O USO DE NANOPARTÍCULAS DE QUITOSANO, REVESTIDAS COM ALGINATO COMO ADJUVANTE DO ANTIGÉNIO DA HEPATITE B NA VACINAÇÃO ATRAVÉS DAS MUCOSAS ORAL E NASAL

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ALGINATE COATED CHITOSAN NANOPARTICLES AS ADJUVANT FOR MUCOSAL VACCINATION WITH HEPATITIS B ANTIGEN.

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ABBREVIATIONS

APC	Antigen-presenting cell
CLSM	Confocal laser-scanning microscopy
CpG ODN	Oligodeoxynucleotide with CpG motifs
CT	Cholera toxin
CTL	Cytotytic T lymphocyte
DC	Dendritic cell
ELISA	Enzyme-linked-immunosorbent-assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GALT	Gastrointestinal-associated lymphoid tissue
HBsAg	Surface hepatitis B antigen
HBV	Hepatitis B virus
IEL	Intra-epithelial lymphocytes
ILF	Isolated lymphoid follicle
ISCOMS	Immune-stimulating complexes
LT	E. coli heat-labile enterotoxin
MALT	Mucosal-associated lymphoid tissue
NALT	Nasal-associated lymphoid tissue
NK	Natural killer cell
PLG	Polylactide-co-glycolide
PMSF	Phenylmethanesulfonyl fluoride
PRRs	Pattern-Recognition Receptors
RPMI 1640	Roswell Park Memorial Institute 1640, culture medium
SEM	Scanning electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
Th	T-helper cell
TLR	Toll-like receptor

TGF-β	Transforming growth factor - β
TNF-α	Tumor necrosis factor
VCAM 1	vascular cell adhesion molecule 1

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RESUMO DA TESE

Introdução: A nova geração de vacinas são, na maioria dos casos, resultado dos rápidos avanços que se têm verificado nos últimos anos na área da biologia molecular e da imunologia, permitindo o uso da biotecnologia na produção de proteínas recombinantes com acção imunológica protectora. A vacina contra o vírus da hepatite B é disso o melhor exemplo. Esta vacina foi licenciada pela primeira vez em 1981, sendo nesta altura um produto derivado do plasma de portadores crónicos da hepatite B. Só mais tarde, em 1986, o mesmo antigénio, uma proteína de superfície do vírus da hepatite B, começou a ser produzido em sistemas de cultura celular. A vacina da hepatite B foi a primeira vacina recombinante a ser licenciada e a sua concepção tem servido de modelo ao desenvolvimento de novas vacinas, nomeadamente em doenças provocadas por vírus. Por outro lado, tem sido constatado pela comunidade científica que estas vacinas recombinantes são em geral antigénios fracos e por isso necessitam de ser administrados conjuntamente com substâncias que ampliem o seu efeito, os adjuvantes. Assim, paralelamente ao crescente desenvolvimento, que se tem verificado nos últimos anos, da tecnologia para o desenvolvimento e produção de novas vacinas, assiste-se também a uma crescente investigação na descoberta de adjuvantes mais seguros e potentes que tornem essas vacinas mais eficazes e estáveis.

O termo “adjuvante” vem do termo latim *adjuvare* que significa ajuda. Recentemente, num livro editado por Virgil Schijns e Derek O’Hagan, dois de entre os maiores especialistas na área das vacinas e adjuvantes, o termo adjuvante é definido tendo em conta o seu efeito biológico. Estes investigadores classificam-nos em duas categorias principais: os adjuvantes cuja função principal é o controlo da biodisponibilidade da vacina nos tecidos linfoides (“*delivery mechanism*”) e os imunopotenciadores que têm uma acção directa sobre componentes da resposta imune inata.

O presente trabalho teve por objectivo desenvolver um sistema nanoparticular (“*delivery mechanism*”), com potencial para a encapsulação de vacinas resultantes da tecnologia recombinante, com a finalidade de transportar e controlar a apresentação do antigénio nos tecidos linfóides.

Tem sido referido por alguns investigadores que o sistema de libertação de vacinas ideal deve ter em conta o fenómeno de maturação da afinidade, que ocorre durante uma resposta imune. Quando a concentração de um antigénio é reduzida, as células com elevada afinidade para os receptores são estimuladas selectivamente. De acordo com este modelo, o padrão do sistema ideal deve imitar os perfis de concentração de antigénios que são observados no decurso de uma infecção natural: elevadas doses de antigénios, nos primeiros dias da administração, seguidos por um período em que há decréscimo da quantidade de antigénios. A biodisponibilidade inicial dos antigénios irá influenciar a extensão da formação da memória das células T, enquanto a subsequente diminuição dos antigénios irá beneficiar o desenvolvimento da maturação da afinidade dos anticorpos.

