ($H\text{-}2^b$) mice were obtained from the National Institute of Health and Environmental Protection and used at 4 to 5 weeks of age. The mice were kept in barrier-contained animal facilities and both sexes were used. The experimental protocols were approved by the Animals Welfare Officer of the National Institute of Public Health and Environmental Protection.

Viruses. The Pitman-Moore (PM) strain of rabies virus (PM/wl-38-1503-3m) was propagated in dog kidney cells (PM-DKCV). The challenge virus standard (CVS) strain of rabies virus was propagated in BHK-21 cells and purified by gradient centrifugation.

Vaccine. The inactivated rabies vaccine (batch R41-A) had been prepared from a concentrated, purified PM virus suspension by inactivation with β-propiolactone according to standard methods (van Wezel *et al.*, 1987). It contained about 200 µg protein per 1 ml dose, of which about 50 µg proved to be glycoprotein as determined by ELISA (Osterhaus *et al.*, 1990).

Cytokines. Recombinant (r) human IL-1α [3×10^5 units (U)/mg protein] and murine rTNF-α (7×10^7 U/mg protein) were diluted in PBS directly before use and injected intraperitoneally (i.p.) 15 min before vaccination on days 0 and 7, at a concentration of 1·3 ng/mouse (i.e. 4×10^2 U IL-1/mouse and 10^2 lytic U TNF/mouse). Human rIL-2 (Proleukin, EuroCetus, 3×10^6 Cetus U/mg, equivalent to $1{\cdot}8 \times 10^7$ International U/mg protein) was reconstituted in sterile water, diluted in PBS and injected i.p. daily on days 0 to 4 and 7 to 11 at a concentration of 1·6 µg/mouse (i.e. 3×10^4 U/mouse). The rat rIFN-γ (4×10^5 U/mg protein), which displays biological activity in both rats and mice, had been produced in Chinese hamster ovary cells and was purified by monoclonal antibody affinity chromatography as previously described (van der Meide *et al.*, 1986). Preparations were assayed in a vesicular stomatitis virus cytopathic effect inhibition assay on rat embryo fibroblasts, and their activity is expressed in U standardized against a laboratory reference. In most experiments 10^3 U IFN-γ/mouse (i.e. 0·25 µg/mouse) was injected i.p. at the indicated times.

Immunizations. Groups of eight to 20 mice were immunized with two injections of an R41-A vaccine dilution (0·25 ml) via the i.p. route on days 0 and 7. On day 14 the animals were challenged by intracerebral (i.c.) injection with 30 µl of CVS-26 rabies virus containing approximately 30 LD_{50}. In the subsequent period the animals were observed for the development of rabies-related symptoms including paralysis. Mortality in the non-vaccinated control animals generally occurred between 7 and 14 days after infection. Other groups of mice received the vaccine in conjunction with cytokine treatment. Titration of the CVS challenge virus was included in each experiment; this is mandatory since reproducibility of LD_{50} values is poor, a problem generally recognized in vaccine potency testing. Therefore only data from the same experiment were compared.

Antibody responses. On days 6 and 13, mice were anaesthetized and bled from the retro-orbital plexus. In individual serum samples rabies virus-specific Ig titres were determined by ELISA and VNAb titres were measured by a rapid fluorescence focus inhibition test (Zalan *et al.*, 1979). The ELISA titre is defined as the reciprocal of the highest serum dilution at which the absorbance was equal to three times the background value.

Proliferative splenocyte responses. C57BL/6 mouse splenocytes isolated 2 weeks after the last immunization were cultured in 96-well round-bottom plates at a density of 3×10^5 cells per well in Iscove's modified Dulbecco's medium containing 1% C57BL/6 mouse serum. Inactivated rabies virus was added at the concentrations indicated in Fig. 3. After 4 days of incubation at 37 °C in a humidified atmosphere containing 5% CO_2, cultures were pulse-labelled with 0·5 µCi [^{3}H]thymidine (Amersham) for 16 h. Cells were harvested on glass filters and the incorporated radioactivity was measured in a Betaplate flat bed scintillation counter (LKB-Wallac).

Statistical analysis. To compare group means of VNAb titres, data were evaluated using the Wilcoxon-Mann-Whitney test. In order to take into account the greater probability of a type I error due to multiple comparisons, the level of significance was pre-set according to Bonferroni's adaptation at $P < [0{\cdot}05/\text{number of comparisons}]$ instead of a fixed $P < 0{\cdot}05$. Group means of splenocyte proliferative responses and Ig isotype ratios of PBS-treated vaccine controls and IFN-γ-treated mice were compared by using the two-tailed Student's t-test. The 50% protective vaccine doses (PD_{50}) were calculated according to Reed & Muench (1938).

Results

Exogenous cytokines augment immunization-induced protective antiviral immunity

Two immunizations of mice with PM-DKCV (dilutions 1:100, 1:1000 or 1:10000) resulted in a dose-dependent resistance to challenge. At the highest vaccine concentration (dilution 1:100) TNF-α and IL-1α improved survival upon challenge, resulting in high (90%) or complete survival, respectively, compared to 40% survival of mock-vaccinated mice (Fig. 1*a*). At a vaccine dilution of 1:1000, TNF-α treatment had no effect on survival, whereas IL-1α treatment caused a prolongation of survival times, with a mean time to death of 9·6 days as compared to 8·3 days in the control group (data not shown). No increase in numbers of long-term survivors was observed (Fig. 1*a*). At the 1:10000 vaccine dilution TNF-α and IL-1α did not influence resistance to challenge.

In contrast, IFN-γ and IL-2 dramatically increased survival upon challenge at all the vaccine concentrations tested (Fig. 1*a*). This effect was still observed in groups of mice injected with a non-protective vaccine dilution of 1:10000. Co-administration of IFN-γ or IL-2 induced 45 and 60% survival, respectively. IFN-γ or IL-2 treatment in non-immunized animals had no effect (data not shown). When compared to the PBS-treated vaccine control group, PD_{50} values were increased 4·2-fold by TNF ($PD_{50} = 10^{2.6}$), 6·8-fold by IL-1 ($PD_{50} = 10^{2.8}$), 50-fold by IL-2 ($PD_{50} = 10^{3.7}$) and 30-fold by IFN-γ ($PD_{50} = 10^{3.5}$); PD_{50} of PBS = 10^2.

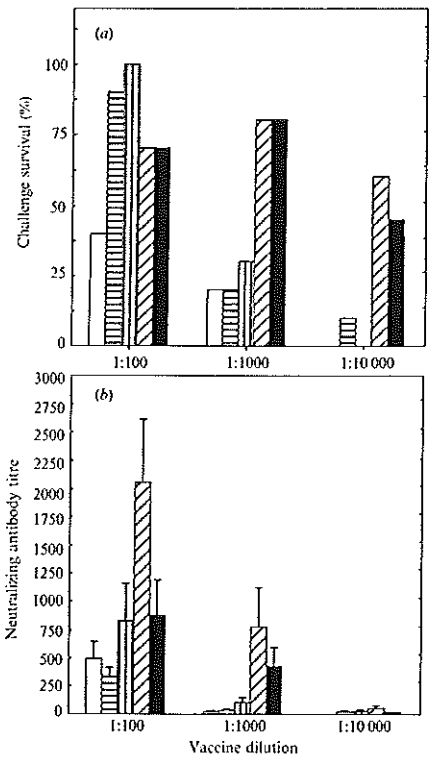


Fig. 1. Effect of TNF-α, IL-1α, IL-2 and IFN-γ on the protective activity (*a*) and VNAb responses (*b*) induced by inactivated rabies virus. NIH mice (10 per group) were immunized i.p. with the indicated dilutions of inactivated rabies virus vaccine (R41-A) at days 0 and 7; 15 min before each immunization the animals were injected i.p. with murine rTNF-α (▤) (1·3 ng/mouse), human rIL-1α (□) (1·3 ng/mouse), rat rIFN-γ (■) (10^3 U = 0·25 μg/mouse) or PBS (□). Another group of mice received human rIL-2 (▨) (3×10^4 U = 1·6 μg/mouse) administered daily on days 0 to 4 and days 7 to 11. The animals were bled on day 13 and challenged i.c. with CVS-26 (34 LD_{50}) on day 14. VNAb titres (mean values ± S.E.M.) were determined for five individual mice per group.

Table 1. *Effect of cytokines on induction of rabies virus-specific serum IgG**

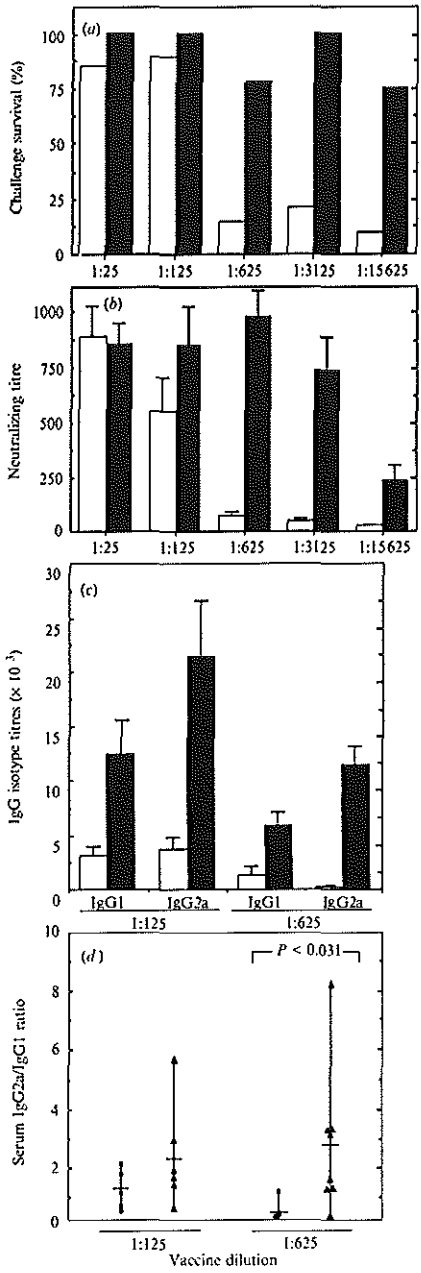
	Virus-specific serum IgG†			
	Vaccine dilution 1:100		Vaccine dilution 1:1000	
Treatment	Day 6	Day 13	Day 6	Day 13
PBS	45 ± 28	732 ± 150	< 10	167 ± 93
TNF-α	192 ± 62	3560 ± 1149	18 ± 7	270 ± 140
IL-1α	208 ± 68	8060 ± 3089	28 ± 11	1332 ± 518
IL-2	560 ± 93	8680 ± 1819	92 ± 44	3380 ± 655
IFN-γ	398 ± 166	3080 ± 773	86 ± 30	1364 ± 580

* NIH mice (five per group) were i.p. immunized with a 1:100 or a 1:1000 vaccine dilution at day 0 and boosted with the same vaccine dose at day 7. The animals were injected with PBS or different cytokines as indicated in the legend of Fig. 1.

† The animals were bled on days 6 and 13. Geometric means (± S.E.M.) of anti-rabies virus IgG titres were determined as previously described (Zalan *et al.*, 1979).

Exogenous cytokines cause an increase in antiviral IgG responses after primary and secondary immunization

The levels of the humoral immune response were determined by measuring antibody titres in sera collected from immunized mice at days 6 and 13, 6 days after primary and secondary immunization, respectively. All cytokines tested augmented rabies virus-specific IgG levels four- to 12-fold within 6 days after primary immunization. At day 13 a five- to 10-fold increase in specific serum IgG response was noted in all cytokine-treated mice (Table 1).



IL-2 and IFN-γ enhance virus-neutralizing antibody responses

VNAb titres were determined in day 13 sera of vaccinated mice. In vaccine control mice the VNAb titres decreased with a lowering of the vaccine dose, which correlated well with survival upon challenge. Neither TNF-α nor IL-1α influenced the VNAb response at any vaccine concentration tested, although both enhanced protective immunity at the 1:100 vaccine dilution (Fig. 1*a*).

At the 1:100 and 1:1000 vaccine dilutions IL-2 and IFN-γ significantly ($P < 0{\cdot}0125$) enhanced VNAb responses (Fig. 1*b*). Remarkably, at the lowest vaccine dilution (1:10000) both mediators failed to do so despite increasing survival upon challenge. These results suggest that enhanced protection by TNF-α and IL-1α at high vaccine doses and by IL-2 and IFN-γ at low vaccine doses is not solely mediated by VNAb but may also result from antibody that does not neutralize *in vitro* or from enhancement of other, probably cell-mediated, immune mechanisms.

INF-γ causes an increase in antiviral IgG2a production

Since IFN-γ emerged as the most practical immunity-enhancing cytokine, requiring only a single injection shortly before vaccination, we examined its activity in a broader range of fivefold vaccine dilutions (1:25 to 1:15625). Again, it increased PD_{50} values 25- to 77-fold. At all vaccine concentrations tested IFN-γ co-administration increased protective immunity (Fig. 2*a*). Enhancement of protection correlated with increased VNAb levels at each vaccine concentration (Fig. 2*b*). At the lowest vaccine concentration, however, once again low VNAb titres were noted despite good protection.

Given the documented role of cytokines in Ig class switching, we found that IFN-γ caused an increase in the levels of both virus-specific serum IgG1 and IgG2a (Fig. 2*c*). Remarkably, the individual isotype distribution in these mice proved to favour specific IgG2a (Fig. 2*d*)

Fig. 2. Effect of IFN-γ on protective immunity (*a*), development of rabies VNAb (*b*) and antiviral IgG1 and IgG2a (*c* and *d*). NIH mice (10 per group) were immunized i.p. with the indicated dilutions of inactivated rabies virus vaccine (R41-A) at days 0 and 7; two hours before each immunization the animals were injected i.p. with rat rIFN-γ (■) (10^5 U = 0·25 μg/mouse) or mock-injected (□). The animals were bled on day 13 and challenged i.c. on day 14 with CVS-26 (10 LD_{50}). (*a*) Percentage of long-term challenge survivors. (*b*) VNAb titres (mean values ± S.E.M.) determined for eight individual mice per group. (*c*) Antiviral IgG1 and IgG2a serum antibody (mean ± S.E.M) determined for four to eight mice per group. (*d*) Individual IgG2a/IgG1 ratios of antiviral antibody of four to eight mice per group. No IFN-γ (●); IFN-γ (▲).

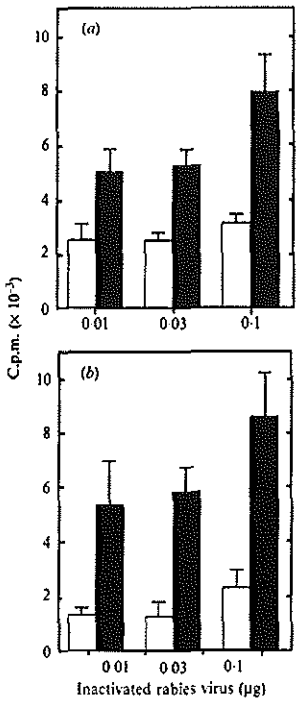


Fig. 3. Effect of *in vivo* IFN-γ administration on *in vitro* proliferative responses of rabies virus-specific splenocytes. C57BL/6 mice (two per group) were given 10^3 U rat rIFN-γ (■) and immunized 2 h later with a 1:100 (*a*) or 1:1000 (*b*) dilution of the inactivated rabies virus vaccine R41-A, on days 0 and 7; all inoculations were via the i.p. route; PBS was used as a control (□). Spleen cells isolated on day 21 were stimulated with different concentrations of inactivated rabies virus antigen. Proliferative response was measured by the incorporation of [^{3}H]thymidine. Results are shown as the net mean±S.E.M. [response −background (medium)] of quadruplicate values obtained from two mice per group.

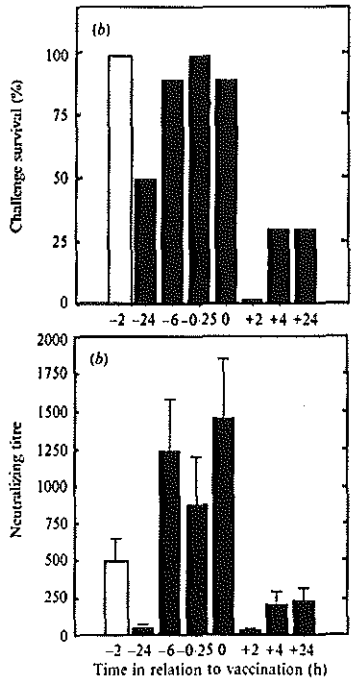


Fig. 4. Effect of the time of IFN-γ administration on inactivated rabies virus vaccine (R41-A)-induced challenge protection (*a*) and VNAb responses (*b*). NIH mice (10 per group) were immunized i.p. with a 1:1000 vaccine dilution on days 0 and 7 and i.p. injected with rat rIFN-γ (■) (10^3 U = 0·25 μg/mouse) at different times before or after immunization as indicated; PBS was used as a control (□). The animals were bled on day 13 and challenged i.c. with 128 LD_{50} CVS-26 on day 14. (*a*) Percentage of long-term challenge survivors. (*b*) VNAb titres (mean values±S.E.M.) determined for five individual mice per group.

whereas the other cytokines did not alter specific IgG2a/IgG1 subclass ratios (not shown).

IFN-γ enhances the priming of rabies virus-specific splenocyte proliferative responses

To determine the effect of *in vivo* administration of IFN-γ on cell-mediated immune reactivity, *in vitro* lymphocyte stimulation tests were performed. To this end, spleen cells isolated 2 weeks after the last immunization were stimulated with PM-DKCV. Fig. 3 shows that *in vivo* IFN-γ administration induced a significant enhancement of the proliferative responses ranging between two- and 3·7-fold ($P < 0·04$), depending upon the concentration of PM-DKCV. These findings demonstrate an IFN-γ-mediated enhancement of the *in vivo* priming of splenocyte responses, which may have contributed to the observed augmentation of protective immunity.