Por outro lado, será útil fazer uma reflexão sobre qual a via de administração mais indicada para a administração de vacinas. Visto que as mucosas, nomeadamente a oral, a nasal, a pulmonar e genitourinária, são os locais por onde entram a maior parte dos microorganismos patogénicos, então a protecção contra esses microorganismos será mais eficiente pela presença de anticorpos nas secreções locais do que no soro. Alguns autores referem mesmo que a imunoglobulina A secretória (sIgA) local previne de forma mais eficiente, não só a colonização dos tecidos das mucosas, mas também a entrada de microorganismos na corrente sanguínea, quando comparada com a acção dos anticorpos sistémicos. Por outro lado, a indução de anticorpos nas mucosas não parece ser possível através da administração subcutânea ou intramuscular, usadas vulgarmente nos esquemas de vacinação. As evidências científicas, até hoje reunidas, indicam que, para haver indução de anticorpos (sIgA) nas mucosas, a administração do antigénio deve ser feita através das mucosas que se encontram revestidas por tecido linfoíde. Por esta razão, parece desejável que a próxima geração de vacinas, particularmente para os microorganismos patogénicos que invadem o organismo através das superfícies das mucosas, deve ser desenvolvida ou optimizada tendo em atenção a potencial indução de uma resposta imune nas mucosas.

Para além da importante vantagem apresentada anteriormente, a administração através das mucosas, particularmente a administração oral, tem sido apontada como a via mais natural para introdução de fármacos no organismo, a de mais fácil acesso e sem os inconvenientes de outras vias de administração, como sejam os riscos de infecção por utilização de agulhas contaminadas, riscos de efeitos hemolíticos ou possível dor durante a administração. Para além disso, a via oral é sem dúvida a melhor

aceite, nomeadamente pelas crianças que são a população alvo da maioria das vacinas. Acresce ainda referir que a vacinação em larga escala, num curto espaço de tempo, seria muito facilitada se tivéssemos vacinas orais em que, para a sua administração, não fossem necessários técnicos especializados. É o caso de situações de bioterrorismo, de ameaças de surtos infecciosos ou, simplesmente, o caso de países não industrializados que, por escassez de recursos humanos e também financeiros, continuam a ter taxas elevadas de prevalência de doenças para as quais já existem vacinas eficazes, como por exemplo de hepatite B.

A administração oral de macromoléculas como o DNA, as proteínas e os peptídeos tem-se mostrado ineficaz, conduzindo a que, em alternativa, esta classe de fármacos seja administrado por uma das vias parentéricas. A limitada biodisponibilidade oral deve-se fundamentalmente a três ordens de razões. Por um lado ao extensivo metabolismo pré-sistémico a que estão sujeitos, devido à degradação enzimática que sofrem antes e durante os processos de absorção, não só ao nível do lúmen, como da mucosa gastrointestinal. A segunda barreira encontrada, prende-se com as fracas características de absorção destes fármacos e, por último, a terceira razão terá a ver com o efeito da primeira passagem pelo fígado (*hepatic first pass effect)*. Consciente destas dificuldades, a comunidade Científica tem procurado novos sistemas terapêuticos, que permitam contornar ou minimizar as condições adversas do meio que impedem a administração oral destas moléculas. Neste contexto, os sistemas de transporte mais estudados, para a encapsulação destas macromoléculas, são as nanopartículas poliméricas e dentro destas são preferidos os sistemas biodegradáveis.

A encapsulação irá proteger o antigénio do meio ácido e rico em enzimas proteolíticas como é o tubo digestivo. Para além das vantagens apontadas, acresce ainda referir que um sistema de transporte polimérico permite uma cedência gradual das moléculas activas, prolongando o seu efeito no organismo e contribuindo assim para a simplificação dos esquemas posológicos. Por outro lado, estes sistemas contribuem igualmente para a simplificação da logística de produção, armazenamento e distribuição de vacinas. Um dos sistemas que tem sido estudado é o de bioadesivos de libertação de fármacos (BDDS- *bioadhesive drug delivery system*). Os Tecnologistas estão confiantes que este novo conceito poderá contornar as dificuldades relacionadas com a libertação oral de peptídeos e análogos de peptídeos. Os BDDS são produzidos com a finalidade de se fixarem ao revestimento mucoso do tracto gastrointestinal. Desta forma,

este sistema deverá exercer uma influência positiva na absorção dos fármacos que transportam. Essa influência deverá fazer-se por vários mecanismos:

- prolongamento do tempo de residência no local de absorção do fármaco, com vista à redução da frequência de administração.

- Intensificação do contacto com a barreira epitelial da mucosa subjacente, com vista ao incremento do transporte através do epitélio.

- Pensa-se que alguns polímeros mucoadesivos têm a capacidade de modular a permeabilidade dos tecidos epiteliais, actuando ao nível das junções das células do epitélio (*tight junctions*).

- Pensa-se igualmente, que alguns polímeros mucoadesivos podem actuar como inibidores de enzimas proteolíticas.

Objectivo: Este trabalho teve como objectivo desenvolver e avaliar *in vitro* e *in vivo* um sistema nanoparticular, com potencial para a encapsulação de vacinas resultantes da tecnologia recombinante, com a finalidade de permitir a sua administração através das mucosas, nomeadamente a mucosa oral e nasal.