IFN-γ administration post-immunization causes a decrease in protective immunity and VNAb responses

The time of IFN-γ injection proved to play a critical role. Under conditions where the vaccine alone induced complete resistance against a low challenge dose (128 LD_{50}), IFN-γ injection in the 6 h interval before

Table 2. *Effect of combined treatment with IFN-γ and IL-2 on vaccine-induced VNAb responses**

Cytokine treatment	Mean VNAb titre (range)† Vaccine dilution 1:10000
PBS control	7 (4 to 16)
IFN-γ	22 (4 to 64)
IL-2	9 (4 to 16)
IFN-γ + IL-2	417 (4 to 1024)

* Groups of mice, i.p. immunized on days 0 and 7 with 0·25 ml of the indicated R41-A vaccine dilution, were treated with either IFN-γ (10^3 U, at −2 h on days 0 and 7), IL-2 (3×10^4 U on days 0 to 4 and days 7 to 11), or both IFN-γ and IL-2, and bled on day 13.

† VNAb titres were determined in a rapid fluorescence focus inhibition test. Results are shown as geometric mean (range) of individual titres for groups of five mice.

vaccination did not influence the levels of protection but stimulated VNAb responses (Fig. 4). In contrast, when IFN-γ was injected 24 h before or 2 to 48 h after vaccination a marked suppression of both parameters was noted. In a similar experiment in which 20% of the control mice survived a high challenge dose (617 LD_{50}), IFN-γ administration before vaccination (0 to 2 h) stimulated challenge resistance. In mice receiving IFN-γ 24 h before immunization or between 2 and 24 h after vaccination, again both the protection and VNAb titres had decreased (data not shown). These findings show that depending on the timing of lymphokine administration, either a stimulation or an inhibition of immune responsiveness could be observed.

Combined treatment with IL-2 and IFN-γ further increases VNAb responses

Since both IL-2 and IFN-γ enhanced vaccine efficacy it was of interest to examine whether they would act additively or synergistically. Combined IFN-γ and IL-2 treatment did not further enhance resistance to challenge as compared with immunization in conjunction with IL-2 only. Only a 3-day increase in survival times was noted under the conditions tested (data not shown). At a low vaccine concentration (dilution 1:10000) neither IFN-γ nor IL-2 alone affected antibody responses. The combined cytokine treatment, however, synergistically enhanced the development of VNAb (Table 2).

Discussion

In this study, we demonstrate that co-injection of each of the cytokines TNF-α, IL-1α, IL-2 and IFN-γ with inactivated rabies vaccine increases the induced protective antiviral immunity. The mechanisms underlying protection against i.c. rabies virus challenge are not fully understood. Inactivated rabies virus induces circulating VNAbs which are directed against the viral surface glycoprotein (G) (MacFarlan *et al.*, 1986). Neither injection of non-neutralizing anti-G and anti-rabies virus nucleoprotein antibody nor inoculation of spleen cells from mice immunized with the inactivated vaccine preparation induced protection against i.c. challenge (Wunderli *et al.*, 1991). Although antigens from inactivated virus preparations are probably processed by the endocytic pathway and presented by class II molecules to $CD4^+$ helper T cells, we could detect $CD8^+$ CTL activity in vaccinated mice, as have other authors (Wiktor *et al.*, 1977; Morgeaux *et al.*, 1989; Sugamata *et al.*, 1990; MacFarlan *et al.*, 1986). Immunoprotection against rabies encephalomyelitis has also been suggested to result from antibody-mediated disruption of viral cell-to-cell spread and inhibition of viral RNA transcription (Dietzschold *et al.*, 1992). Destruction of the blood–brain barrier following i.c. challenge allows the inoculum to be distributed to the periphery (Cairns, 1950). Part of the i.c. inoculated virus may activate vaccine-sensitized peripheral T cells before being neutralized by circulating antibodies. Therefore, the contribution of cell-mediated immune mechanisms in the elimination of virus-infected cells in the central nervous system should also be considered in our system.

The establishment of immunization-induced immunity is under the control of distinct cytokine cascades. In mice, physiological cytokine production reflects the expansion of functionally distinct T helper cell subsets: Th-1 cells produce TNF, IL-2 and IFN-γ and give rise to cellular reactions, whereas Th-2 cells produce IL-4, IL-5, IL-6 and IL-10 and lead to antibody production. The subset-specific cytokines stimulate expansion of their own subset while inhibiting the other (Fiorentino *et al.*, 1989); activation of Th-1 or Th-2 subsets is often mutually exclusive. Thus, administration of particular cytokines during or shortly before the immunization period may be expected to influence the physiological cytokine balance and may inhibit or stimulate antigen-specific immune reactions.

In immunized control mice (no cytokine added) we found a correlation between protection and induction of VNAb, as reported before by Wunderli and co-workers (1991). In mice immunized in conjunction with a cytokine however, discrepancies between VNAb titres and protection were noted. Both TNF-α and IL-1α, when given shortly before the vaccination, stimulated protection at the highest vaccine concentration (1:100 dilution) without increasing VNAb titres. In contrast, both IL-2 and IFN-γ stimulated protective immunity which correlated well with increased VNAb responses. Notably, at the lowest vaccine concentrations tested

(1:10000 and 1:15625 dilution), which gave minimal or no protection, both cytokines prolonged survival in the absence of increased VNAb titres (Fig. 1 and 2). These data suggest that other protective immune mechanisms are stimulated. These are antigen-dependent and specific, since injection of the cytokines alone did not confer protection. Indeed, virus-specific IgG responses were augmented four- to 12-fold as early as 6 days after primary immunization and five- to 10-fold after secondary immunization in cytokine-treated mice. These non-neutralizing antibodies may be expected to contribute to protective immunity.

The mechanism of the *in vivo* cytokine actions is difficult to define because of their numerous and complex activities. The effects resulting from early cytokine administration demonstrate a modulatory activity at the beginning of the immune response. Indeed, the commitment to a Th-1 or Th-2 response appears to occur very soon after antigen exposure (Scott, 1991). Although inactivated rabies virus by itself can stimulate secretion of IFN-γ (Celis *et al.*, 1986) and IL-2 (Ertl *et al.*, 1989) by T helper cells *in vitro*, it is unknown whether *in vivo* Th-2-characteristic lymphokine secretion is stimulated as well and eventually to a larger extent. Since the PM-DKCV induces both VNAb responses and cell-mediated immunity, including CTL priming and delayed type hypersensitivity (not shown), it is difficult to speculate about induction of either T helper subset.

In contrast to the moderate effects of TNF-α and IL-1α, the Th-1-derived lymphokines IFN-γ and IL-2 vigorously stimulated vaccine potency and VNAb responses at high vaccine doses. IL-2, given daily over a 5-day period, probably acts by stimulating the proliferation of antigen-reactive T cells. IL-2 is the principal lymphokine produced by naive helper T cells (Swain *et al.*, 1991); it acts as a growth factor for both Th-1 and Th-2 cells (Williams *et al.*, 1991), and has a direct role in the induction of B cells (Zubler *et al.*, 1984). Our IL-2 data are consistent with the findings of Nunberg and co-workers (1989) who suggested that IL-2 acts by increasing the cellular immune response to the rabies vaccine. However, because of the requirement for repeated injections the use of IL-2 as an immunological adjuvant is not practical for routine pre-exposure vaccination protocols.

In contrast, IFN-γ is a practical and potent modulator. It is the key regulatory Th-1 lymphokine which favours Th-1-type responses and antagonizes Th-2 reactions. Recent studies in murine *Leishmania* infection models have demonstrated that a single injection of IFN-γ, when included in the parasite inoculum, enhanced Th-1 and decreased Th-2 responses in BALB/c mice (Scott, 1991). Early injection in our system provided enhanced challenge protection, increased VNAb responses and augmented the priming of rabies virus-reactive splenocyte proliferation. In the mouse, the lymphokine is known to promote IgG2a secretion from IgG2a-committed B cells *in vitro* (Snapper & Paul, 1987; Mosmann & Coffman, 1989; Bossie & Vitetta, 1991). Our data show that *in vivo* IFN-γ administration facilitates antiviral IgG2a synthesis. This Ig isotype mediates complement-dependent virus destruction and antibody-dependent cell-mediated cytotoxicity. The augmented antibody responses and elimination of infected cells may have contributed to the increased protective immunity observed. If we assume that physiological IFN-γ production requires a few hours to become established, our finding that post-vaccination IFN-γ injection decreases immune responsiveness is surprising. A similar time-dependent inhibitory effect has been noted with an experimental malaria vaccine (Heath & Playfair, 1991). In this model IFN-γ adjuvanticity correlated with increased expression of MHC class II molecules but was inversely related to lymphocyte influx (Heath *et al.*, 1991). The stimulatory effect of IFN-γ may therefore also result from improved antigen presentation or uptake.

Th-1-like responses are believed to play a major role in the control of most acute virus infections (Mosmann & Coffman, 1989). However, immunization-induced Th-2 reactions resulting in high antibody titres may protect by immediate neutralization of the viral inoculum. Since combined treatment with IFN-γ and repeated daily injections of IL-2 further stimulated VNAb production this regimen may be especially valuable to increase the efficacy of vaccination by preventing systemic spread in those infections which depend upon viraemia during pathogenesis, which is not the case for rabies virus infections.

Previous studies have demonstrated the beneficial effects of type I IFN injection in mice and monkeys previously infected with rabies virus at the same site (Harmon *et al.*, 1975; Baer *et al.*, 1977). Further studies to evaluate whether the observed cytokine modulation also improves systemic post-exposure immunization in rabies infection are in progress. If so, this application would be of major importance for the protection of humans after accidental exposure.

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III. OMV and iscom compared in a bacterial system: *Neisseria meningitidis* as a model

Chapter III.1

Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine.
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Vaccine, 1996, **14:**1001-1008

Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine

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An experimental serogroup B meningococcal vaccine was prepared from two genetically engineered strains; each expressing three different class 1 outer membrane proteins (OMPs) (PorA). The two strains expressed the subtypes P1.7,16;P1.5,2;P1.19,15 and P1.5^c,10;P1.12,13;P1.7^h,4, respectively. Outer membrane vesicles (OMV) were prepared from these strains by deoxycholate extraction, mixed with aluminiumphosphate as adjuvant and formulated to final vaccines. The class 1 OMPs represent ca *90% of the protein in the vaccine. The vaccine was found safe for human use and induced a bactericidal immune response in mice against five of the six wild type strains, which served as donors for the various porA genes. Copyright © 1996 Elsevier Science Ltd.*

Neisseria meningitidis serogroup B strains are responsible for a large number of clinical cases of bacterial meningitis in the Netherlands and worldwide[1,2]. General immunization of the population appears to be the only means to reduce the mortality and morbidity due to meningococcal disease, beyond antibiotic therapy.

The B capsular polysaccharide, being the first choice as a vaccine component, was found to be nonimmunogenic[3]. After chemical modification and conjugation to a carrier protein the polysaccharide was found to be immunogenic in animals[4]. Results with volunteers are not yet described. The issue of safety, however, needs to be addressed. The B polysaccharide is structurally identical to saccharide containing components such as N-CAM in man and mammals. Currently more experience has been obtained with vaccines which are based on meningococcal outer membrane proteins (OMPs). A number of large scale efficacy trials have been carried out with meningococcal OMPs either purified[5] or in an outer membrane vesicle (OMV) formulation[6]. In each instance, the OMPs were derived from wild type strains and the vaccines were designed to combat occurring epidemics. These vaccines, contain multiple OMPs derived from one meningococcus. These OMPs are mainly the class 1, 2 or 3, 4 and 5 proteins. Such first generation OMP vaccines demonstrated efficacies around 50–80% except in infants were their efficacy is negligible[7].

In vitro bactericidal antibody assays were found to be partly strain specific, revealing a particular importance of the class 1 OMP[8]. These findings are in line with immunogenicity and protection data in mice and in rats[9]. The extent of heterogeneity of the class 1 OMP is limited[10] which motivated us to develop multivalent class 1 OMP containing meningococcal OMV vaccines. We constructed a number of vaccine strains which are able to express three class 1 OMPs[11,12]. The expression of Class 3 OMP was eliminated because this protein contributes little to the induction of bactericidal antibodies in mice and man[8,9]. The B-polysaccharide and lacto-*N*-tetraose structures are deleted since those are shared with host structures and immune responses against them are poor and may even be deleterious. The expression of Opc is left untouched, since this protein may induce cross reactive bactericidal antibodies[8], although the expression is poor by many case isolates[13]. The heterogeneity and variability of Opa proteins appears very high[14] so we did not attempt to realise a high expression, although some Opa is left in our vaccine. The six PorA proteins chosen, which constitute about 90% of the vaccine protein, were chosen on the basis of epidemiological data from the Netherlands[1] but also cover the majority of a global strain collection[10]. A major issue to be resolved remains the occurrence

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of microheterogeneity, i.e. point mutations/deletions, within PorA and the cross reactivity of human polyclonal antibodies to such heterogeneity[15]. This issue will be addressed by clinical studies with the vaccine described. In addition to PorA, other major OMP exist that bind/induce bactericidal antibodies such as Tbp-2 and FrpB. These proteins reveal strong heterogeneity[16,17] which currently is not understood in enough detail. Meningococcal vaccine development in essence is still a matter of debate. We have made a first choice for a set of proteins on the basis of combined epidemiological, genetical, structural and immunological data.

In this paper we describe the production and control of an experimental hexavalent class 1 OMP containing (P1.7,16;P1.5,2;P1.19,15; P1.5^c,10;P1.12,13;P1.7^h,4) OMV vaccine using two vaccine strains. Bacteria are treated with deoxycholate (DOC) to extract OMVs. The OMVs were isolated and mixed with aluminumphosphate as adjuvant. The vaccines made thereof were found to be safe and immunogenic and suitable for human use. A safety study with adult volunteers has been carried out and will be presented in a follow up paper[18].

MATERIALS AND METHODS

Bacterial strains

Two *N. meningitidis* strains PL16215 (CPS$^-$, P1.7,16,5,2,19,15) and PL10124 (CPS$^-$, P1.5^c,10,12,13,7^h,4)[12] were used for the production of the OMV-vaccine.

Vesicle production

Hexavalent meningococci-B vaccine was produced using two different *N. meningitidis* trivalent strains PL16215 and PL10124, each producing three different class 1 proteins.

The cultivation medium was meningococci medium according to Frantz, with main components: L-glutamic acid, L-cysteine, glucose and yeast extract[19]. Cells were cultivated at 36°C for 18 h, in a 135 l volume.

The cell harvest was concentrated by centrifugation by continuous flow centrifugation (Westfalia separator) and the cells were resuspended in NaCl buffer. The cell suspension was homogenized for 30 min and the total wet weight of the suspension was determined The cell suspension was centrifuged for 60 min at 2900 *g*. The cell pellet was resuspended in 0.1 M Tris–10 mM EDTA-buffer, 7.5 times wet weight. Extraction of the vesicles was performed by the addition of 1/20th volume of 0.1 M Tris, 10 mM EDTA, DOC (100 g l^{-1}) buffer. Vesicles were separated from cell debris at 20000 *g* at 4°C for 1 h (Centrikon T-1170). The supernatant containing the vesicles was concentrated by ultracentrifugation at 125000 *g* at 4°C for 2 h. The OMV pellet was resuspended in 0.1 M Tris, 10 mM EDTA, DOC (5 g l^{-1}) buffer and the suspension was centrifuged again at 125000 *g* at 4°C for 2 h. The concentrated OMV were resuspended in 3% sucrose solution[20]. The two trivalent bulk products were mixed in equimolar amounts based on class 1 OMP protein content with $AlPO_4$ as adjuvant. Throughout the process thiomersal (100 mg l^{-1}) was added as preservative.

Electron microscopy and immunogold labelling

OMVs were ultrasonically treated to disperse the vesicles and were attached to Formvar/carbon-coated nickel grids. Grids were washed with a 0.01 M PBS supplement with 0.5% BSA and 0.1% gelatine (PBG) and the vesicles on the grids were fixed briefly with 1% glutaraldehyde in PBS and negatively stained with 1% potassium phosphotungstate pH 6.0. The grids were examined in a Philips EM400T electron microscope at 80 kV.

For immunogold labelling grids were washed with 5% BSA+1% normal goat serum in 0.01 M PBS followed with PBG to avoid aspecific background labelling. The grids were incubated at 4°C for 16–18 h with anti-OMP monoclonal antibodies diluted in PBG. After washing with PBG the vesicles were labelled with goat-anti-mouse IgG conjugated to 6 nm colloidal gold (GAMG6) (Aurion, Wageningen, The Netherlands), diluted 1:20 in PBG, for 4 h at 20°C. Control incubations were carried out with irrelevant monoclonal antibodies also diluted in PBG.

The grids were washed again with PBG and the vesicles on the grids were fixed briefly with 1% glutaraldehyde in PBS, washed in PBG, and negatively stained as described.

Protein determination

Protein concentration was measured according to Peterson[21]. Briefly vesicles were solubilized in the presence of 0.15% (w/v) DOC for 10 min. Proteins were precipitated by the addition of 72% (w/v) trichloroacetic acid (TCA) and centrifuged at 3000*g* for 15 min at 4°C. The pellet was dissolved in distilled water. The protein content was determined using copper–tartrate–carbonate and Folin Ciocalteu's phenol reagent. The absorption at 750 nm was determined after 30 min incubation at RT and compared to a bovine serum albumin standard (Pierce, USA).