Com este propósito foram desenvolvidas nanopartículas de quitosano às quais, numa primeira fase, foi adsorvida a vacina e, subsequentemente, efectuado um revestimento com o alginato de sódio. O alginato de sódio, ao contrário do quitosano, é um polímero aniónico, característica que teoricamente facilitaria o revestimento. Por outro lado, tem a particularidade de ser insolúvel em meio ácido e, portanto, teoricamente poder conferir às nanopartículas resistência ao pH baixo do estômago. Finalmente, as suas propriedades mucoadesivas, em teoria, terão um papel importante para o aumento do tempo de retenção das nanopartículas no tubo digestivo.

O quitosano é um polímero derivado da quitina, um polissacarídeo presente na natureza em crustácios (p. ex. no camarão) e em algumas espécies de fungos. Este polímero é obtido por desacetilação da quitina, podendo aparecer no mercado com diversos graus de desacetilação, assim como com diversos pesos moleculares. Estruturalmente, o quitosano é um poli(2-amino 2-deoxi D-glucopyranose), no qual as unidades de repetição estão ligadas por ligações β 1-4. Possui grupos hidroxil e grupos amino reactivos, que por modificação química nos permitem a obtenção de derivados com propriedades diferentes, possibilitando diversas aplicações. O polímero é largamente usado, não só na indústria farmacêutica (excipiente, composição de

produtos para emagrecimento), como na indústria Alimentar, na Agricultura, na indústria de cosméticos, tratamento de águas, etc.

O interesse por este polímero tem sido crescente nos últimos anos, principalmente pela sua abundância na natureza, pelas suas propriedades físico-químicas, por ser biodegradável e por não apresentar toxicidade. Diversos estudos evidenciam as suas propriedades mucoadesivas, a sua capacidade de aumentar a permeabilidade das membranas e de inibir a acção de algumas enzimas, tornando-o um forte candidato à produção de microsferas mucoadesivas.

Deve no entanto realçar-se que o quitosano é pouco solúvel a pH 6,5, condicionando a sua actividade de promotor de absorção, em especial, nas mucosas com este pH de que é exemplo uma parte significativa do intestino.

Primeiro Capítulo: No primeiro capítulo são apresentados os resultados do desenvolvimento e optimização das nanopartículas de quitosano revestidas com alginato. Na literatura, encontram-se alguns trabalhos que descrevem a produção de sistemas multiparticulares. Estes consistem basicamente na encapsulação em microsferas de alginato, de partículas de quitosano, com vista à protecção destas partículas durante a sua passagem pelo estômago. Estes sistemas, normalmente com tamanhos unitários superiores a 10 μm, destinam-se a libertar a proteína no intestino para que esta seja depois absorvida. O presente trabalho teve como objectivo o desenvolvimento de um sistema constituído por nanoparticulas transportadoras de antigénios destinadas a ser internalizadas pelas células M das placas de Peyer do intestino e não a libertar a vacina na mucosa gástrica, onde esta poderia ser facilmente destruída pelas enzimas ai presentes. É frequentemente descrito na literatura que estes sistemas nanoparticulares devem ser constituídos por partículas inferiores a 10 μm, para que possam ser internalizados pelas células M. Por conseguinte, e dado que com estas características nada havia descrito na literatura, foi necessário, na primeira fase do trabalho, fazer o desenvolvimento do método que permitisse a obtenção das referidas nanopartículas, com tamanho inferior a 10 μm.

O método de obtenção das nanopartículas de quitosano consiste na precipitação do quitosano, após a adição de uma solução de sulfato de sódio, sob agitação e sob o efeito de ultrasons. Nesta fase do trabalho foram optimizadas a concentração de quitosano em solução e o volume de solução de sulfato de sódio a adicionar. O resultado deste estudo permite concluir que soluções com 0,25% de quitosano

conduzem à formação de partículas, quando são adicionados 3,5 ml de uma solução de sulfato de sodio a 10%. As nanopartículas preparadas deste modo apresentaram um tamanho médio de 643 ± 171 nm e um potêncial zeta de +37 ± 4 mV (zetasizer 3000 HSA).

Após a liofilização das nanopartículas, estas são posteriormente re-suspendidas numa solução tampão fosfato e incubadas com a solução de uma vacina modelo, a ovalbumina, à temperatura ambiente (20º C) e sob agitação moderada. Foram experimentadas diferentes concentrações de ovalbumina e de suspensão de partículas, tendo-se verificado que 0,5% de ovalbumina e 1% de partículas de quitosano produziam os melhores resultados de eficiência de encapsulação e de capacidade de encapsulação para estas partículas. Verificou-se ainda que a adsorção é imediata e os períodos de incubação até 120 minutos não resultaram num aumento da eficiência e da capacidade de encapsulação destas partículas, quando comparados com o momento inicial da adição da ovoalbumina às nanopartículas.