Monoclonal antibodies

Monoclonal antibodies (MoAbs) against individual subtype specific epitopes[10], different OMPs and mutant lipopolysaccharide (LPS) were used in ELISA, immunoblotting and immunogold EM. A list of the MoAbs used and the antigens which they recognise is given in *Table 1*.

SDS–PAGE, quantitation of protein bands and immunoblotting

Vaccine samples were analysed on 10% polyacrylamide gels in the presence of 2% (w/v) SDS[22]. After electrophoresis proteins were stained by silver staining[23] or by 0.1% (w/v) Coomassie Brilliant Blue staining. The relative amount of class 1 OMP in the vaccine was determined by staining the gel using Coomassie Brilliant Blue. The intensity of Coomassie Brilliant Blue stained protein bands was determined by gelscanning and the relative amount of class 1 OMP is calculated using Imagemaster (Pharmacia, Sweden) software and given as percentage of the total protein content.

Unstained proteins were transferred to nitrocellulose sheets by electroblotting. Proteins were visualized using

Table 1 The MoAbs and the meningococcal antigens that they recognize

MoAb	Protein specificity
MN14C11.6	P1.7
MN5C11G	P1.16
MN22C2.55	P1.5
MN16C13F4	P1.2
MN3C5C	P1.15
MN20F4.17	P1.10
MN20B9.34	P1.4
MN20A7.10	P1.12
MN24H10.75	P1.13
MN15A14H6	Class 3 OMP
MN3B9F	Class 4 OMP
15-1P5.5	Class 5 OMP, specificity 5.5
S3141	Opc
4A8B2	L3 LPS
MN31D8.51	galE LPS

These MoAbs have been used for ELISA, immunoblotting analysis and immunogold EM

type specific MoAb (*Table 1*) or sera derived from mice and Protein-A-HRP conjugate (Amersham, UK).

Identity test by ELISA

The identity of the vaccine was determined by identification of characteristic epitopes using ELISA. Starting at a concentration of 0.3 μg class 1 OMP per well ^{2}log dilution's of the vesicles were coated in PBS 0.01 M pH 7.2 on PVC 96-well plates (Greiner) overnight at room temperature. The coated plates were incubated at 37°C for 1 h with subtype-specific monoclonal antibodies in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA (*Table 1*). The binding of MoAbs was demonstrated using biotinylated goat-anti-mouse-IgG (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 1 h and streptavidin horseradish peroxidase (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 30 min. Binding of the conjugate was visualized using TMB/ethanol substrate. Binding of specific monoclonal antibody should be demonstrated, whereas binding of non-specific antibody (directed against subtypes of the other strain) should be absent.

LPS determination

The LPS content was determined by SDS-PAGE[24]. Vesicles were dissolved in a buffer containing 2% (v/v) β-mercapto-ethanol and 2% (w/v) SDS and boiled for 5 min followed by proteinase-K (2.5 mg ml^{-1}, Boehringer) treatment for 1 h at 60°C. The treated samples were separated by SDS–PAGE with a 14% polyacrylamide gel. LPS was oxidized with 0.4% (w/v) periodic acid and visualized by silver staining. Unknown LPS samples were compared with an LPS standard in the same gel. LPS concentration was also determined by gaschromatography[25].

Endotoxin determination

The biological activity of the endotoxin was determined in the limulus amebocyte lysate (LAL) assay. The lipid-A part of the endotoxin molecule can activate the gelation of the limulus lysate. The OMV vaccine was incubated in a fivefold dilution with a fixed concentration of limulus lysate (Heamachem Inc., St. Louis, USA) at 37°C for 45 min. The endotoxin activity was visualized using Bromthymol Blue. Unknown activity of the endotoxin was compared with *Escherichia coli* standard endotoxin (FDA, Bethesda, USA).

Inactivation and sterility control

Trivalent bulk products were tested for absence of live organisms by incubation of the product on gonococcal agar and Trypton Soy Broth (TSB) medium for 3 days at 37°C in a 5% CO_2 atmosphere.

To test sterility on final lot 5 ml was added to 180 ml Clausen and thioglycolate and incubated for a minimum of 13 days at 20–25°C for Clausen and 30–32°C for thioglycolate. No growth should be observed.

Immunological responses

Immunogenicity testing was performed in NIH mice. Eight week old female NIH mice were immunized subcutaneously with 10 μg total protein (1:10th human dose) of OMV vaccine with 2 mg ml^{-1} $AlPO_4$. After 28 days mice were euthanized and blood samples were collected.

Sera were tested for the presence of subtype specific antibodies using an OMV based ELISA, by immunoblotting and with a subtype specific bactericidal assay. Reference sera for all experiments were obtained from mice which were hyperimmunized with killed wild type bacteria.

For OMV based ELISA, plates were coated with a fixed concentration (0.025–0.1 μg total protein per well) of OMV derived from a single subtype [H44/76 (P1.7,16), 2996 (P1.5,2), MC51 (P1.19,15), 870227 (P1.5^c,10), 870446 (P1.12,13), 892257 (P1.7^h,4)] in PBS 0.01 M pH 7.2 overnight at room temperature. After washing serum samples were titrated in a ^{2}log dilution in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA and incubated at 37°C for 1 h. The binding of antibodies was demonstrated using biotinylated goat–anti-mouse-IgG (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 1 h and streptavidin–horseradish peroxidase (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 30 min. Binding of the conjugate was visualized using TMB/ethanol substrate.

Bactericidal antibodies were determined in subtype specific bactericidal assays. Bacterial strains as mentioned above were used to determine subtype specific antibodies except for P1.7^h,4 in which case 880049 was used as target for bactericidal antibodies. Briefly 25 μl of bacterial suspension (10^5 ml^{-1}) were incubated with baby rabbit complement (Pel-Freez Clinical Systems, Brown Deer, Wisconsin, USA) and ^{2}log dilutions of immune serum at 37°C for 1 h in a volume of 100 μl. Seven microlitres of this mixture was pipetted onto gonococcal agar plates in triplicate and incubated overnight at 37°C in a 5% CO_2 atmosphere. The serum bactericidal titre was reported as the highest reciprocal serum dilution yielding 50% killing of bacteria.

RESULTS

Production

Over a period of 2 years six independent OMV production runs were carried out, three productions

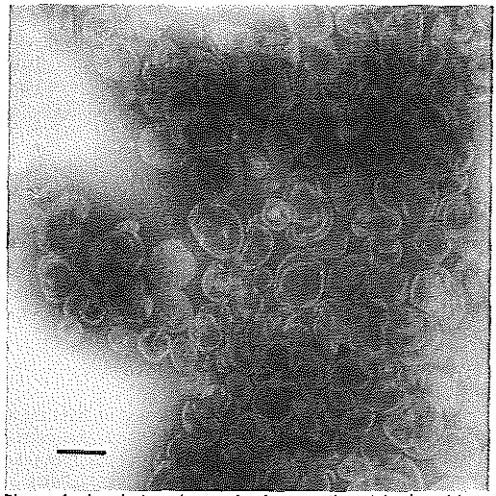

Figure 1 An electronmicrograph of a negative stained vesicle preparation of PL16215. The bar represents 100 nm

runs were performed of each experimental vaccine strain on a 135 l scale as described. After extraction and purification vesicles could be demonstrated in all preparations by negative contrast EM, an example of which is shown in *Figure 1*.

Total protein yield of the OMV production was determined after extraction and purification. Total protein yield ranged from *ca* 250 to 1000 mg in the final product. The differences in yield did not influence the composition or immunogenicity of the of the OMV (data not shown).

Vaccine characterization and identity tests

The composition of the OMV vaccine was characterized using different immunochemical and biochemical techniques.

As shown in *Figure 1* vesicles can be visualized using negative contrast staining and electron microscopy. OMV size ranged from 50 to 150 nm in different preparations. Intactness of the vesicles in these preparations ranged from 25 to 60% of the vesicles. The remaining vesicles were partly broken ("half moon structures"). Only very little amorphous granular material was found. Aggregates of vesicles were also observed frequently especially after the addition of sucrose.

Protein content was estimated by the method described by Peterson[21]. To demonstrate the presence of all relevant class 1 OMP epitopes an ELISA method was used. Vesicles or bacteria were coated on PVC plates and the presence of the relevant epitopes are characterized using subtype specific MoAbs (*Table 1*). The results of a typical assay are shown in *Figure 2*. All relevant epitopes can be demonstrated in both trivalent strains and in the OMVs which were made from these strains. In strain PL10124 OMV a reaction is observed of the $P1.7^{h}$ epitope with the P1.7 specific monoclonal, this reaction is normally absent in bacteria of the same strain (*Figure 2b*). It is known that this epitope is present in the original strain, but as a hidden epitope which cannot normally be detected[26]. It is clear from this figure that it is possible to discriminate between vesicles obtained from different strains. Immunogold electron microscopy using subtype specific monoclonal antibodies revealed that the relevant epitopes were present on the outside of the vesicles (*Figure 3*) and that vesicles derived from the two trivalent strains can be identified on basis of the class 1 protein (*Figure 4*).

When analysed on SDS–PAGE (*Figure 5*) the three class 1 OMPs which were expressed by PL16215 can be separated and their MW can be calculated. Using immunoblot analysis we have been able to demonstrate the origin of the three class 1 OMPs (data not shown). The MW of these proteins differ from that in the original strains due to the cloning procedure which is carried out[12]. Class 1 OMPs in PL10124 OMV can only be separated in two bands. The band with the lowest MW in PL10124 contains two different class 1 OMPs as was demonstrated by immunoblotting (data not shown). Class 4 and class 5 OMP are also present in the vesicles. A protein of 82 kD is also present in small amounts (<1%) in all OMV preparations.

The relative amount of class 1 OMP in the OMVs can be calculated using densitometry. Coomassie Brilliant Blue stained SDS-polyacrylamide gels were scanned using ImageMaster software. The relative amounts of the different OMPs in the OMV vaccine is given in *Table 2*. These results demonstrate that a relatively high portion of total protein in the vesicles is class 1 OMP which are responsible for the induction of bactericidal antibodies. The bactericidal antibodies follow the typical serosubtype characteristics. For clinical research two vaccine lots were prepared that contain 50 and 100 μg total protein per dose (0.5 ml). In *Table 2* the composition of the OMV vaccine of the 100 μg dose is given.

Taken together these results demonstrate that the vesicles are of the desired structure and composition i.e. contain intact proteins with the relevant epitopes in a high concentration.

Safety tests

Regarding the safety of the OMV the following parameters were considered.

- Inactivation of the bacteria which are used to prepare the OMV.
- Sterility of the intermediate and the end products.
- Determination of the toxicity related with the presence of bacterial endotoxin by LAL assay, LPS determination and pyrogenicity testing in rabbits.
- Abnormal toxicity of the hexavalent vaccine.

All safety tests were performed according to international guidelines or requirements [European Pharmacopeia (EP) and WHO]. For OMV based vaccines no specific requirements have been formulated by the EP or WHO. The only comparable vaccine is the Norwegian Folkehelsa vaccine based on H44/76 OMV[20]. This vaccine has been proven to be safe and effective.

Endotoxin determination

The bacterial LPS is a component of the bacterial outer membrane. Moreover, LPS is a structural component of the OMV and, although it is a toxic molecule, it

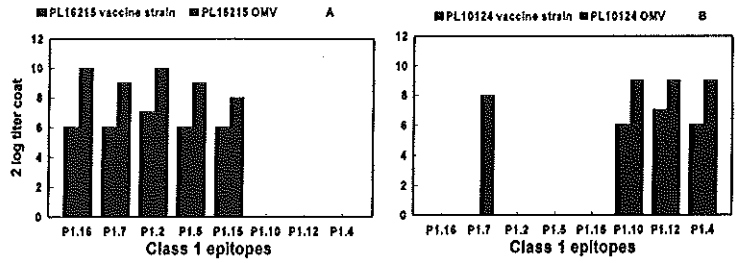

Figure 2 Presence of subtype specific epitopes in the two vaccine strains PL16215 and PL10124 and vesicles derived thereof as measured in ELISA. (A) Results obtained with PL16215 bacteria and OMV, (B) the results obtained with PL10124 bacteria and OMV

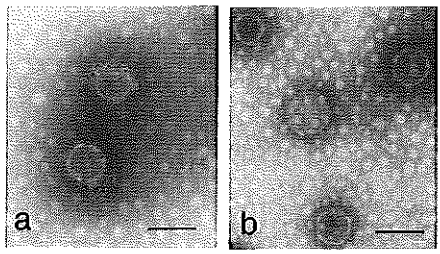

Figure 3 An electronmicrograph of an immunolabelled vesicle preparation of PL16215 (A). The P1.2 epitope is visualized on the surface of the vesicles by immunogold labelling using the monoclonal antibody MN16C13F4 (anti P1.2). Numerous 6 nm gold particles surround the vesicles (B). Control incubation with the monoclonal MN24H10.75 (anti P1.13) showed no gold particles. Bars indicate 100 nm

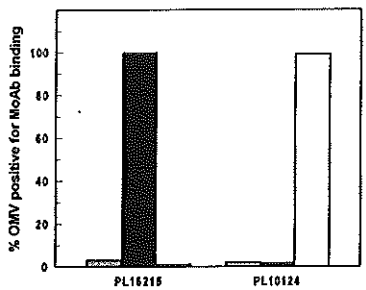

Figure 4 Vesicles of the PL16215 and PL10124 strains were labelled for P1.2 and P1.13 with the immunogold method. The percentage of OMV positive for the two class 1 epitopes is given. Approximately 300 vesicles were analysed per sample. □, Control incubation without subtype specific MoAb; □, a-P1.2 incubation; and ■, a-P1.13 incubation. Almost 100% of the vesicles of each strain were positive for the correct class 1 epitope

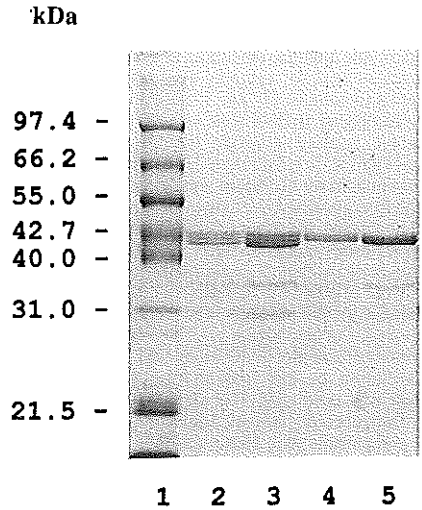

Figure 5 10% SDS–PAGE pattern of different vaccine bulk products. Class 1 OMP are *ca* 40 kDa. Lane 1, low molecular weight markers; lane 2, OMV PL16215 2 μg total protein; lane 3, OMV PL16215 4 μg total protein; lane 4, OMV PL10124 2 μg total protein; lane 5, OMV PL10124 4 μg total protein

Table 2 The composition of the hexavalent vaccine lot containing 100 μg of total protein: one human dose contains 0.5 ml

Substance	Concentration
Protein	200 μg ml^{-1}
Class 1 (6 subtypes)	89%
Class 4	7%
Class 5 (5c and 5.5)	3%
82 kDa	<1%
pH	6.9
DOC	22 μg ml^{-1}
B-PS	Absent
LPS (GalE)	20 μg ml^{-1}
$AlPO_4$	2 mg ml^{-1}
Sucrose	30 mg ml^{-1}
Thiomersal	0.7 mg ml^{-1}

is at the same time being studied as a possible vaccine adjuvant. Therefore we have carefully monitored the LPS content and the biological activity of the LPS in the vesicles. LPS free vesicles cannot be prepared since the presence of LPS in the OMV is necessary for the stability of the OMV vaccine. The LPS/protein ratio is lowered during the production process by partly replacing LPS by DOC. The LPS content was determined on the intermediate products by SDS–PAGE and silver-staining[24]. Results ranged from 2.5 to 10% LPS relative to the protein content. Both European Pharmacopoeia and WHO failed to provide requirements.

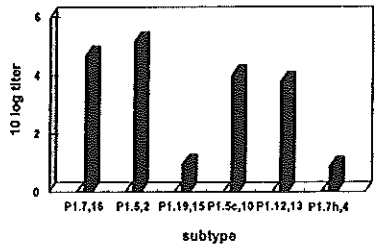

Figure 6 Immunogenicity of a hexavalent vaccine lot. The antibody response was measured in an OMV based ELISA against different subtype strains and is given as a reciprocal log titre of two fold serum dilutions. □, Pre-vaccination titres; ■, post-vaccination titres after 28 days

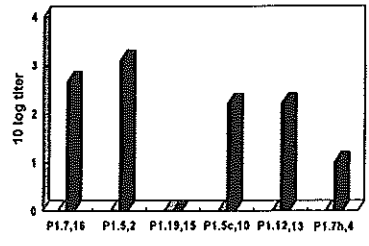

Figure 7 Bactericidal activity of sera of mice immunized with hexavalent vaccine lot. Results are given as reciprocal log titre of two fold serum dilutions. □, Pre-vaccination titers; ■, post-vaccination titres after 28 days

The biological activity of the endotoxin was determined in the limulus amoebocyte lysate (LAL) assay. Hexavalent final lot samples were tested in a fivefold dilution and contain 977 and 195 EU ml^{-1}, respectively, when compared with *E. coli* standard endotoxin. The endotoxin activity is within the range of DPT/polio vaccines and is therefore regarded as safe. Moreover, the endotoxin activity of LPS in vesicles is much lower than that of free purified LPS[20] (own observations).