O quitosano é um polímero solúvel em soluções ácidas, nomeadamente em ácido acético e em ácido clorídrico. As partículas de quitosano, obtidas por precipitação do polímero com sulfato de sódio, são pouco resistentes em meio ácido, particularmente a 37ºC. Por esta razão, foi decidido revestir estas nanopartículas já com a ovoalbumina adsorvida, tendo-se escolhido o alginato de sódio. Dado que as partículas de quitosano são carregadas positivamente e o alginato tem uma carga predominantemente negativa, a hipótese para o desenvolvimento de um método foi esperar que o revestimento se concretizasse por interacção electrostática. Com esta finalidade foram experimentadas diferentes composições de sistemas (solução de alginato/suspensão de partículas de quitosano). Na maioria dos sistemas verificou-se uma imediata agregação das partículas, tendo-se observado que os sistemas mais estáveis tinham na sua composição 0,5% de alginato e 0,2% de partículas de quitosano. Estes sistemas foram mantidos 20 minutos sob agitação moderada e, no final, o alginato que não reagiu foi eliminado, centrifugando a suspensão das nanopartículas revestidas. As nanopartículas foram posteriormente ressuspendidas numa solução de cloreto de cálcio.

As imagens obtidas por microscopia electrónica de varrimento permitiram concluir que, para além do tamanho médio anteriormente registado e avaliado com recurso ao zetasizer, existe igualmente um número elevado de nanopartículas com um tamanho médio de cerca de 100 nm, antes do revestimento. A maior parte, porém, apresentou um tamanho entre os 300 e os 600 nm, após o revestimento.

Consequentemente, as nanopartículas revestidas terão um tamanho adequado para ser internalizadas pelas células M das placas de Peyer no intestino.

Dado que a vacina modelo se encontrava predominantemente adsorvida à superfície das partículas, foi necessário, durante o processo de revestimento, monotorizar a sua libertação a partir das nanopartículas de quitosano. Verificou-se que a capacidade de encapsulação das nanopartículas revestidas diminuiu ligeiramente, quando comparada com as partículas não revestidas, resultando numa capacidade de encapsulacão de 35% (w/w).

A confirmação da presença do revestimento de alginato de sódio foi feita através da análise das nanopartículas por calorimetria diferencial de varrimento (DSC) e por espectrofotometria de infravermelho (FTIR). A inversão dos valores de + 37 mV, encontrados para o potencial zeta das nanopartículas antes de serem revestidas, para -34.9 mV, após o processo de revestimento com o alginato de sódio, veio reforçar o pressuposto da presença do alginato à superfície das nanopartículas. Por último, neste capítulo é ainda apresentado e discutido o resultado dos primeiros estudos de libertação, feitos em fluido intestinal simulado (USP XXIV), usando uma proteína modelo (albumina do ovo), a partir de suspensões de partículas revestidas e de partículas não revestidas. O perfil de libertação da proteína foi alterado após o revestimento, tendo-se verificado que o revestimento permitiu evitar a sua dessorpção rápida nos primeiros minutos do ensaio. Por conseguinte, este ensaio veio mais uma vez confirmar não só a presença do referido revestimento, mas também realçar a utilidade do revestimento no controlo da libertação da proteína a partir das nanopartículas.

Segundo Capítulo: No segundo capítulo são apresentados os resultados de uma série de experiências que tiveram como objectivo principal um conhecimento mais aprofundado das nanopartículas desenvolvidas. Paralelamente, faz-se uma avaliação do potencial destas nanopartículas para poderem vir a ser usadas em estudos *in vivo*. Assim, foram feitos estudos adicionais de libertação da proteína-modelo, a partir das nanopartículas em diversos meios, como o de tampão fosfato pH=7,4; pH=6,8 e pH=5,5, em fluido gástrico simulado, em água e em tampão HEPES pH=7,4.

Os resultados são significativamente diferentes para partículas revestidas e para partículas não revestidas, tendo-se ainda verificado que as partículas não revestidas são instáveis em determinados valores de pH do meio, libertando imediatamente toda a proteína. De uma forma geral, o revestimento retardou a libertação da albumina e,

particularmente nos ensaios feitos em água e em tampão HEPES, só 5% da proteína tinha sido libertada ao fim de 3,5 horas. Também a força iónica do meio (tampão) parece ter influência na cinética de libertação da substância activa a partir das nanopartículas revestidas com alginato.

Ainda neste capítulo são apresentados e discutidos os estudos de citotoxicidade das nanopartículas revestidas. Neste estudo foram usadas culturas primárias de células do baço de ratinhos Balb/c. Foram testados, individualmente, os polímeros quitosano e alginato de sódio, assim como as partículas revestidas. A avaliação da morte celular foi feita por dois métodos diferentes: num, usando o iodeto de propídio para marcação das células mortas e fazendo a contagem das células viáveis e das células mortas por citometria de fluxo; no segundo, as células mortas foram coradas com o azul tripano e observadas ao microscópio. O ensaio efectuado com MTT (tetrazolium assay) permitiu avaliar o estado metabólico das células. Verificou-se que, por qualquer um dos métodos, tanto o quitosano como o alginato, como ainda as nanopartículas revestidas, nas concentrações estudadas, não apresentam toxicidade para as células.

Finalmente, ainda no segundo capítulo, foram descritos e discutidos os estudos de internalização das nanopartículas de quitosano revestidas com alginato, nas células M e placas de Peyer do intestino do rato (Wistar).

Na literatura, estão descritos vários trabalhos fazendo referência à capacidade de partículas hidrófobas e partículas positivamente carregadas poderem ser internalizadas pelas células M do intestino. No caso presente, das nanopartículas revestidas com alginato, existem partículas carregadas negativamente, por isso é importante verificar se também estas terão aquela capacidade. Para o efeito, foram realizados estudos no rato, através de uma pequena cirurgia que permitiu a colocação da suspensão das nanopartículas directamente no duodeno. As partículas permaneceram no intestino durante duas horas e no final os ratos foram sacrificados. O intestino delgado foi removido e lavado com tampão fosfato salino (pH 7.5), as placas de Peyer foram posteriormente removidas e preparadas para poderem ser observadas no microscópio confocal. Verificou-se que as nanopartículas revestidas foram capazes de ser internalisadas nas placas de Peyer. Este resultado, embora qualitativo, mostrou que a utilização destas nanopartículas *in vivo* poderia ser promissora.

Terceiro Capítulo: No terceiro capítulo são apresentados os resultados de estudos *in vitro* efectuados com culturas primárias de células extraídas do baço do ratinho com o

objectivo de determinar se a presença do quitosano ou do alginato na cultura celular, induziria algum tipo de estimulação nos linfócitos.

Paralelamente e funcionando como controles positivos foram ainda ensaiados nas mesmas condições a “concanavalin A” e o “CpG ODN”. A activação das células foi avaliada pela expressão do marcador CD69 e pela maior ou menor capacidade das células proliferarem na presença dos compostos. O CD69 é um receptor que é expresso à superfície dos linfócitos quando estes são estimulados. Este receptor tem a particularidade de poder ser expresso após 2 horas de exposição a um mitogénio e atingir o pico entre as 18 e as 24 horas de exposição. Foi mostrado, neste trabalho, pela primeira vez, que a presença do quitosano ou do alginato induz à expressão do CD69 nos linfócitos B numa percentagem elevada e um pouco inferior nos linfócitos T do tipo CD4+, sendo o alginato, dos dois polímeros, o que mostrou o menor efeito. Relativamente aos linfócitos T do tipo CD8+, apenas o quitosano mostrou capacidade para induzir a expressão deste receptor. A indução do receptor CD69+, tendo sido generalizada nos linfócitos, não foi no entanto acompanhada pela proliferação dos mesmos. Paralelamente, com este estudo foi confirmado que o CpG ODN (1826), usado como adjuvante nos estudos *in vivo* a seguir apresentados, é essencialmente um activador de linfócitos B.

Nos capítulos quarto, quinto e sexto são apresentados e discutidos os estudos, *in vivo,* de immunização no ratinho Balb/c, usando a vacina da hepatite B. Nestes capítulos, as nanopartículas revestidas são avaliadas quanto à sua capacidade para poderem funcionar como adjuvantes da vacina da hepatite B recombinante. Paralelamente é avaliada a associação de um outro adjuvante, o CpG ODN.

As nanopartículas foram usadas para a encapsulação da vacina da Hepatite B e para a encapsulação do CpGODN. A eficiência de encapsulação para o antigénio de superfície do vírus da hepatite B foi 85.9 ± 4.7% e para o CpG ODN foi 98.8% ± 4.7%.

Quarto Capítulo: No quarto capítulo são apresentados e discutidos os resultados da resposta imune, observada após a administração oral da vacina encapsulada. A vacina foi administrada em solução (grupo de referência) ou encapsulada nas partículas revestidas, associada ou não com o imunopotenciador CpG ODN. Este, por sua vez, foi administrado encapsulado a alguns grupos e noutros foi dado em solução. A primeira imunização com 10 μg da vacina foi seguida de um reforço, 3 semanas depois com a mesma dose da vacina. Os ratinhos foram sacrificados 10 dias depois do reforço.

Apenas o grupo vacinado com a suspensão das nanopartículas carregadas com o antigénio (grupo I) e o grupo vacinado com uma mistura de nanopartículas, umas carregadas com o antigénio e outras carregadas com o CpG (grupo VI), apresentaram valores de parâmetros imunológicos diferentes dos verificados no grupo de ratinhos não imunizados. Ambos os grupos mostraram valores significativamente maiores de expressão do CD69+ em linfócitos CD4+ e CD8+ e, paralelamente, valores de CD69+ significativamente inferiores em linfócitos B. Os mesmos grupos apresentaram os valores mais elevados de proliferação de linfócitos do baço colocados em cultura na presença da concanavalina A. Finalmente, foi nos mesmos grupos, muito embora com a presença dentro dos grupos de ratinhos que não responderam à vacina, que se observou a produção de anticorpos específicos contra o antigénio de superfície do vírus da hepatite B, no sangue (IgG e isotipos) e na mucosa do intestino (sIgA) dos ratinhos. No entanto, de entre os dois grupos, o VI foi o que apresentou um número maior de ratinhos que responderam e caracterizou-se por apresentar uma resposta imune do tipo Th1, desejável no caso do vírus da hepatite B.