To determine the pyrogenicity of the OMV vaccine a study in rabbits was performed. Samples of a hexavalent vaccine final lot (which contains the highest amount of LPS) were injected i.v. in rabbits (1 ml kg^{-1}) at 1:100, 1:300 and 1:1000 dilution and temperature rise was monitored. Furthermore a placebo which contains only aluminumphosphate and thiomersal, was injected at 1:100 dilution. For the 1:100 vaccine dilution a repeat of the test was necessary. The other dilutions passed the test. Temperature rises of 1:300 and 1:1000 dilutions were comparable with those obtained with the placebo (data not shown). 1:1000 dilution of the vaccine corresponds with the requirements for 23-valent pneumococcal polysaccharide vaccine and mono-, bi- and tetravalent meningococcal polysaccharide vaccines.

Immunological responses

Immunological responses were studied in NIH mice. Sera obtained from these mice at day 28 after immunization were analysed for the presence of anti meningococcal antibodies by ELISA, immunoblotting and bactericidal assays. Serial dilutions of sera from mice immunized with OMV vaccine revealed subtype specific responses in ELISA and bactericidal assays (*Figure 6* and *Figure 7*). Antibody responses against all individual subtypes which are included by the vaccine can be demonstrated on plates coated with vesicles derived from the original strains. Some epitopes seem to be more immunogenic than others. The P1.19,15 and $P1.7^{h}$,4 subtypes induced very low responses. Since an ELISA only demonstrates the elicitation of antigen binding antibodies but not of functional bactericidal antibodies the sera were also tested in a bactericidal assay. The results of these experiments are given in *Figure 7*. In this assay no anti-P1.19,15 (test strain) antibodies with bactericidal activity could be demonstrated, but all other strains were killed in the presence of complement.

In immunoblot experiments antibodies were demonstrated mainly directed at class 1 OMP but also against other OMP that were present in the vesicle (data not shown). The 82 kDa protein which was present only in small amounts (<1%) induced high antibody titres in mice as determined in immunoblotting. This reaction has also been observed in post-vaccination sera from humans after vaccination with H44/76 vesicles[27]

DISCUSSION

In the bacterial vaccine world, whole cells (inactivated or attenuated), toxoids and capsular polysaccharides (preferentially conjugated to proteins) are established vaccine principles. In addition to this there is a need for OMP based vaccines. Most research and clinical work is dedicated to meningococcal OMP vaccines. The OMV principle combines easy production procedures with potent delivery adjuvant characteristics. The OMP content of OMV vaccines can be manipulated by recombinant DNA technology and LPS in its various forms can be used as an adjuvant. Therefore, the meningococcal OMV vaccine deserves further attention as a meningococcal vaccine development and as a general principle.

We describe the preparation of an experimental hexavalent class 1 OMP containing OMV vaccine. Bacterial OMPs need to be presented in a membrane like structure to remain in or regain proper conformation. We have chosen an OMV formulation for vaccine purposes since this is a production friendly way of preparing immunogenic OMP containing vaccines. In our hands when OMV had disintegrated into AGM (amorphous granular material) the OMP were still immunogenic but the antibodies induced had very low bactericidal activity[28]. The likely explanation for this phenomenon is epitope competition at the B lymphocyte level, leading to a response against surface exposed epitopes (i.e. bactericidal antibodies) in case of an OMV vaccine and to a response against non-exposed epitopes in case of an AGM type vaccine. Alternative approaches for the OMV vaccine principles are artificial membrane like structures such as liposomes or ISCOMs (immune stimulating complexes). In that case the OMPs need to be purified and reintegrated into membrane like structures. Such procedures are more complicated in production terms as compared to the OMV principle.

We have used modified meningococci for our vaccine purposes. The vaccine strains did not express class 3 OMP, B polysaccharide and the lacto-*N*-neotetraose structure. In addition to class 1 OMP which represent *ca*

90% of the protein, class 4 and class 5 OMPs and some 82 kDa OMP are present in the vaccine. Each strain expressed three different class 1 OMPs. The advantages of combining three different class 1 OMP in one strain as compared to mixing OMV derived from three individual strains are obvious. The relative amount of class 1 protein is enhanced and thus it is possible to make a vaccine with a relatively low protein content (100 μg per dose) that contains a high amount of target protein. Moreover the LPS content in a vaccine that is a combination of six OMV preparations would be much higher than in the vaccine described.

The OMVs derived from these strains, were found to be stable over time (18 months) and after thermodegradation experiments (data not shown). In each strain class 1 OMPs are synthesized in equimolar amounts and OMVs of the two individual strains are mixed to contain equal amounts of all class 1 OMPs. The choice for the class 1 OMP was driven by findings in man and animals which demonstrate this protein to be a major target for bactericidal antibodies[9]. The heterogeneity of the class 1 OMP appears to be limited[10]. The P1.19,15 subtype in the trivalent OMV was non-immunogenic in the animal model tested for bactericidal antibody induction. Immunization of NIH mice with OMV containing only the P1.19,15 subtype and no other class 1 OMP did induce bactericidal antibodies (data not shown). The decreased immunogenicity of the P1.19,15 epitope in the trivalent vaccine in mice is now studied in our laboratory, however, an anti-P1.19,15 response could be demonstrated in some human adult volunteers i.e. fourfold rise in bactericidal antibody titre[18]. Whether it is immunogenic in children after a first immunization remains to be investigated.

The vaccines were also found to be safe for human use although LPS is present in a 10% w/w ratio as compared to OMP. It is known that the OMV structure has a shielding effect on the endotoxin properties of LPS[20]. Taken together the results with regard to safety of the vaccine do not deviate much from the data obtained with the Folkehelsa vaccine[20]. Since this vaccine proved to be safe in adolescents we do not expect adverse reactions on the basis of these observations.

A phase I study in adults with this vaccine has been carried out[18] and phase II studies in children and infants have started.

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Chapter III.2

Immunogenicity of various presentation forms of class 1 outer membrane protein of *Neisseria meningitidis* in mice.
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Vaccine in press

ABSTRACT

In this study we compare different vaccine formulations containing meningococcal class 1 outer membrane protein (PorA); purified PorA, outer membrane vesicles (OMV) and immune-stimulating complexes (iscom). Bactericidal antibodies could be generated by the OMV and iscom formulation but not with purified PorA using either $AlPO_4$ or Quil-A as adjuvant. OMV and iscom formulations revealed similar immunogenicity when tested in a dose response manner, with respect to bactericidal as well as OMV-binding antibodies. The anti-OMV IgG subclass response induced by PorA in OMV formulation was found in all subclasses IgG1, IgG2a, IgG2b, IgG3. OMP-iscoms induced very high IgG1 anti-OMV antibodies but almost no IgG3 response.

Also, OMP-iscoms appeared to be a potent inducer of antibodies directed against linear peptides corresponding to surface exposed loops of PorA. In addition, iscoms as well as purified PorA with Quil-A as adjuvant (but not with $AlPO_4$) induced high levels of antibodies against purified PorA. In summary, in addition to the OMV formulation, only iscoms containing PorA are able to generate an anamnestic and bactericidal antibody response.

INTRODUCTION

Neisseria meningitidis is an encapsulated bacterial species which causes invasive diseases such as meningitis with incidences that can sometimes reach epidemic levels. Infectious diseases caused by encapsulated bacteria can be prevented by immunization with vaccines generating antibodies to the capsular polysaccharides. In contrast to the group A and C capsular polysaccharides, the group B meningococcal polysaccharide induces poor or negligible bactericidal antibodies in humans (1). One of the explanations for this poor immunogenicity is the similarity of poly-α- 2,8-neuraminic acid, which composes the B polysaccharide, with sialic acid moieties on human cells (2). In addition to the presence of anti-capsular antibodies, bactericidal and opsonizing antibodies to noncapsular meningococcal surface antigens have been demonstrated in humans after meningococcal disease (3,4). For this reason many investigators have chosen to develop a vaccine against group B meningococcal disease based on outer membrane proteins (5-7, 8, 9, 10,11,12, 13-15,16).

A large number of proteins can be identified in the outer membrane (17). Most isolates contain the PorA protein, PorB (class 2/3 OMP), Rmp (class 4 OMP), Opc (class 5c OMP), and several Opa (class 5 proteins). Many other OMPs have been recognized amongst which iron-limitation inducible OMPs, such as LbpA and LpbB, lactoferrin binding proteins; FrpB (70 kD), function unknown; TbpA and TbpB, transferrin binding proteins. The antigenic heterogeneity of PorA and PorB forms the basis of typing of meningococcal strains into subtypes and serotypes, respectively (18). Monoclonal antibodies with *in vitro* bactericidal activity in the presence of

complement have been raised against PorA and PorB, of which PorA specific antibodies were the most effective (13). In an infant rat passive protection model it was demonstrated that antibodies to class 1 proteins are strongly protective (19). Opa proteins have been shown to be very immunogenic, though they do have a high variability and are therefore of less interest for vaccine development. Opc protein also induces bactericidal antibodies, however most case isolates do not express this protein (20). TbpB and FrpB also induce a strong bactericidal antibody response (21,22). Most results so far demonstrate potent bactericidal antibodies to be directed against strain- or type specific epitopes within the various OMPs (PorA, TbpB, FrpB, PorB, Opa).
Anti-LPS polyclonal and monoclonal antibodies raised in animals have been shown to have bactericidal activity (22-24). Also, in sera of patients bactericidal anti-LPS antibodies could be demonstrated (25,19). However, LPS is not suitable for vaccine development in its natural form. Saccharide-protein conjugates or liposomal LPS are currently being investigated as potential LPS vaccines (26,27).
The first generation of meningococcal OMP vaccines, predominantly containing PorA and PorB were derived from wild-type strains and designed to be used in area's dominated by a single strain or clone. These vaccines demonstrated efficaciesabove 4 years of age in the range of 50-80% (5,6,11,28,16). Although antibodies were induced no efficacy was demonstrable in infancy (6). The in vitro bactericidal activity was found to be strain-specific for the greater part (29) although cross reactivity is observed after boosting (12, 20). It is clear from these vaccine studies that OMP vaccines need to be improved. One way to improve OMP vaccines lies in the area of vaccine formulation. Previously, it was demonstrated that the presentation form of PorA was of great importance for the generation of bactericidal antibodies (7,30,13). In this study we compare three presentation forms of the meningococcalPorA: i) purified OMP with $AlPO_4$ or Quil-A as adjuvant, ii) OMP in outer membrane vesicle (OMV) formulation with intrinsic LPS and added $AlPO_4$ as adjuvant and iii) OMP in immune-stimulating complex (iscom) (31,32) formulation with intrinsic Quil-A as adjuvant.

MATERIALS AND METHODS

Animals

8 to 12 week-old female NIH/RIVM mice (random outbred strain) were used. They were bred and kept at the National Institute of Public Health and Environment.

Antigens

OMP of *Neisseria meningitidis* serogroup B strain HIII-5 (B:-:P1.7,16:L3,8), a PorB-negative variant of H44/76, was purified basically as described for the purification of gonococcal porin protein IB by Teerlink et al. (33). Lyophilized meningococci were extracted with Zwittergent 3-14 in 0.5 M $CaCl_2$. The extract was precipitated with 20% ethanol and freed from ethanol and $CaCl_2$ by repeated dilution and ultrafiltration. Further purification was achieved by ionexchange chromatography (DEAE

Sepharose) and gel permeation chromatography (Sephacryl S-300). Fractions were analyzed by SDS-PAGE, and PorA containing fractions were pooled. The purified protein was stored at 4°C with 0.2 % sodium azide (w/v) as a preservative.

OMP containing iscoms were prepared according to the procedure described for gonococcal porin protein 1B containing Iscoms (34,35). Briefly, the purified OMP was ethanol precipitated and solubilized in octylglucoside (Sigma) in Tris buffered (10 mM) saline (140 mM) pH 7.4. Solubilized OMP and Quil-A (Spikoside, Iscotec, Sweden) were added to lipid micelles of phosphatidylethanolamine type IIIA (Sigma) and cholesterol (Sigma). The weight ratio of OMP : Quil-A : Phosphatidyl-ethanolamine : cholesterol was 2:20:5:5. The micelles were dialyzed against the Tris/NaCl buffer at 4°C. The Iscoms were purified on a sucrose gradient 10-60% in Tris/NaCl buffer. and the iscom fraction was dialyzed against Tris/NaCl. Incorporation of OMP was confirmed by SDS-PAGE of the gradient fractions. The iscoms were structurally identified by negative contrast electron microscopy. The presence of PorA was demonstrated by SDS-PAGE of the OMP-iscom and immunoblotting with monoclonals recognizing the P1.7 and P1.16 epitope. Both monoclonals recognized a 43 kD protein band in the immunoblot and no degradation products could be demonstrated.
OMV of *Neisseria meningitidis* serogroup B strain H44/76 and of a multivalent serosubtype *Neisseria meningitidis* strain PL16215 were prepared by deoxycholate extraction of fermentor grown bacteria. OMV were obtained after high speed and ultra centrifugation and were subsequently adsorbed to $AlPO_4$ (36).
PL16215 is a genetically engineered H44/76 strain that expresses three different PorA proteins, P1.7,16, P1.5,2 and P1.19,15 and does not express lactoneotetraose, PorB and B-polysaccharide (10).
OMV were structurally identified by negative contrast electron microscopy. A variable size of 10-100 nm was found for these vesicles. The presence of PorA, Rmp, Opa5.5 and Opc was demonstrated by SDS-PAGE and immmunoblotting. Approximately 30% of total protein present in H44/76 OMV was found to be PorA. For PL16215 OMV, approximately 90% of total protein was found to be PorA, with equimolar distribution of all three PorA subtypes, implying the P1.7,16 OMP to be present at about 30% of total protein. Both OMV preparations contained approximately 10% of LPS (w/w protein) as was demonstrated with silver staining after SDS-PAGE.

Immunization scheme
Groups of 5 to 6 mice were immunized subcutaneously with 0.25 ml solutions of variable doses (ranging from 0.33-10 μg or 30 μg) of OMP adsorbed to $AlPO_4$ (RIVM), OMP with Quil-A, OMP-iscom, H44/76 OMV adsorbed to $AlPO_4$ or PL16215 OMV adsorbed to $AlPO_4$ in saline. A subcutaneous second immunization with the same dose of antigen was given to each animal 28 days after the first immunization. Blood samples were collected from the retro-orbital plexus at regular

intervals after each immunization. The blood was allowed to clot at room temperature, the serum was withdrawn and stored at -20°C until use. From every individual serum collected on day 42 from a given experimental group a 10 µl sample was used to prepare pooled samples. These pooled samples were used for analysis of antibody responses on this day. All other assays were performed with individual serum samples of experimental groups in which antibody responses could be detected in pooled samples.

Serum antibody assays

Antibody titers to H44/76 OMV , 2996 OMV(B:2b:P1.5,2:L3,8) and purified HIII-5 OMP were determined by ELISA as described below.

Antibody titers to OMV of H44/76 or 2996 and to HIII-5 OMP were measured on highly activated immunoassay microtiter plates (Flow, Irvine, United Kingdom) coated overnight at roomtemperature with 5 µg/ml of total protein of OMV or 2 µg/ml of purified class 1 OMP in 0.01 M phosphate buffer pH 7.4. Plates were washed with tap water-Tween 80 (0.03 % (w/v)). All plates were then incubated for 2 h at 37 °C with three-fold serial dilutions of serum samples in PBS containing 0.1 % (w/v) Tween-80. Binding of specific antibodies was demonstrated with peroxidase conjugated goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL), which detect IgM, IgG, IgG1, IgG2a, IgG2b or IgG3, in PBS-Tween-80 0.1 % (w/v) and skimmed milk powder 0.5 % (w/v) for 90 min at 37 °C. Plates were washed again and incubated at room temperature with 100 µl of the peroxidase substrate 3,3',5,5'-tetramethylbenzidine (0.1 mg/ml; Sigma)-0.01% H_2O_2 (Merck) in 0.11 M sodium acetate buffer pH 5.5. After 10 minutes, the reaction was stopped by adding 100 µl of 2 M H_2SO_4. The A_{450} was registered on a Biokinetics microplate reader EL312e. The data from each plate were analyzed by using the KinetiCalc V2.03 calculation programme. All IgG antibody titers were expressed as percentages of a reference hyperimmune serum either for H44/76 OMV, 2996 OMV or P1.7,16 OMP.

Antibody titers towards the serotype-specific variable domains in loop 1 and loop 4 of the P1.7,16 class 1 OMP were determined by using a peptide ELISA. Biotinylated 18-mer peptides corresponding to the amino acid sequence of surface exposed loop 1 (QLTEAQAANGGASQVKV) and loop 4 (YTPAYYTKDTNNNLTLVP) of the P1.7,16 class 1 OMP were bound to avidine coated plates. The ELISA was further proceeded as described above.

SDS-PAGE and Western blotting

H44/76 OMV preparation was separated on a 12 % SDS PAGE gel at a constant amperage of 30 mA/gel for approximately 1 hour. Sample buffer was added to the OMV preparation and the mixture was heated for 5 min. at 100°C. A total protein concentration of 10 µg protein was loaded on the top of a lane. After running the gels, the separated proteins were transferred to nitrocellulose sheets in a BioRad Trans blot

semi dry transfer cell at a constant current of 0.8 mA/cm^2 for 1 hour. The nitrocellulose sheet was cut into 4 mm strips. The nitrocellulose strips were washed twice with Tris buffered saline 0.01 M, pH 7.2 containing 0.5% (w/v) Tween-80 (TBST). After washing, the strips were incubated with a 1:500 dilution of pooled serum samples in TBST. As controls, strips were incubated with monoclonals recognizing the surface exposed loops P1.7 or P1.16, class 4 protein, class 5 protein and 5C protein. Subsequently, the strips were blocked by incubation in TBST and 0.5 % (w/v) skimmed milk powder for 1 hour at room temperature. After washing, the strips were incubated for 1 hour at roomtemperature with peroxidase labeled goat anti-mouse IgG (Southern Biotechnology Associates) diluted 1:10,000 in TBST with 0.5 % (w/v) skimmed milk. The strips were washed again and subjected to coloring with substrate.