Quinto Capítulo: No capítulo quinto são apresentados e discutidos os resultados da resposta imune, observada após a administração nasal da vacina encapsulada. A vacina foi administrada em solução (grupo de referência) ou encapsulada nas partículas revestidas, associada ou não com o imunopotenciador CpG ODN. Este, por sua vez, foi administrado encapsulado a alguns grupos, e noutros foi dado em solução. A primeira imunização com 10 μg (15 μl) da vacina foi seguida de mais dois reforços, com 3 semanas de intervalo entre as imunizações, com a mesma dose da vacina. Os ratinhos foram sacrificados 4 semanas depois do último reforço. Comparando a resposta imune observada nos ratinhos dos grupos, imunizado com uma solução nasal contendo o antigénio ou vacinado com a formulação comercial pela via subcutânea, com a resposta imune obtida com a suspensão nasal das nanopartículas revestidas, contendo o antigénio da hepatite B, verificou-se que o último grupo, ao contrário dos primeiros, apresentou, nas secreções nasais e vaginais, quantidades detectáveis da imunoglobulina A secretora, específica do antigénio de superfície do vírus da hepatite B. Esta observação vem comprovar o que já se sabia relativamente à vacina comercial, administrada subcutaneamente, dado que esta não induz a produção de anticorpos nas mucosas. Por outro lado, para que se verifique a indução de anticorpos nas mucosas não basta que o antigénio seja aplicado directamente na mucosa, a presença de um

adjuvante será sempre necessária para a vacina da hepatite B. Com este estudo, foi igualmente observado que a vacina encapsulada nas partículas revestidas mostrou ser eficiente na indução de anticorpos nas mucosas. A presença de anticorpos específicos (IgG) no soro dos ratinhos só foi observada quando às formulações foi adicionado um imunopotenciador o CpG ODN.

Sexto Capítulo: No capítulo sexto são apresentados e discutidos os resultados da resposta imune, observada após a administração subcutânea da vacina encapsulada nas nanopartículas revestidas. Com este estudo pretendeu-se avaliar se as nanopartículas revestidas teriam ou não um efeito adjuvante, quando administradas com o antigénio de superfície do vírus da hepatite B pela via subcutânea. Nomeadamente, pretendeu-se avaliar a concentração de anticorpos específicos no soro, visto que uma resposta imune nas mucosas era pouco provável após uma administração parentérica. A vacina foi administrada em solução (grupo de referência) ou encapsulada nas nanopartículas revestidas, associada ou não com o imunopotenciador CpG ODN. Este, por sua vez, foi administrado encapsulado no grupo III e em solução no grupo II. A primeira imunização com 10 μg da vacina foi seguida, três semanas depois, por um reforço com uma dose igual. Os ratinhos foram sacrificados quatro semanas após o reforço. O grupo de ratinhos vacinados, em que o antigénio tinha sido encapsulado nas nanopartículas revestidas (Grupo I), apresentou uma concentração média elevada de IgG, específica no soro, com uma clara predominância de anticorpos específicos, típicos de uma resposta do tipo Th2. Esta concentração foi aproximadamente 5 vezes superior à concentração média calculada no grupo de ratinhos vacinados com a solução do antigénio sem adjuvante. Relativamente à resposta imune celular, nomeadamente à concentração da IL-4 e do IFN-γ, secretados pelos linfócitos do baço do ratinho colocados em cultura, os valores médios não apresentaram diferenças entre estes dois grupos. Foi depois investigada a resposta imune induzida após a administração de uma suspensão contendo o antigénio encapsulado nas nanopartículas e o adjuvante CpG ODN em solução (grupo II). Muito embora, neste último grupo, o valor médio dos anticorpos específicos não tenha sido estatisticamente diferente do valor do grupo I (Grupo com o antigénio encapsulado nas nanopartículas), o tipo de resposta imune foi diferente. Observou-se um aumento de anticorpos do tipo IgG2a e uma diminuição dos anticorpos do tipo IgG1, resultando uma resposta imune mista Th1/Th2. Fazendo ainda a comparação destes mesmos grupos (Grupos I e II), em que a vacina foi administrada

encapsulada, observou-se um aumento da produção de IFN-γ no grupo que continha o CpG ODN em solução (Grupo II). Finalmente, foi ainda constituído um terceiro grupo de ratinhos aos quais foi administrada uma formulação mais simples que consistiu numa suspensão de nanopartículas de quitosano não revestidas às quais foram previamente adsorvidos o CpG ODN e o antigénio de superfície do vírus da hepatite B. Os resultados apresentados não demonstraram uma clara superioridade desta formulação, tendo-se verificado que a resposta imune foi, neste caso, do tipo Th2, apesar da presença nesta formulação do CpG ODN.

Considerações finais: neste trabalho foi apresentado um novo sistema de libertação constituído por nanopartículas poliméricas. As nanopartículas são constituídas por um núcleo de quitosano onde foi posteriormente adsorvido o antigénio e finalmente revestido com alginato de sódio. O método desenvolvido permite encapsular, com elevada eficiência, antigénios do tipo proteico em condições reconhecidamente não agressivas, minimizando assim a possibilidade de perda de acção biológica por parte da vacina.