Serum bactericidal assay

Neisseria meningitidis serogroup B strain H44/76 (B:15:P1.7,16:L3,8) and the recombinant engineered Men B strain A8-6t (B:15:P1.5,2:L3,8) (30) were used in this assay. The lyophilized strains were rehydrated with PBS and a small sample of this suspension was inoculated onto (gonococcal) GC agar plates containing 1% IsoVitaleX. After overnight incubation at 37°C in 5 % CO_2 the colonies were harvested with a sterile polyester swab and suspended in Mueller Hinton Broth (MHB) with 15 % glycerol. Working seed lots of 1.5 ml were stored at -70°C. A sample of this working seed lot was tested for surface expression. To eliminate phase variation a fresh aliquot was plated on GC agar plates containig IsoVitaleX for each experiment. A scrape was taken form the frozen seed lot and streaked onto a GC agar plate with IsoVitaleX. After overnight incubation at 37°C in 5% CO_2 colonies were harvested in 2 ml of sterile MHB. Approximately 100-200 µl of the stock bacterial cell suspension was added to 20 ml of sterile MHB pre-equilibrated at room temperature to yield an A_{600} between 0.07 and 0.08. The culture flask was then incubated for approximately 2 hours at 37°C with 160 rpm shaking until A_{600} was between 0.23-0.24. This yielded approximately into 1.10^9 CFU/ml. The bacterial cells were diluted in sterile 50 mM phosphate buffer pH 7.2 containing 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.5 % (w/v) BSA (assay buffer) until a concentration of 1.10^5 CFU/ml was reached (bacterial working concentration).

All mouse sera to be tested were heat inactivated for 30 minutes at 56°C. Pooled sterile baby rabbit serum (3-4 weeks old; lot 11814; Pel Freeze Clinical Systems, Brown Deer, Wisconsin) without bactericidal activity against the strains to be tested was used as exogenous complement source in this assay. A sterile polystyrene U bottom 96 well microtiter plate was used for the micro serum bactericidal assay. The total volume of each well of the plate was 50 µl with 25 µl of serially diluted serum in assay buffer (final starting dilution 1:4), 12.5 µl of bacteria, 12.5 µl of complement (final concentration 20 % (v/v) in assay buffer). Controls included samples with buffer, bacteria and complement and samples with serum, bacteria and buffer. A known positive serum sample (either monoclonal or polyclonal was included in each assay;

the acceptable limit of variability was 1 well dilution). After all components were added to each well of the plate a 10 µl aliquot was taken of the control samples with buffer, bacteria and complement and pipetted onto a dry square formed GC agar plate containing 1% IsoVitaleX and allowed to run in lanes down the plate. The microtiter plate was then incubated for 1 hour at 37°C. The GC agarplate with 1% IsoVitaleX was incubated 18 hour at 37°C in 5% CO_2. After 1 h incubation a 7 µl aliquot was taken from each well of a lane using a multichannel pipet and spotted onto a dry square formed GC agar plate containing 1% IsoVitaleX. After 18 hr incubation at 37°C in 5 % CO_2, the colonies on time zero and 60 min-incubation plates were counted. The average number of CFU at time zero was used as 100%. The serum bactericidal titer was reported as the reciprocal of the serum dilution yielding ≥ 50 % killing.

RESULTS

Protein composition of the antigens

The antigen preparations that were used in this study were analyzed on 10% polyacrylamide gels in the presence of SDS (Figure 1). PL16215 OMV (lane 2) contain class three porA's, Rmp, Opa, Opc and trace amounts of an 82 kD OMP. H44/76 OMV (lane 3) contain class 1, 3, 4 and 5 OMP and the 82 kD OMP. In the batch of the purified OMP used in this study only one single band with a molecular weight of app. 43 kD (PorA) was detected. In OMP-iscom the 43 kD OMP was the only protein that could be detected.

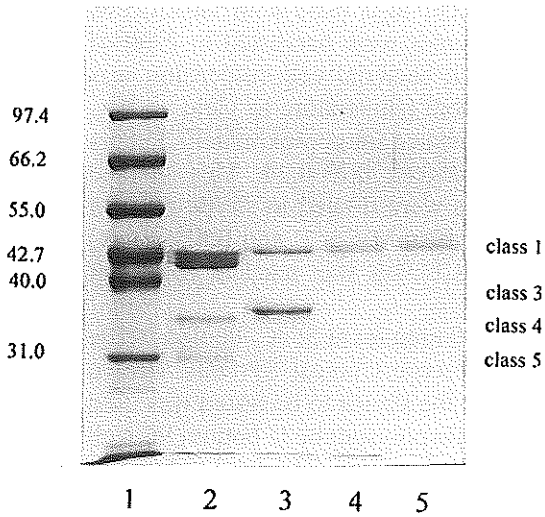

figure 1: Separation of antigens on 10 % polyacrylamide gel in the presence of SDS. Proteins were stained with Coomassie Brilliant Blue. 1: marker proteins, 2: PL16215-OMV, 3: H44/76 OMV, 4: purified meningococcal OMP, 5: OMP-Iscom

Primary and secondary antibody responses generated with various presentation forms of class 1 outer membrane protein

At day 28 after primary immunization, the highest percentage of responding mice for anti-H44/76 OMV IgG antibodies were found in groups of mice immunized with 0.33-10 µg of H44/76 OMV (six out of six) as shown in table 1.

Preparation and dose (µg)	Mean IgG titer SEM	
	day 28	day 42
PL16215 OMV + $AlPO_4$		
0.33	1± 1 (1/6)	3± 9 (1/6)
1.0	1± 1 (2/6)	4± 9 (4/6)
3.3	3± 3 (3/6)	24± 3 (6/6)
10.0	6± 2 (6/6)	41± 1 (6/6)
30	8± 2 (6/6)	194± 4 (6/6)
H44/76 OMV + $AlPO_4$		
0.33	11± 2 (6/6)	30± 1 (6/6)
1.0	13± 2 (6/6)	45± 2 (6/6)
3.3	20± 1 (6/6)	62± 2 (6/6)
10.0	22± 2 (6/6)	168± 2 (6/6)
P1.7,16 + $AlPO_4$		
0.33	N.D.	2± 2 (3/6)
1.0	N.D.	2± 14 (3/6)
3.3	N.D.	1± 2 (1/6)
10.0	N.D.	1± 2 (1/6)
P1.7,16 + Quil A		
0.33	N.D.	1± 1 (0/5)
1.0	N.D.	1± 1 (0/6)
3.3	N.D.	1± 1 (0/6)
10.0	N.D.	1± 1 (0/6)
P1.7,16 in ISCOM		
0.33	1± 1 (0/6)	22± 2 (6/6)
1.0	2± 3 (5/6)	137± 2 (6/6)
3.3	6± 4 (4/6)	258± 3 (6/6)
10.0	2± 3 (2/5)	185± 3 (5/5)
Saline with $AlPO_4$	1± 1 (0/6)	1± 1 (0/6)
Saline with Quil A	1± 1 (0/6)	1± 1 (0/6)
Saline with ISCOM empty	1± 1 (1/6)	1± 1 (1/6)
Saline	1± 1 (0/6)	1± 1 (0/6)

table 1: Anti-H44/76 OMV IgG antibody response after immunization with different antigen presentation forms of P1.7,16.Experimental groups of mice were immunized with an increasing dose of various presentation forms of P1.7,16. A second injection was given in a 4 week interval. Individual sera were analyzed at day 28 after first immunization and at day 14 after secondary immunization. Data are presented as geometric mean SEM of 5 to 6 individual mice as indicated. IgG antibody levels are expressed as percentage of reference serum. The number of responding mice is indicated between brackets. N.D.= not done.

After a boost immunization, all mice receiving OMP-iscom and H44/76 OMV adsorbed to alum had developed IgG antibodies binding to H44/76 OMV. OMP-Iscoms induced higher titers at low doses of antigen.
After primary immunization a dose of 1.0-10 μg of IgG PL16215 OMV or OMP-iscoms was also sufficient to induce seroconversion in most but not all mice. After a boost immunization some mice immunized with the lowest concentrations of PL16215 OMV (0.33 μg and 1.0 μg adsorbed to alum) still did not respond whereas at the higher doses of this antigen all mice responded with equally high titers as mice immunized with H44/76 OMV. Immunization with purified OMP adsorbed to alum or in the presence of Quil-A induced only low IgG anti-OMV antibody titers after a boost immunization indicating that no response against surface exposed OMP epitopes had developed.
Anti-H44/76 IgM antibody responses after immunization with the various presentation forms were rather low (data not shown).

Antibodies to purified class 1 outer membrane proteins and to peptides corresponding with the main surface exposed loops of class 1 OMP
At day 42 , 14 days after boost immunization all groups had developed IgG titers against purified OMP as measured in ELISA as shown in table 2. The highest IgG response was observed in sera of mice immunized with 3.3-10 μg of OMP mixed with Quil-A or 1, 3.3 or 10 μg of OMP-iscoms. The highest response against peptides mimicking surface exposed loop 1 and loop 4 of the P1.7,16 OMP was measured after immunization with OMP-iscoms.

Relatively high anti-OMP IgG antibodies, as compared with OMV immunization, could be detected in sera of mice immunized with OMP with added Quil-A, especially since these sera did not contain any measurable anti-OMV antibodies (Table 1).
Small amounts of IgG anti-loop 1 antibodies could be measured in sera of mice immunized with H44/76 OMV, PL16215 OMV and OMP- iscom. A relatively higher proportion of IgG anti-loop 4 antibodies could be detected in the same serum samples, especially the Iscom formulation at a dose of 3.3 μg.
Immunization with purified class 1 OMP adsorbed to alum or in the presence of Quil-A induced no or low IgG anti-loop 1 or loop 4 antibodies, again indicating that the response measured in these mice is not directed against surface exposed OMP epitopes.
Analysis of IgM antibody levels to class 1 OMP and to peptides in pooled serum samples of each group showed only limited increase of IgM antibodies as compared to the groups of mice immunized with adjuvant alone (data not shown).

Subclass distribution of anti-H44/76 OMV IgG antibodies induced by the various presentation forms
Immunization with OMP-Iscoms induced remarkebly high titers of anti-OMV IgG1 at

low doses (1 and 3.3 μg) but relatively low titers of all other IgG subclasses tested (Table 3). Both OMV preparations induced anti-OMV antibodies of the IgG1, IgG2a, IgG2b and IgG3 subclasses. As expected, anti-OMV titers measured in mice immunized with purified OMP were low for all subclasses.
It should be noted that the distribution of the subclass antibodies not only varied depending on the dose, but also that a large variation in subclass contribution in the anti-H44/76 OMV antibody response was observed among individual mice within one experimental group.

Preparation and dose (µg)	Anti-P1.7,16	Anti-loop 1 P1.7	Anti-loop 4 P1.16
PL16215 OMV + $AlPO_4$			
0.33	6± 5 (4/6)	2± 2 (4/6)	3± 4 (3/6)
1.0	2± 3 (2/6)	1± 1 (0/6)	2± 3 (2/6)
3.3	35± 3 (6/6)	1± 2 (1/6)	14± 5 (5/6)
10.0	64± 3 (6/6)	3± 20 (1/6)	34± 2 (6/6)
30	110± 6 (6/6)	4± 3 (3/6)	35± 4 (6/6)
H44/76 OMV + $AlPO_4$			
0.33	5± 2 (4/6)	2± 2 (2/6)	4± 4 (4/6)
1.0	10± 4 (5/6)	2± 3 (1/6)	12± 4 (5/6)
3.3	21± 3 (6/6)	1± 2 (1/6)	25± 3 (6/6)
10.0	47± 3 (6/6)	3± 2 (5/6)	24± 3 (6/6)
P1.7,16 + $AlPO_4$			
0.33	14± 3 (6/6)	1	6± 3 (4/6)
1.0	41± 11 (5/6)	1	10± 7 (5/6)
3.3	61± 3 (6/6)	1	2± 5 (1/6)
10.0	69± 2 (6/6)	1	1± 2 (1/6)
P1.7,16 + Quil A			
0.33	21± 3 (5/5)	1	1± 1 (0/5)
1.0	71± 2 (6/6)	4	1± 1 (0/6)
3.3	180± 2 (6/6)	6	1± 1 (0/6)
10.0	215± 3 (6/6)	3	1± 1 (0/6)
P1.7,16 in ISCOM			
0.33	31± 6 (5/6)	4± 4 (4/6)	13± 2 (6/6)
1.0	280± 2 (6/6)	3± 12 (4/6)	103± 3 (6/6)
3.3	232± 2 (6/6)	2± 2 (4/6)	538± 3 (6/6)
10.0	322± 2 (5/5)	16± 16 (4/5)	37± 11 (4/5)
Saline	1	1	1

table 2: IgG antibody response to class 1 OMP and to peptides corresponding with the main surface exposed loop 1 and loop 4 of class 1 OMP. Groups of 5 to 6 mice were immunized twice (4 week interval between immunization) with increasing doses of various antigens presentation forms of P1.7,16. IgG antibody levels to class 1 OMP and to peptides corresponding with surface exposed loop 1 (P1.7) and loop 4 (P1.16) outer membrane protein were determined 14 days after second immunization. Data are presented as geometric mean SEM and are expressed as percentage of the standard serum.

Preparation and dose (µg)	IgG1	IgG2a	IgG2b	IgG3
PL16215 OMV with $AlPO_4$				
0.33	2± 4 (2/6)	4± 10 (2/6)	4± 5 (3/6)	4± 8 (2/6)
1.0	7± 3 (5/6)	1± 1 (0/6)	6± 7 (3/6)	1± 1 (0/6)
3.3	41± 5 (6/6)	1± 1 (0/6)	10± 2 (6/6)	4± 9 (2/6)
10.0	90± 3 (6/6)	10± 18 (6/6)	12± 4 (6/6)	20± 9 (4/6)
30.0	34± 8 (6/6)	3± 6 (6/6)	77± 4 (6/6)	6± 25 (3/6)
H44/76 OMV with $AlPO_4$				
0.33	4± 3 (4/6)	1± 2 (6/6)	18± 2 (6/6)	1± 2 (1/6)
1.0	37± 6 (6/6)	4± 9 (6/6)	34± 2 (6/6)	15± 13 (4/6)
3.3	41± 5 (6/6)	117± 7 (6/6)	94± 3 (6/6)	9± 3 (5/6)
10.0	90± 3 (6/6)	65± 9 (6/6)	142± 2 (6/6)	108± 12 (5/6)
P1.7,16 with $AlPO_4$				
0.33	10± 13 (3/6)	1± 1 (1/6)	1± 1 (1/6)	1± 1 (0/6)
1.0	20± 18 (5/6)	1± 1 (1/6)	2± 3 (1/6)	4± 7 (2/6)
3.3	2± 6 (1/6)	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)
10.0	3± 7 (2/6)	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)
P1.7,16 with Quil A				
0.33	1± 2 (0/5)	1± 1 (0/5)	1± 1 (0/5)	1± 1 (0/5)
1.0	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)
3.3	1± 2 (1/6)	1± 1 (1/6)	1± 1 (1/6)	1± 1 (1/6)
10.0	2± 2 (2/6)	3± 2 (2/6)	1± 1 (0/6)	1± 1 (0/6)
P1.7,16 in ISCOM				
0.33	17± 4 (5/6)	3± 2 (0/6)	2 ± 2 (2/6)	1± 2 (1/6)
1.0	152± 2 (6/6)	57± 9 (4/6)	12± 11 (5/6)	3± 5 (2/6)
3.3	410± 3 (6/6)	16± 15 (6/6)	7± 4 (6/6)	2± 2 (3/6)
10.0	70± 9 (5/5)	42± 21 (5/5)	20± 6 (5/5)	2± 3 (2/5)
Saline with $AlPO_4$	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)
Saline with Quil A	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)
Saline with ISCOM empty	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)
Saline	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)	1± 1 (0/6)

Table 3. Subclass distribution of anti-H44/76 OMV IgG antibodies in NIH mice induced by different antigen presentation forms of P1.7,16. Individual sera of 5 to 6 mice per experimental group were analyzed for anti-H44/76 OMV IgG antibodies two weeks after the second immunization. Antibody levels are given as geometric mean SEM and are expressed as percentage of a reference serum containing anti-H44/76 IgG1, IgG2a, IgG2b and IgG3 antibodies. Number of responding mice and number of mice tested after each immunization are indicated.

Bactericidal activity of antibodies induced by various presentation forms of P1.7,16

Immunization with PL16215 OMV induced the highest anti -H44/76 bactericidal antibody titers at day 42 (Table 4).

The mean bactericidal titer of antibodies generated after immunization with H44/76 OMV and OMP-Iscom were comparable. Titers induced with purified OMP preparations were low in pooled serum samples.

An increase in bactericidal activity was measured with an increasing dose of antigen, with respect to PL16215 OMV, saturation was demonstrated with H44/76 OMV and

OMP-Iscom. To investigate whether the bactericidal antibodies were directed against PorA P1.7,16 OMP, pooled serum samples of each of these three groups were analyzed for bactericidal activity towards a recombinant engineered group B meningococcus strain A8-6t (B:15:P1.5,2:L3,8), expressing PorA (P1.5,2) of strain 2996 (Table 4). Bactericidal activity towards this strain could only be demonstrated in sera of mice immunized with PL16215 OMV, which contains P1.5,2 PorA in addition to P1.7,16 PorA. These data demonstrate the PorA specificity of the bactericidal response.