As nanopartículas revestidas mostraram ter um efeito adjuvante relativamente à vacina da hepatite B quando administradas pela via subcutânea. A adição do imunopotenciador CpG ODN à suspensão das nanopartículas, contendo o antigénio da hepatite, permitiu modificar o tipo de resposta imune de Th2 para uma resposta mista Th1/Th2, mais adequada no caso do vírus da hepatite B.

A administração, através das mucosas oral e nasal, da vacina da hepatite B encapsulada nas nanopartículas de quitosano revestidas permitiu confirmar a opinião de muitos especialistas na área da vacinologia, que defendem que não será suficiente a inclusão do antigénio em sistemas de libertação de nanopartículas, principalmente quando se trata de um antigénio fraco. Para se obter uma resposta imunológica adequada, para além da encapsulação do antigénio, vai ser ainda necessário adicionar um imunopotenciador. De facto, os melhores resultados foram obtidos com formulações que continham na sua composição o CpG ODN. Para finalizar, o sistema nanoparticular desenvolvido mostrou ser igualmente útil para a encapsulação do imunopotenciador em formulações orais. Em contraste, na mucosa nasal, o imunopotenciador produziu melhores resultados quando foi administrado em solução, pelo que a futura utilização destas nanopartículas para a encapsulação de moléculas que demonstrem uma elevada

afinidade para o quitosano, como o caso do CpGODN, estará condicionada a uma optimização conveniente destas nanopartículas.

As nanopartículas de quitosano, revestidas com alginato provaram ter um efeito adjuvante com o antigénio da hepatite B pela via subcutânea. Por conseguinte, os próximos estudos serão feitos com o objectivo de avaliar se o sistema nanopartícular poderá substituir, com vantagem, o actual adjuvante (compostos de alumínio), da vacina da hepatite B. Para finalizar, o efeito adjuvante, observado para a vacina da hepatite B, deverá ser avaliado com outras vacinas, de preferência mais fortes, não só pela via subcutânea mas também através das mucosas oral e nasal.

THESIS ABSTRACT

It has been long known that protection against pathogens invading the organism via mucosal surfaces correlates better with the presence of specific antibodies in local secretions than with serum antibodies. The most effective way to induce mucosal immunity is to administer vaccine directly to the mucosal surface. The development of vaccines for mucosal application requires antigen delivery systems and immunopotentiators that efficiently facilitate the presentation of the antigen to the mucosal immune system.

In the more recent years, several polymeric delivery systems have been investigated to deliver vaccines to the mucosa, while protecting them from adverse conditions that could affect their bioactivity. There are also indications that these delivery systems act as adjuvants, increasing the immunogenicity of poor immune response antigens. One of the problems in vaccine formulation is the structural and/or conformational change of antigens during preparation or storage. Therefore, the preservation of antigen stability during encapsulation and release is essential for the development of successful controlled release vaccine delivery systems.

Taking this into consideration, the main aim of this thesis was to develop a multiparticulate delivery system, appropriate to protect the antigen from the adverse conditions on the mucosal surfaces, particularly in the gastrointestinal tract. Simultaneously, these particles should be taken up by M- cells of Peyer's patches in the gut, and M-cells from the nose-associated lymphoid tissue (NALT).

Chitosan and sodium alginate are biodegradable and biocompatible natural polysaccharide polymers with a good safety profile. Both materials are polyelectrolyte polymers and they are of opposite charges. Furthermore, these polymers allow the absence of organic solvent during the particles preparation. This aspect is particularly important for the encapsulation of vaccines, which might be denatured even by limited exposure to organic solvents.

The adsorption of the antigen to previously manufactured empty chitosan microspheres has been shown to be a very mild process since it can be done in phosphate buffered saline. For vaccines it seems to be the more appropriate method.

However when the particles are non porous, like in this case the antigen will be linked at the surface. This is probably one of the major drawback of the adsorption method because the antigen will be exposed to enzymes of mucosal surfaces. This problem is believed to be overcome by coating the particles with a polymer resistant to those adverse conditions. Therefore, the developed delivery system is composed of a chitosan core, to which the antigen was adsorbed, being both subsequently coated with sodium alginate. The majority of these coated nanoparticles have a diameter between 300 and 600 nm and a negative charge.

In this work, the capability of these coated nanoparticles for being internalized in rat Peyer's patches as well as their cytotoxicity were evaluated. With the help of a surgery, the alginate coated particles loaded with ovalbumin fluorescein were placed directly into the duodenum and two hours later the rats were sacrificed, the small intestine removed and the Peyer's patches isolated for CLSM analysis. The confocal images of the Peyer's patches undoubtedly showed the presence of coated particles in this specific secondary lymphatic tissue. Additionally, cytotoxicity studies performed with spleen cells have confirmed the non-toxicity of these polymers as well as the non-toxicity of the coated and uncoated chitosan nanoparticles.