Antigen preparation form and dose (µg)	H44/76 B:15:P1.7,16:L3,8	A8-6t B:15:P1.5,2:L3,8
PL16215 OMV + $AlPO_4$		
0.33	362± 8 (6/6)	512
1.0	322± 11 (6/6)	2048
3.3	912± 6 (6/6)	2048
10	2580± 2 (6/6)	4096
30	3251± 2 (6/6)	2048
H44/76 OMV + $AlPO_4$		
0.33	128± 5 (6/6)	<16
1.0	1024± 10 (5/6)	<16
3.3	1448± 2 (6/6)	<16
10	512± 26 (5/6)	<16
P1.7,16 + $AlPO_4$		
0.33	32	
1.0	57± 19 (3/6)	
3.3	32	
10	64	
P1.7,16 + QuilA		
0.33	64	
1.0	64	
3.3	32	
10	64	
P1.7,16 in ISCOM		
0.33	102± 10 (5/6)	<16
1.0	912± 2 (6/6)	<16
3.3	1824± 4 (6/6)	<16
10	1176± 4 (5/5)	<16
Saline + $AlPO_4$	32	
Saline + QuilA	32	
Saline + ISCOM empty	32	
Saline	32	<16

table 4: Bactericidal titers against N.meningititis strain H44/76 and A8-6t in sera of mice immunized with different antigen presentation forms of meningococcal OMP. Bactericidal titers are expressed as the SEM ± SD of the reciprocal dilution yielding in 50% killing of the bacteria. Number of responding mice is given in brackets. When pooled sera were tested no SD or number of responding mice are shown.
Only mice that responded to strain H44/76 were analyzed for bactericidal activity to strain A8-6t.

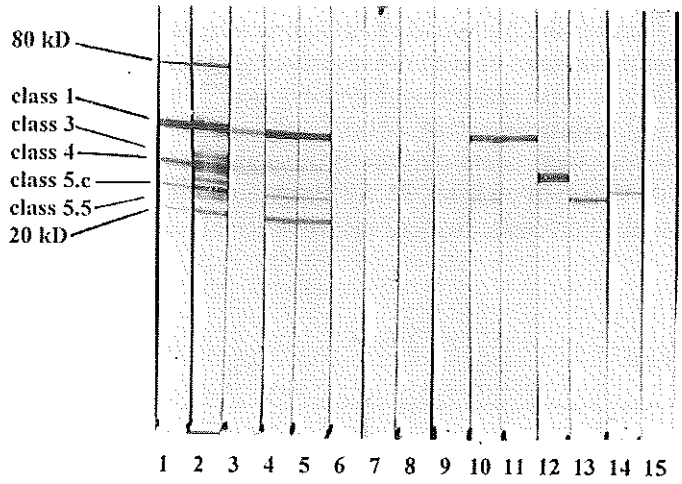

figure 2: Reactivity of antisera and Moabs on Western blots to separated proteins from H44/76 OMV. Strips 1-9 were incubated with immune sera raised against the following antigens; 1; PL16215 OMV, 2; H44/76 OMV, 3; OMP with AlPO4, 4; OMP with Quil-A, 5; OMP-iscom, 6; AlPO4, 7; Quil-A, 8; iscom matrix, 9; PBS. lanes 10-14 were incubated with monoclonal antibodies directed against the following meningococcal OMP; 10;α-P1.16, 11; α-P1.7, 12; α-class 4 OMP, 13; α-class 5.5 OMP and 14; α-class 5.1 OMP. Strip 15 was incubated with goat-anti-mouse IgG HRP conjugate only.

Reactivity of antisera on Western blots to separated proteins from H44/76 OMV

The specificity of antibodies in pooled serum samples (500 times diluted), two weeks after second immunization was determined by Western blotting against H44/76 OMV (Figure 2). H44/76 OMV contain all major OMP that are found in the wild type strain PorA, PorB, Rmp, Opa5.5, Opc and 5c and trace amounts an 82 kD OMP (Figure 1). As shown in figure 2 after immunization with all different antigen preparations anti-class 1 antibodies could be demonstrated in sera. Only in mice immunized with H44/76 or PL16215 OMV preparations a response could be demonstrated against the 82kD OMP. Although this protein was only present in very low amounts in the OMV preparations it induced a strong antibody response. Also a response against a 20 kD OMP, most likely H.8, could be demonstrated following immunization with the OMV preparations. Only H44/76 immunization induced anti class 3 OMP antibodies since this antigen is not present in the other antigen preparations. In general responses observed in immunoblotting experiments correspond with antibody responses in human vaccinee sera after vaccination with H44/76 OMV (37).

Sera from mice immunized with purified meningococcal OMP and OMP-iscom reacted with class 1 OMP but also with class 4, class 5 OMP and the 20 kD OMP . These results indicate that the purified OMP fraction and the OMP-iscom contained trace amounts of OMP other than class 1 OMP, and that these OMP were immunogenic in the presence of adjuvant. In Western blot no difference could be

observed between OMP-iscom or purified OMP in combination with Quil-A. $AlPO_4$ is a weak adjuvant for class 5 OMP and the 20 kD OMP. Control immunizations ($AlPO_4$, Quil-A, iscom matrix or saline) did not lead to detectable antibody levels.

DISCUSSION

In this paper we demonstrate that meningococcal OMV and Iscoms containing meningococcal OMP are equally potent inducers of bactericidal antibodies in mice. We show that OMP-iscoms induce fourfold higher IgG1 titers as compared with OMV preparations. IgG2a, IgG2b and IgG3 titers were lower in mice immunized with OMP-iscoms as compared with OMV preparations. Moreover, immunization with OMP-iscoms results in the induction of 15-fold higher IgG responses directed against a peptide which mimicks a surface exposed class 1 OMP specific bactericidal epitope (loop 4).
Anti-OMP antibodies could also be generated by immunization with purified OMP in the presence of alum or Quil-A. However these antibodies , did not show complement mediated killing and could not bind to OMV.

Sera raised to either meningococcal OMV and to OMP in iscom demonstrated comparable complement mediated killing of meningocci *in vitro*, OMV containing mainly PorA, being the most potent inducer of bactericidal antibodies. The bactericidal activity of antibodies is dependent on the amount, specificity, affinity, subclass and ratio of complement activating and non-complement activating antibodies (38,39,40,41). OMP-iscom induced mainly IgG1 whilst OMV induced antibodies of all IgG subclasses. Whether this has been of any influence on the total amount of bactericidal activity measured in sera raised to OMP in iscom is not known. The correlation between anti-OMV IgG antibodies and bactericidal activity to meningococcal strain H44/76, as measured in individual sera of mice immunized with either various doses of PL16215 OMV, H44/76 OMV or OMP-iscom, was rather low (0.65, 0.29 and 0.47 respectively). The different isotype pattern induced by iscom with a preference for the IgG1 subset suggest a stronger Th-2 type response.
Bactericidal antibodies generated with OMV might be directed to PorA, PorB, or Opa/Opc. In immunoblot (Figure 2) it was demonstrated that all antigen adjuvant combinations induced an anti-P1.7,16 response and antibodies directed against Rmp. Antibodies binding to other meningococcal OMP (82kD, Opa/Opc) were detected in pooled sera of mice immunized with PL16215 OMV, H44/76 OMV and OMP-iscom. Anti-PorB antibodies could only be demonstrated after immunization with H44/76 OMV since this is the only antigen preparation containing these antigens. The bactericidal antobodies induced were dependent upon the subtype specific epitopes of PorA as demonstrated by the use of isogenic target strains containing P1.5,2-PorA instead of P1.7,16-PorA (Table3). A correlation of 0.61 exists between bactericidal activity against H44/76 and serum IgG reactivity with peptides corresponding with surface exposed loop 4 of PorA. Murine PorA specific bactericidal antibodies are able

to bind to loop 1 and loop 4 linear peptides (30). Presumably these sera contain a rather large proportion of functional antibodies, reactive with native surface epitopes.
These findings support the idea that only antibodies to conformational surface exposed epitopes are able to show complement mediated killing (7,30).
Sera raised to meningococcal OMV and to OMP-iscom showed similar reactivities to OMV in ELISA and to linear peptides corresponding to surface exposed loops 1 and 4 in ELISA. For both presentation forms, antibodies were mainly formed to peptides corresponding to loop 4 but OMP-iscoms induced much higher levels of such antibodies. Another possibility is that loop 4 is better recognized by the immune system since it is more exposed as compared to loop 1 and is more exposed in iscoms as compared with OMV. These data suggests that the OMP in Iscom is present in an orientation that resembles that of the OMP in the bacteria and in vesicle formulation, although Iscoms are micellar rather than vesicular structures (35). Kersten et al. demonstrated that the chymotrypsin susceptibility of OMP of *Neisseria gonorrhoeae* in iscom and in OMV was similar (33). The finding that Iscom vaccines induced much higher responses to purified class 1 OMP and loop 4 as compared to OMV vaccines may indicate that incorporation of purified class 1 OMP into Iscom leads to a different conformation. Antibodies to the purified class 1 OMP as observed by Western blot and ELISA were found in sera of mice immunized with all antigen presentation forms of P1.7,16. Data from ELISA showed that anti-purified OMP antibody responses were highest in sera of mice immunized with OMP mixed with Quil-A and OMP- iscom.
Quil-A was found to have a better adjuvant effect for the induction of an immune response to purified OMP as compared to $AlPO_4$.

OMP-iscom also induced a stronger anamnestic response as compared to OMV. These observations confirm results of other studies in which a T-cell independent polyclonal Bcell activating character of OMP was suggested (7,40). It is also in line with results from clinical trials showing rapid antibody declines and poor boosting responses induced by OMV vaccines.However, meningococcal OMP do contain strong T-cell epitopes (43, 44). Different activation of T helper cells such as leading to different activation of Th_1 and Th_2 cells by AlPO4 (OMV vaccines) or Quil-A (iscom vaccines) may also explain the difference in isotype distributions and primary responses.

In conclusion the conformation and presentation of PorA is important for the induction of antibodies able to kill meningococci. The immunogenicity of meningococcal PorA in Iscom is comparable to PorA in OMV. The dose of specific antigen (P1.7,16) needed to induce maximal immune responses was equal for PL16215 OMV, H44/76 OMV and P1.7,16 in iscom. The PL16215 OMV was the most potent inducer of bactericidal antibodies, OMP-iscom induced a stronger anamnestic response. Both OMV and iscom are multimeric presentation forms and possess intrinsic adjuvant activity (Quil-A and LPS respectively). Each of them has advantages and disadvantages (4,32, 15). A major point in favour of OMV is that they can be prepared

more easily as compared to Iscoms. A possible advantage of iscoms is that they are very stable structures when stored frozen or freeze-dried. In our hands a meningococcal OMV vaccine has been stable at 4 °C for more than three years without loss of structural integrity or immunogenicity in mice. OMV vaccines to *Neisseria meningitidis* serogroup B strains are being studied in humans at the moment (5,29,20). The use of OMP in iscom might be an alternative vaccine formulation capable to induce antibodies with bactericidal activity.

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Summarizing discussion

Summarizing discussion

Basic knowledge of the mechanisms underlying the effects of adjuvants and adjuvant systems, is important for the development of vaccines against several infectious diseases for which no effective vaccines are available and against newly emerging infectious diseases (Kaufmann, 1997). More efficient antigen presentation forms are therefore required for the presentation of viral, bacterial and parasite antigens (Nossal, 1997). Also for vaccines which require high amounts of antigen to induce adequate immune responses, like rabies vaccine, adjuvation is needed to reduce the cost (Tsarev et al., 1997). In this thesis the evaluation of two different antigen presentation forms, iscom and OMV, which are both tested in human trials at present (personal commmunication Osterhaus; Peeters et al.,1996), is described. Each of these presentation forms has its advantages and disadvantages and neither one of them can be considered the only and ideal vehicle to present all possible antigens. Not all vaccines need to be adjuved or require a specific presentation form. Live vaccines in general can do without any adjuvant (Bernstein, 1998). Inactivated human polio vaccine contains about 1 µg formaldehyde inactivated virus of three different serotypes (Salk et al., 1982). Without the addition of any adjuvant this vaccine has proven to be very efficient and safe (Salk et al., 1984). In comparison β-propiolacton inactivated rabies virus contains about 200-300 µg viral protein, and is adjuved with the classical adjuvant used in humans, Alum (AlOH or $AlPO_4$) (van Wezel et al., 1978). In rabies vaccine production the amount of RV antigen that can be produced is a bottleneck, thus more efficient ways of presentation are urgently needed. Bacterial vaccines are usually administered in high doses and adjuvated with alum. Moreover some components of bacterial vaccines, e.g. pertussis vaccine, contain LPS which possesses high adjuvant activity (Nurmine and Olander, 1991). However, this component is also responsible for most of the negative side effects that are observed with DPT vaccines. In order to develop better presentation forms for non-replicating vaccines we studied the mechanisms underlying the immune stimulating effects of both iscoms and OMV. To this end we analysed iscom as a model for viral and bacterial antigen presentation and OMV as a model for bacterial antigen presentation.

In chapter II.1. a labelling method for RV antigen and RV-iscoms is described which enabled us to investigate the localization of these antigens *in vivo* after immunization. Experiments revealed that two different subsets of macrophages are involved in the uptake and handling of these antigens. Labelled ß-propiolacton inactivated RV (RV-BPL) was found predominantly in the marginal zone macrophages and the red pulp macrophages whereas labelled RV-iscoms were localized in a small subset called marginal metallophilic macrophages (MMM). Iscom matrix containing no protein antigen but only Quil-A , cholesterol and phospholipids were also found in this

particular subset suggesting that either the iscom structure or the presence of Quil-A is responsible for this phenomenon. Until now the involvement of this subset in antigen uptake (and processing) had not been demonstrated . Targeting to specific APC, such as those MMM, is considered to be one of modes of action of adjuvants. Targeting of antigens to mucosal surfaces is described in chapter II.2. The specific uptake of iscom in M-cells in Peyers patches in the gut was studied *in vitro*. M-cells are APC draining the gut lumen and are involved in the generation of a mucosal immune response. This response is not essential for the protection against rabies but is considered important for other pathogens. Particulate antigens used as a control like inactivated virus or polystyrene microparticles were not taken up at all or very badly in the M-cells in Peyers patches. RV-iscom and *lactobaccillus* were taken up very efficiently by M-cells. Attempts to localize the antigens after oral administration were unsuccessful, probably due to degradation of the antigens in the stomach or, more likely for lipid structures, bile salts in the gut itself. Therefore for oral immunization much higher amounts of antigen are required to induce relevant immune responses (Mowat et al., 1993). Again, the findings described in this chapter and chapter II.1. suggest specific targeting of iscom to certain antigen presenting cells. One of the approaches that can be used to study the role of these phagocytosing cells *in vitro* or *in vivo* is the removal of these cells from the immune system. In chapter II.3. a method is described for the *in vitro* removal of macrophages from (mixed) cell populations. The method, which describes the use of chlodronate containing liposomes to eliminate macrophages is an adaptation of a method that has been described to kill macrophages *in vivo* (van Rooijen and van Nieuwmegen, 1984). Murine macrophages are eliminated from spleen cell preparations within 4 hours. It is demonstrated that at least *in vitro* this method has no adverse effect on the percentage of B- and T-cells in spleen cell populations. Moreover, no direct effect on B hybridoma cells could be measured with regard to proliferation or antibody production. In a system where human EBV transformed B-cells were used to present antigen to specific T-cells it was demonstrated that incubation of either B- or T-cells with chlodronate liposomes did neither influence antigen presentation by the B-cells nor the capability of the T-cells to proliferate after stimulation. Chlodronate containing liposomes were also used to remove spleen and liver macrophages *in vivo*.

The effect of depletion of splenic macrophages on the uptake of, and immune response against, different presentation forms of RV antigen is described in chapter II.4. RV-BPL and iscom containing RV antigen were used to immunize macrophage depleted and control mice. In the absence of phagocytic cells in the spleen, antigen is still trapped in the red pulp and to a lesser extent in the periarteriolar lymphocyte sheaths (PALS) for both antigen presentation forms. The localization pattern in the main area of immune response induction, the follicles, was unaltered after macrophage depletion. Functionally, the depletion of splenic and liver macrophages had no influence on the induction of specific antibody responses in both RV-BPL or RV-iscom immunized mice, even though the latter presentation form was clearly

associated with specific localization in the MMM. In RV-BPL immunized mice, macrophage depletion had no influence on proliferative T-cell responses. However, macrophage depleted mice that were immunized with RV-iscom showed a significant decrease in proliferative T-cell responses. These results confirm existing ideas on the spleen as a physical filter rather than an induction site for humoral responses and shed new light on the efficient role of iscom as antigen presenting moieties in relation to their specific *in vivo* localisation patterns and only partial macrophage dependency.

One of the possible advantages of using iscom for the presentation of viral antigens is their potential to induce MHC class I restricted CTL. In chapter II.5. priming of mice for CTL is described after immunization with both RV-iscom and RV-BPL. *In vitro* experiments revealed that these CTL are restricted by MHC class I molecules on the surface of the infected target cells. It is a remarkable finding that the inactivated form of the RV antigen is able to induce CTL even without the addition of any adjuvant. Although this has been described before in the RV system the finding is in contrast with results from other authors which indicate that induction of CTL require the presentation of antigen in particulate form or in the presence of suitable adjuvants. It shows that the use of iscoms for this particular antigen has no qualitative advantage, although it does have a quantitative effect, with regard to the induction of RV specific CTL after immunization.
A different approach to enhance RV specific responses after vaccination is described in chapter II.6. The *in vivo* administration of exogenous cytokines TNF-α, IL-1α, IL-2 or IFN-γ on the immune response against rabies vaccine in mice was examined. Each of the cytokines increased RV specific IgG responses after primary and secondary immunization. IL-2 and IFN-γ were very potent and led to an 77 and 50 fold increase in PD50 values respectively with a concomitant enhancement of virus neutralizing antibodies. When administered together IL-2 and IFN-γ synergistically enhanced VNAb responses. The timing of cytokine administration proved to be very important as it was demonstrated that IFN-γ was most effective when administered prior to vaccination. When IFN-γ was administered 2 -24 hours after vaccination VNAb responses and protection were dramatically lowered.

In chapter III another antigen presenting system is described that is used to present meningococcal outer membrane proteins (OMP). Using meningococci manipulated to express three different subtypes of the class 1 OMP, outer membrane vesicles (OMV) were prepared. These vesicles are similar to iscoms in that they are a multimeric presentation form with a built-in adjuvant. The adjuvant in this case is the bacterial LPS that is a normal component of the outer membrane and during the formation of the OMV is necessary to maintain the structure and stability of these OMV. Chapter III.1. describes the production and characterization of a hexavalent OMV vaccine and its immunogenicity in mice. The vaccine described in this study was tested in a phase I

study in humans and is currently tested in a phase II trial in infants. It was demonstrated that the OMV vaccine has no negative side effects and that the subtypes incorporated in the vaccine were immunogenic in humans (Peeters et al., 1996; Rouppe van der Voort et al., 1997b and 1998).
Neisseria meningitidis OMP were also incorporated in iscom which made it possible to compare iscom and OMV as antigen presenting systems. Chapter III.2. describes the immunogenicity of different presentation forms of (purified) meningococcal class 1 OMP. In this study OMV and iscoms are compared with soluble OMP using Quil-A and $AlPO_4$ as adjuvant. It is clear from this study that solubilised neisserial class 1 OMP are not able to induce a bactericidal antibody response. Both iscom and OMV were shown to induce functional antibody resposne although differences in isotype distribution were observed.

Potential of iscom and OMV based subunit vaccines
Table 1 shows some aspects of the two antigen presenting moieties studied in this thesis, both adjuvant systems have advantages and disadvantages. From this table it becomes clear that both adjuvant systems, which are presently being evaluated in human trials, have a great potential of becoming routinely applied in human and veterinary medicine to combat and contain infectious diseases. Before discussing the advantages of both approaches, it should be realized that each suffers from the general drawback of non-replicating vaccines, that the amount of the immunizing antigen required is relatively high. However, due to the potent and long lasting immune responses that appear to be induced by both presentation forms, the amount of antigen needed is relatively low as compared to that needed for other non replicating vaccines based on inactivated viruses and microorganisms alone.

Production aspects of iscom and OMV vaccines
One of the prerequisites for vaccine production is that production processes can be executed under good manufacturing practice (GMP) conditions.
Antigens to be presented in iscom and OMV are usually produced by biological fermentation processes followed by down stream processing (DSP) procedures which lead to the final vaccine. When producing iscom vaccines, DSP typically consists of extraction and purification of the relevant antigen structures followed by incorporation in the iscom matrix. DSP in relation to OMV vaccines consists of extraction of the vesicles and purification/concentration which simultaneously removes large amounts of (free) LPS. OMV are relatively simple to produce since they can be obtained by a simple extraction. Cultivation of viruses and bacteria can be upscaled in fermenters for efficient antigen production.
The observation that lower amounts of antigen induce good immune responses when incorporated into iscom could be useful in cases where antigen production is a limiting factor. During the formation of iscom however usually not all antigen is incorporated so this step should be optimized to take full advantage of the lower amount of antigen

needed. Optimal incorporation conditions will have to be determined for each antigen. Antigen production and immunogenicity are limiting factors of the production of RV vaccines especially when produced on continuous cell lines. Maybe a switch to iscom vaccines could promote the acceptance of cell substrates e.g. baby hamster kidney (BHK) cell lines for viral production. In general iscom are of interest where there is a limited amount of target antigens that can easily be incorporated or when specific immune responses are required that can not be induced otherwise. Large scale production of iscom is under development. Upscaling of iscom production is necessary if iscoms are to be considered as candidate for pediatric vaccines. A commercial veterinary influenza vaccine has been on the market for several years now, demonstrating the potential of this vaccine technology (Mumford et al., 1994).

	iscom	**OMV**
Induction of B cell responses		
-biologically active (virus neutralization, bactericidal)	+	+
-long lasting	+	NT
-also in the presence of pre-existing specific antibody	+	+/NT
Induction of potent T cell responses		
-MHC class II restricted CD4+	+	+
-MHC class I restricted CD8+ (CTL)	+	NT
-also in the presence of pre-existing specific antibody	+	NT
Induction of immunity upon local application		
-mucosal immunity	+	NT
-systemic immunity	+	NT
Induction of protective responses	+	+/NT
Absence of toxicity		
-accepted for registered vaccine	+	+
Suitable for large scale production		
-well defined production technology	+	+
-very stable vaccine	+	+
-sterile filtration possible (0.22μm)	+	-

table 1: shows the relevant immunological, toxicological, and economical aspects of iscom and OMV based vaccines. NT: not tested.

It may not always be necessary to incorporate the antigen in the iscom structure as it was shown that influenza antigen in combination with Quil-A matrix elicits equally high antibody responses as influenza antigen incorporated in iscom (Lovgren and Sjolander, 1996). For primary responses the amount of Quil-A required was higher when antigen and Quil-A were not present within the same structure.
An advantage of iscom is that they are very stable structures that can be stored for prolonged periods of time when frozen or freeze dried. Another advantage of iscom is that unlike OMV they can be sterilized by filtration (0.22 μm).

One of the issues in development of OMV vaccines are the DSP requirements. Vesicles are easy to obtain but hard to purify. The procedures described in chapter III.1. involve high- and ultra-speed centrifugation steps that can be used for large scale production. Unlike iscom, OMV are not formed after mixing of individual components. Instead they are formed naturally by some bacterial strains as so called blebs. The biological function of these blebs is unknown but it has been speculated that the bacteria use blebs to fool the immune system. Blebs may bind antibodies and complement and in this way some of the invading pathogenic bacteria can survive in a hostile environment. Blebbing can be promoted by the addition of DOC. Treatment of bacteria with DOC results in the massive formation of OMV and at the same time in the complete inactivation of the bacterium. Purification of OMV that form spontaneously during the cultivation process would require an additional inactivation step to guarantee safety of the vaccine. Temperature inactivation (56°C) works very well with the *N.meningitidis* vaccine strains (data not shown). However it is not known how the antigenicity is affected of the antigens present in the OMV, not whether temperature inactivation has any influence on stability of the vaccine. With respect to inactivation DOC extraction guarantees a safe vaccine.
Also there are some steps in the described process that are susceptible to microbial contamination from the environment. This is of some concern since, unlike iscoms, OMV can not be sterilized by 0.22 μm filtration. Therefore these steps should be validated or replaced by steps in which the risk for contamination is absent. Replacement with other purification methods e.g. ultrafiltration and column chromatography is under development and may result in the possibility to produce batches of preferably at least 50000 doses each to satisfy the needs of vaccination programs. The consistent production of suitable biomass is central to an effective production process. Optimization and upscaling of large scale fermentation processes are the means to achieve this. Such a process has been described for a group B *Neisseria meningitidis*, including a chemically defined medium and event based harvest conditions, resulting in consistent production of 5 g/l biomass under GMP conditions (Fu et al., 1995).
Real time stability studies have shown that the experimental OMV vaccines are very stable structures in $AlPO_4$ adjuvant. Four years after production these OMV are still

similar to the original product with regard to structure (EM), antigen composition and immunogenicity in mice. OMV are very much like the surface of the original pathogen. They lack the capsular polysaccharide (CPS) but contain all the OMP and LPS. Since not all OMP are relevant for protection it was decided to knock out these genes and replace them with class 1 OMP genes (van der Ley et al., 1995). In this way it was possible to construct vaccine strains that express class 1 OMP but no or relatively low amounts of other OMP.
Studies will have to be initiated to investigate whether OMV can be combined with other pediatric (meningitis) vaccines. Other vaccines will most likely be polysaccharide-protein conjugate vaccines that are stable as freeze dried preparations. It is not known whether OMV can be freeze dried and retain their immunogenicity.

T cell responses induced by iscom immunization
It has been demonstrated in many papers that iscom are efficient inducers of T-cell immunity. Both MHC class I and class II restricted reponses can be shown after immunization with iscom vaccines.
Functional class I restricted CTL may add to or may even be necessary for the induction of protection against most viruses and against other intracellular pathogens. CTL could be demonstrated against a wide variety of pathogens after immunization with iscom (Takahashi et al., 1990; Jones et al., 1988; Hsu et al., 1996; Tarpey et al., 1996; Heeg et al., 1991). The induction of MHC class I restricted CTL appears to be associated with the use of Quil-A as an adjuvant (Newman et al., 1992) since liposomes that contain Quil-A and ovalbumin also support the generation of a CTL response (Lipford et al., 1994). The Quil-A containing iscom are targeted to different cellular subcompartments involved in the generation of peptide/MHC complexed that are recognized by the T-cells (van Binnendijk et al., 1992; Heeg et al., 1991; Villacres et al., 1998). Iscom can target the antigens to both endosomal and cytosolic pathways for antigen presentation.
Efficient internalization of the antigen containing iscom also is dependent on the nature of the incorporated antigen. Iscoms containing influenza virus membrane proteins were internalized 50 times more efficiently than ova containing iscom (Villacres et al., 1998).
Within the T helper subset the generation of either Th1 or Th2 type responses can determine the outcome of infection. Iscom are able to induce both type of responses to some extent (Sjolander et al., 1997; Maloy et al., 1995; Villacres-Eriksson, 1995). This is not always beneficial for the induction of protection, for example in the case of vaccination against leishmaniasis (Sjolander et al., 1998b).
After vaccination with influenza virus iscom moderate to strong IL-2, IL-4 and IFN-gamma responses could be demonstrated (Sjolander et al., 1997). As demonstrated in chapter II.6. of this thesis, IL-2 and IFN-gamma have a strong positive effect on the immune response against rabies vaccine. It is therefore tempting to conclude that the

stronger lymphokine responses after vaccination with iscom vaccines are responsible for the higher immune responses. However in IL-4 and IFN-gamma knock out mice normal immune responses were observed after both parenteral and mucosal immunization with ovalbumin containing iscom (Smith et al., 1998).

T-cell responses after immunization with OMV vaccines

One of the goals of OMV vaccine development was to be able to induce a T-cell dependent immune response and memory for the immunizing antigen. Isotype switch, level of immunoglobulin synthesis, affinity maturation and the development of a secondary booster response are all T- cell dependent phenomena. To study whether vaccine induced T-cell responses were generated two independent approaches were followed. In a small scale efficacy study in three human voluteers it was found that proliferative T-cell responses could be measured after the first and second immunization (Rouppe van der Voort et al., 1997b). After a third booster immunization no T-cell responses could be measured in any of the volunteers vaccinated with the Dutch vaccine. Naess (1998) demonstrated high boostable T-cell responses that correlated well with the development of specific IgG titers in volunteers. The differences between the Norwegian OMV vaccine and the Dutch OMV vaccine are many. Differences that may be associated with differences in the immune responses are: wild type LPS versus GalE mutant LPS, differences in protein composition especially the different amount of Opa and Opc, and the use of AlOH instead of $AlPO_4$ adjuvant (Table 2). An increase in the presence of CD45RO+ (memory) T- cells could be measured as well. Wiertz et al. (1991 and 1996) demonstrated that unvaccinated, primed by infection, healthy individuals possess T-cells that recognize peptides corresponding to class 1 and class 5 OMP sequences. If other proteins than class 1 are necessary for the induction of memory they should be included in the vaccine strain.

OMV but not purified PorB protein could recall responses in B10 mice that had been immunized with peptides corresponding to PorB sequences (Delvig et al., 1997). In experiments in mice OMV derived from H1-5, a meningococcal strain that lacks class 1 OMP, induced T-cell proliferative responses that were even higher than those obtained with H44/76 OMV immunization (own observations). Further studies are necessary to determine the nature of the T-cell response against antigens incorporated in OMV. Specificity of the T-cells involved should be studied as well as the question whether they belong to the Th1 or Th2 subset. Establishment of *in vivo* or *in vitro* measurements of lymphokine patterns will provide some insight in this matter. In this respect the role of (modified) LPS and the way in which it influences responses is interesting. LPS or LPS derivatives mainly induce Th1 type responses (Johnson et al., 1990) in mice wereas aluminum adjuvants are known to induce Th2-type responses (Mancino and Ovary, 1980).

Meningococcal group B OMP are used as carrier for peptides and polysaccharide antigens to render them T-cell dependent. Proteasomes containing these OMP can

serve as carrier as well (Levi et al., 1995).
In a recently executed clinical trial in adult volunteers it was demonstrated that OMV are immunogenic even without the addition of alum adjuvant but that responses after a booster vaccination were higher when the vaccine was absorbed as compared with unabsorbed OMV. Absorption of OMV to AlOH reduced pyrogenicity in rabbits. A combination of serogroup C polysaccharide and OMV proved to be highly immunogenic, even without AlOH (Rosenqvist et al., 1998).

Contents	Dutch Vaccine	Norwegian Vaccine
Total protein	100 µg	25 µg
High mol. wt.	2% (80 kD)	5% (70 + 80 kD)
PorA	89%-90%	33%
Subtypes	P1.7,16;5,2;19,15 P1.5[c],10;12,13;7[b],4	P1.7,16
PorB serotype 15	absent	38%
Rmp	7%	12%
Opa/Opc	3%	15%
LPS, immunotype	10%, GalE (truncated)	7%, wildtype (L3, L8)
Phosholipid	nd	8%
B CPS	absent	< 0.065 µg
DOC	11 µg	4 µg
$AlPO_4$	1.5 mg	-
$Al(OH)_3$	-	1.67 mg
Sucrose	10 mg	9 mg
Thiomerseal	0.05 mg	0.05 mg

PorA: class 1 outer membrane protein (OMP)
PorB: class 2/3 OMP
Rmp: class 4 OMP (reduction modifiable protein)
DOC: deoxycholate
LPS: lipopolysaccharide
B CPS: group B capsular polysaccharide
Opa/Opc: class 5 and class 5-like OMPs
nd: not determined

table 2: Vaccine composition of one human dose (0.5 ml) of the Dutch and Norwegian OMV vaccines. (with permission taken from: Meningococcal vaccines. A continuous crusade? (1998) E. Rouppe van der Voort, thesis, Free University Amsterdam).

Induction of protection by iscom

Protection against a variety of bacterial, parasitic, and viral diseases, can be demonstrated after vaccination with iscom vaccines. Comparison with other adjuvants demonstrate that responses after iscom vaccination are usually higher, and also different with regard to induction of specific cellular immunity (Coulter et al., 1998; Rimmelzwaan et al., 1997; Ben-Ahmeida et al., 1993; de Vries et al; 1994). Protective levels of immunity against influenza virus infection could be demonstrated after parenteral or local application (Ghazi et al., 1995; Ben-Ahmeida, 1994; Rimmelzwaan et al., 1997).
Cross reactive protection against influenza virus could be demonstrated and was

associated with the induction of cross reactive CTL (Sambhara et al., 1998).
Protection against measles virus infection, in the presence of preexisting antibodies, in macaques was demonstrated (van Binnendijk et al., 1997; Osterhaus et al., 1998). This demonstrates that iscom may be attractive candidate vaccines in cases were maternal antibodies are present.
From the data presented in this thesis one may conclude that the adjuvant effect of antigen incorporated in iscom is quantitative rather than qualitative when responses are compared with an unadjuvated inactivated RV antigen. Less antigen is needed to induce immune responses and protection against RV infection. In studies performed by Fekadu (Fekadu et al., 1992) it was shown that RV-iscom are superior for post exposure treatment when compared with a widely used human diploid cell vaccine. Protection could be induced with glycoprotein containing iscom in mice.
Iscom vaccination does not allways lead to protection against infection or disease (Hulskotte et al., 1995). After immunization of cats with feline immunodeficiency virus iscoms, virus neutralizing antibodies could be demonstrated but the animals were not protected against a challenge with the virus (Huisman et al., 1998). After vaccination enhancement of infectivity may be seen instead of protection (Siebelink et al., 1995) which is probably attributable to virus specific antibodies (Robinson et al., 1989; Schutten et al., 1994).

Induction of protection by OMV
A number of efficacy trials with OMV based meningococcal vaccines have been conducted since 1987 in Cuba, Brazil, Norway and Chile. Estimated efficacy varies between 50% and 83% largely depending upon age (Bjune et al., 1991; Cassio de Moraes et al., 1992). Bactericidal and opsononizing antibodies, specific for all off the OMP, could be demonstrated after immunization with the Norwegian vaccine (Aase et al., 1995; Rosenqvist et al., 1993, 1995; Sjursen et al., 1990; Delvig et al., 1995; Guttormsen et al., 1993). Complete protection however was not provided by the vaccine since some of the vaccinees contracted the disease (Wedege et al., 1998). Immunization with the Dutch vaccine gave rise to class 1 OMP specific antibodies (Rouppe van der Voort et al., 1996, 1997a). The vaccine described in this thesis is currently being tested in infants and children, but no data are available yet on protective levels that can be reached. The approach to include multiple subtypes into a single vaccine should give a broad coverage against infection with meningococci.
Recently it was demonstrated that after intranasal administration of the Norwegian vaccine persistent local and systemic bactericidal antibodies (Haneberg et al., 1998a, 1998b) OMV can be used as adjuvant/carrier system for intranasal application as well (Levi et al., 1995).

Toxicological aspects of iscom and OMV based vaccines
Large scale application of most adjuvants is hampered by toxicological problems that may occur after application. As mentioned earlier both iscom and OMV contain

adjuvants that are potentially harmful but may also be essential for their adjuvant activity.

Low doses of Quil-A in iscom are preferable because of the toxic nature of Quil-A. Quil-A in matrix or iscom is less toxic than Quil-A in its free form (Ronnberg et al., 1995). Mixing of antigen and Quil-A matrix may work well for antigens that have a multimeric structure by themselves (e.g. inactivated viruses) (Lovgren and Sjolander, 1996) but almost certainly will not work with monomeric antigens (peptides or recombinant proteins), but it may be an alternative for RV-iscom. For other viral antigens (measles virus, HIV) the quality of the immune response may be more relevant.
Quil-A is the unique component that is absolutely necessary for the formation of iscom. Quil-A is a mixture of *Quillaia* saponins extracted from the bark of the tree *Quillaja saponaria Molina*. Purification of Quil-A components has led to the insight that it is composed of fractions that differ in toxicity and adjuvant activity. Studies by Kensil (for review see Kensil, 1995) have led to the widely studied component QS-21 that can be purified from crude Quil-A. Kersten showed that of the individual fractions that can be obtained after separation of crude Quil-A by HPLC only very few peaks are able to form iscom structures (Kersten, 1990, thesis Utrecht).
Crude Quil-A was purified in three components and each of these components was tested for its ability to form iscom matrix and for toxicological properties. Fractions were isolated (QH-A and QH-C) that contribute to the formation of iscoms and have low toxicity (Ronnberg et al., 1995; Behboudi et al., 1995,1996,and 1997). An attempt to chemically detoxify QH-B, a Quil-A fraction with high adjuvant and toxic activity, resulted in a fraction with low toxicity but that was unable to form classical iscom structures (Ronnberg et al., 1997). Iscoms with a defined quillaja triterpenoid formulation named QH703 are presently tested in human trials (Morein et al., 1998a and b). From these results it becomes clear that iscom vaccines with acceptable side effects can be realized.

LPS is an important component of the OMV structure. It is necessary for the integrity of the lipid bilayer. If too much LPS is removed during down stream processing protein aggregates are formed instead of vesicles. These aggregates were found to be non immunogenic. On the other hand one would like to develop a vaccine, which is intrinsically low in toxicity. It was shown that LPS, when present in vesicles or liposomes, has greatly reduced toxicity. OMV demonstrate low pyrogenicity in rabbits (Frederiksen et al., 1991; own observations). Furthermore in a placebo controlled clinical trials only mild side effects were observed (Nøkleby, 1991; and Peeters et al., 1996). Studies on the genes that are involved in the biosynthesis of LPS may provide insight in how to genetically alter the lipid-A core to render it less toxic while still retaining its adjuvant properties. Chemically modified monophosphoryl lipid-A (MPL)

has a reduced toxicity but high adjuvant activity. It is not known whether this would be the same for meningococcal lipid-A which possesses two phosphate groups. Another possibility to reduce toxicity might be the addition or removal of one or more fatty acid side chains of the lipid-A structure. It remains to be established whether meningococcal LPS biosynthesis genes can be altered to result in the of LPS with reduced toxicity while still retaining its adjuvant activity.

It has always been assumed that LPS is an important component of the Neisserial outer membrane and hence of the OMV structure. LPS is supposed to be necessary for the integrity of the membrane. Recently however viable Neisseria isolates have been described that are free of LPS in their membrane (Steeghs et al., 1998). If however, too much LPS is removed during DSP the vaccine no longer contains vesicles but merely aggregates of proteins. Since the conformation of these proteins is not identical to the membrane bound can be expected to be lower and less functional. On the other hand one will want a vaccine to be intrinsically low in toxicity. It was shown that LPS when present in vesicles or liposomes has greatly reduced toxicity.
The contribution of LPS as an adjuvant in OMV vaccines still has to be established. It seems very likely however that the high immunogenicity of antigens in OMV can partly be explained by the fact that antigen and adjuvant (LPS) are incorporated in the same particle. It has been demonstrated that the length of the carbohydrate chain of the LPS has a drastic effect on the bactericidal response to PorA (class1 OMP) in mice (Andersen et al., 1997a). This demonstrates that adjuvant activity of LPS in vesicles is not determined by the Lipid-A part alone. Purified meningococcal serogroup B OMP that were complexed to group C polysaccharide, a LPS free OMP vaccine, was used in a vaccine study in Chile. This vaccine contains no LPS and the proteins are not in their natural conformation. Significant protection could be demonstrated in volunteers 5 to 21 years of age but not in younger children. The experiments described in chapter III.2. of this thesis show that these responses are comparable with the same antigens incorporated in iscom with Quil-A as an adjuvant. In these experiments however OMV were adjuvated with alum. Studies involving liposomes containing antigen in the absence or presence of LPS can answer these questions. A prerequisite is that liposomes can be prepared in which antigen and LPS are incorporated in the membrane rather than being captured inside the liposomes.
In evaluating the role of LPS in OMV vaccines it should be considered that anti LPS antibodies play a role in the protection against bacterial infections as well. It has therefore been postulated that OMV vaccines should contain various LPS immunotypes (Andersen et al., 1997b).

Concluding remarks
Iscom and OMV are interesting candidates for antigen presentation in the development of new generations of vaccines. Iscoms are more widely studied than OMV and have shown a great potential as antigen presentation form for a wide variety of protein

antigens. Antigens derived from viruses, bacteria, mycoplasmatales (Abusugra et al., 1997) and parasites have been successfully incorporated into iscom (for review see Morein., 1998b). Relevant B- and T-cell responses and protection have been described against wide variety of antigens in various animal systems. Iscom can induce relevant immune responses in the presence of maternal antibodies (van Binnendijk et al., 1997; Osterhaus et al., 1998a). Polyvalent subunit vaccines incorporating virus, bacterial, and parasitical epitopes in the same particle are feasible (Sjolander et al., 1998a). Moreover iscoms are also suitable for mucosal vaccination (Hu et al., 1998). Side effects described are limited and the purification of Quil-A components with low toxicity will lead to even safer iscom vaccines (for reviews see Morein et al., 1998a, 1998b and Osterhaus et al., 1998b).

In view of the above, iscom should be seen as an antigen presentation/adjuvant system with great vaccine potential especially for those viral infections and diseases for which at present no efficacious vaccines are available.

OMV vaccines are more restricted in their potential application. In this thesis vaccine candidates were studied using homologous expression of membrane proteins in *N.meningitidis*. The literature describes also meningococcal OMP as carrier/adjuvant for foreign antigen structures (van de Verg et al., 1996). Recently it was demonstrated that OMV can be used for mucosal vaccination. The induction of T-cell responses has been demonstrated but their relevance has to be established. Other bacteria are known to produce vesicles and could be used as vaccine strains. Recombinant DNA techniques could allow the heterologous expression of bacterial, viral and parasitic proteins in bacterial OMP. Theoretically in this way OMV could develop into broad range vaccine carriers.

The magnitude and effectiveness of the induced immune responses varies greatly with the choice of adjuvant and antigen, which emphasizes the need to study different vaccine/adjuvant formulations. Taken together the results presented in this thesis indicate that iscom and OMV are serious presentation forms for new candidate vaccines against a number of viral and bacterial diseases.

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Samenvatting

Gedurende het hele leven wordt de mens geïnfecteerd met een grote variëteit aan microorganismen en virussen die ziekten, soms met lethale afloop of blijvende restschade, kunnen veroorzaken. Ons lichaam beschikt over meerdere afweermechanismen om aan deze infecties het hoofd te bieden. Door middel van vaccinatie is het mogelijk het immuunsysteem op specifieke wijze voor te bereiden op de (eventuele) infectie met een pathogeen agens. Ongetwijfeld het grootste succes van vaccinatie is het wereldwijd uitbannen van het menselijk pokkenvirus geweest. In Nederland is door het grootschalig vaccineren van kinderen op jonge leeftijd het voorkomen van de typische kinderziekten fors teruggedrongen. Dit proefschrift beschrijft studies aan een tweetal "nieuwe" wijzen van vaccinformulering die mogelijk een bijdrage kunnen leveren aan het elimineren van infectieziekten waartegen momenteel nog geen effectieve vaccins bestaan.
Klassieke vaccins bestaan vaak uit verzwakte of "gedode" microorganismen of virussen die een immuunrespons opwekken die uiteindelijk leidt tot bescherming. De huidige strategieën binnen de vaccinontwikkeling zijn er op gericht steeds beter gedefinieerde antigenen in gezuiverde vorm te gebruiken in vaccins, om zodoende in staat te zijn gericht immuunresponsen te stimuleren. Een algemeen nadeel van deze aanpak is dat deze zogenaamde sub-unit vaccins meestal slecht werkzaam zijn, tenzij er stoffen aan toegevoegd worden die het immuunsysteem extra stimuleren. Stoffen die in staat zijn op deze wijze te helpen bij een immuunrespons noemt men adjuvantia (afgeleid van het Latijnse woord *adjuvare* dat helpen betekent). Gezuiverde sub-unit vaccins worden slecht herkend door het immuunsysteem omdat ze "te klein" zijn. Door meerdere sub-units in één deeltje te brengen kan de immuunrespons verbeterd worden. De antigeenpresentatiesystemen die in dit proefschrift beschreven worden, combineren, in hetzelfde deeltje, meerdere sub-units en een adjuvant. De onderzochte multimere presentatievormen zijn immuun-stimulerende complexen (iscom) en buitenmembraan vesikels (OMV).

In hoofdstuk II.1. wordt een methode beschreven om geinactiveerd hondsdolheid (rabies) virus (RV) en iscoms zichtbaar te maken in weefselcoupes met behulp van een fluorescentiemicroscoop. Hierdoor werd het mogelijk te bestuderen welke cellen er kort na immunisatie betrokken zijn bij de opname en verwerking van de antigenen in deze vaccins, in muizen. Het bleek dat er twee verschillende soorten macrofagen in de milt een rol spelen. Opvallend was dat iscoms, ook als ze geen antigeen bevatten, voornamelijk teruggevonden werden in de zgn. marginale metallofiele macrofaag (MMM), een celtype waarvan tot dusverre geen betrokkenheid bij de opname en verwerking van antigeen was aangetoond. Het sturen van de antigenen naar specifieke celpopulaties is een van de mogelijke werkingsmechanismen van adjuvantia. Een goede bescherming tegen pathogene microorganismen start op de plaats waar deze het lichaam binnen treden. Vaak zijn dat de slijmvliezen van de neus, longen en darm

(mucosa). Technisch gezien is dit een grensvlak tussen binnenkant en buitenkant van het lichaam. Deze slijmvliezen beschikken over een eigen immunologisch afweersysteem met daarin specifieke cellen die betrokken zijn bij de opname van (vreemde) antigenen.
In hoofdstuk II.2. wordt beschreven hoe iscoms in de darm opgenomen worden door zgn. M-cellen. De opname van iscoms geschiedt veel efficiënter dan de opname van andere (in grootte vergelijkbare) partikel antigenen (RV en polystyreenbolletjes). Alleen *lactobacillen* werden op vergelijkbare wijze opgenomen in de M-cellen. De bovengenoemde experimenten ondersteunen het mogelijk gebruik van iscom voor orale vaccinatie.
In hoofdstuk II.3. wordt een methode beschreven om *in vitro* macrofagen te elimineren. Aangetoond werd dat de beschreven methode, die gebruikt maakt van liposomen die gevuld zijn met een voor macrofagen toxische stof (chlodronaat liposomen), geen effect heeft op het percentage B- en T-cellen. Ook werd aangetoond dat de B- en T-cellen na behandeling functioneel blijven voor wat betreft antilichaamproductie, antigeen presenterende capaciteit en antigeen specifieke proliferatie.
Om te bestuderen of de fagocyterende cellen die in hoofdstuk II.1. beschreven zijn van belang zijn voor de ontwikkeling van de immuunrespons werd de boven beschreven methode toegepast om *in vivo* lever en milt macrofagen te elimineren. In hoofdstuk II.4 werd aangetoond dat de depletie van de macrofagen geen invloed had op de localisatie van RV antigeen of iscom in de follikels in de milt. Antigenen werden nog steeds weggevangen in de rode pulpa en de periarteriolaire lymfeschede. Er was geen invloed te meten op de specifieke antilichaam respons. Er was wel een significante afname te meten van de proliferatieve T-cel responsen na immunisatie met iscom in muizen die met chlodronaat liposomen behandeld waren. Deze resultaten ondersteunen geldende ideeen dat de milt met name een filterende functie heeft en geen rol in de inductie van de humorale immuunrespons.
In hoofdstuk II.5. wordt beschreven dat zowel immunisatie met RV-iscom alsook met geinactiveerd rabies vaccin in staat zijn RV specifieke cytotoxische T-cellen (CTL) op te wekken. Met behulp van depletie experimenten werd aangetoond dat deze CTL MHC klasse I gerestricteerd zijn. De bevinding dat geinactiveerd rabies vaccin CTL activiteit kan opwekken is niet nieuw echter immunisatie met RV-iscom is efficiënter, dwz er is minder antigeen nodig, voor het opwekken van CTL activiteit in vergelijking met rabies vaccin. De conclusie uit deze studie is dat iscom voor dit specifieke antigeen geen kwalitatief betere respons geeft maar wel een kwantitatief voordeel. Deze bevinding is van belang omdat voor de huidige rabies vaccins antigeenproductie een beperkende factor is.
Een andere benadering om de respons tegen RV te versterken is beschreven in hoofdstuk II.6. De in vivo toediening van cytokinen TNF-α, IL-1α, IL-2 en IFN-γ op de immuunrespons tegen rabies vaccin werd bestudeerd. Elk van de cytokinen had een positief effect op de RV specifieke IgG respons. IL-2 en IFN-γ bleken het meest

effectief met respectievelijk 77 en 50 voudige verhoging van de beschermende waarde. De adjuverende werking van IL-2 en IFN-γ werd versterkt als zij beiden gelijktijdig toegediend werden. Het tijdstip waarop deze cytokinen werden toegediend bleek cruciaal. IFN-γ was het meest effectief als het voor de vaccinatie werd toegediend. Wanneer IFN-γ 2 tot 24 uur na het rabies vaccin werden toegediend namen bescherming en de specifieke antilichaamproductie af.

In hoofdstuk III wordt een ander antigeen presentatie systeem beschreven dat gebruikt wordt om *Neisseria meningitidis* serogroep B (een van de veroorzakers van hersenvliesontsteking) buitenmembraan eiwitten te presenteren. Van gemanipuleerde meningoccocen werden OMV gemaakt die geformuleerd werden tot een vaccin. Deze OMV hebben met iscom gemeen dat zij meerdere copieën van het antigeen bevatten en een ingebouwd adjuvant, in dit geval het bacteriële lipopolysaccharide (LPS). LPS is een normaal bestanddeel van de bacteriele buitenmembraan.
Hoofdstuk III.1. beschrijft de productie en karakterisering van een hexavalent OMV vaccin dat bescherming kan bieden tegen zes verschillende varianten (subtypen) van meningococcen B. Het vaccin dat in dit hoofdstuk beschreven wordt is inmiddels in diverse klinische studies getest. Een van de bescherming inducerende antigenen werd tevens ingebouwd in iscom waardoor het mogelijk werd de beide antigeen presentatie structuren te vergelijken.
In hoofdstuk III.2. worden iscom, OMV en gezuiverd antigeen met Quil-A en AlPO4 als adjuvant vergeleken in een studie in muizen. Alleen iscom en OMV konden beschermende antilichamen opwekken in muizen. Gezuiverde antigenen wekten wel een specifieke antilichaamrespons op, maar deze antilichamen waren niet in staat om *in vitro* meningococcen te doden. Deze resultaten geven aan dat in iscom en OMV de betreffende antigenen in de juiste conformatie aanwezig zijn om een functionele immuunrespons op te wekken.
De resultaten in dit proefschrift geven aan dat zowel iscom als OMV serieuze presentatie vormen zijn voor nieuwe kandidaat vaccins tegen een groot aantal virale en bacteriële infectieziekten.

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Ivo.

Curriculum Vitae

De auteur van dit proefschrift werd geboren te Boxtel op 2 juli, 1959. In 1978 werd het diploma VWO behaald aan het Rythoviuscollege te Eersel. In datzelfde jaar werd begonnen met de studie medische biologie aan de Vrije Universiteit te Amsterdam. In 1986 werd het doctoraalexamen behaald met als hoofdvak Moleculaire Microbiologie en als bijvakken Immunologie en Antropogenetica.
Vanaf 1986 tot 1997 was hij werkzaam bij het Rijksinstituut voor de Volksgezonheid en Milieu te Bilthoven bij het Laboratorium voor Controle van Biologische producten. In deze periode werden de experimenten die in dit proefschrift beschreven staan uitgevoerd in samenwerking met diverse andere laboratoria binnen en buiten het RIVM. Sinds februari 1997 is hij werkzaam binnen het DLO Instituut voor Dierhouderij en Diergezondheid te Lelystad bij de afdeling Quality Management.

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