The suitability of the alginate coated chitosan nanoparticles for the encapsulation of the surface hepatitis B antigen, a recombinant protein and the immunopotentiator, the CpG ODN was also investigated in this work. In order to optimize the encapsulation conditions, different ratios of hepatitis B vaccine to chitosan nanoparticles and CpG ODN to chitosan nanoparticles were investigated. The systems with the highest loading efficacy were used for further studies. It was found that hepatitis B antigen (HBsAg) and CpG ODN were efficiently associated with alginate coated chitosan nanoparticles. The loading efficacy of hepatitis B vaccine on coated nanoparticles was 84.1 ± 3.0 % (mean ± STDEV) and the mean of the loading capacity was 83.9 μg of HBsAg/ mg of dry chitosan nanoparticles ± 2.77 (mean ± STDEV). The loading efficacy of CpG was 97.0 ± 1.3% and the loading capacity was 97.0 ± 0.03 (μg of CpG ODN/ mg of dry chitosan nanoparticles). Furthermore, SDS-PAGE and blotting of the released antigen from the particles in buffer phosphate (37ºC) demonstrated that no important modifications in the structure of the antigen were produced during the entrapment process.

The role of the alginate coated chitosan nanoparticles, as adjuvant for hepatitis B vaccine was investigated by oral, intranasal and subcutaneous administration routes.

Subcutaneously vaccinated mice with the antigen associated with the nanoparticles showed a high anti-HBsAg IgG titer, with the majority of antibodies being Th2 profile. This immune response was 5.3-fold higher than the mean value found for the reference group immunized with the saline solution of the antigen. However, concerning cellular immune response, no important differences were observed concerning the antigen-specific splenocyte proliferation or the secretion of Th1 (IFN-γ) and Th2 type cytokines (IL-4) between both groups, the group vaccinated with the antigen associated with the nanoparticles and reference group. Moreover, in the same study it was proven the usefulness of adding together in the same formulation the antigen associated with coated nanoparticles with the immunopotentiator CpG ODN in solution. The redirection of the Th2 profile for a mixed Th1/Th2 immune response was observed, which make this formulation promising for further studies not only with HBsAg but also with other antigens.

Mucosal vaccination studies were conducted with similar formulations, however different results were obtained. The oral vaccination with coated nanoparticles containing HBsAg and coated nanoparticles containing CpG ODN has shown to be able not only to induce anti-HBsAg sIgA in the gut, but also systemic specific antibodies. Although the seroconversion rate had not been excessively high, it was, however, higher than the seroconversion rate observed in mice group vaccinated just with the antigen associated with nanoparticles. Moreover, the intranasal vaccination just with the antigen associated with the nanoparticles induced mucosal antibodies, but a systemic immune response was not observed. A systemic immune response was induced only in mice intranasally vaccinated with formulations containing the immunopotentiator, CpG ODN.

Therefore, these results showed that alginate coated chitosan nanoparticles have an adjuvant effect for the hepatitis B antigen when administered subcutaneously, however for the mucosal routes the association of an immunopotentiator is required. As future prospects, the evaluation of the adjuvant effect of this new delivery system will be performed with different antigens.

CHAPTER

1

GENERAL INTRODUCTION AND AIM OF THE THESIS

ABSTRACT

The aim of this thesis is to design a new nanoparticulate mucosal vaccine delivery system that protects the loaded subunit vaccine during its passage through the gastrointestinal and nasal mucosa and facilitates the internalization of the vaccine into the mucosal-associated lymphoid tissue.

This introductory chapter provides an overview of the events within mucosal tissues that lead to protective mucosal immune responses. The understanding of those biological mechanisms together with knowledge of the technology of vaccines and adjuvants provides guidance on the technical aspects of mucosal vaccine design. Therefore, this chapter also provides some technical information related to modern adjuvants. This is not an exhaustive review since the objective of this chapter is to make information available related to the biomaterials and adjuvants used in this thesis in order to enable a clear understanding of the work. Moreover a small introduction is provided in each of the following chapters in order to bring the area under discussion into focus.

1.1 A BRIEF HISTORY OF VACCINES AND ADJUVANTS

The Scientific era of vaccinology started in the early eighteenth century, with the introduction in Europe of an ancestral Chinese practise of preventing severe natural smallpox by inoculating pus from smallpox patients. This procedure was introduced to England for the first time by a farmer named Benjamin Justy who inoculated his family with cowpox pus to prevent smallpox, and the first clinical investigations were eventually conducted in 1796 by the English practitioner Edward Jenner [1]. During the fifteenth century in China, healthy people acquired immunity to smallpox by sniffing powdered smallpox pustules, by inserting them into small cuts in the skin (a technique called variolation) [2], or finally by the oral administration of fleas from cows with cowpox. These are the first reports of a mucosal vaccination practice [3].

A great expansion of biomedical sciences and vaccinology occurred in the 19th century with the enormous contribution by Louis Pasteur of the first attenuated vaccine (vaccine = vacca; latin for cow), and with the achievements of Robert Koch, Emil von Behring, the first recipient of the Medicine Nobel Prize, and Paul Ehrlich. Between World

Wars I and II, many studies were carried out which led to the description of most kinds of humoral immunologic phenomena [1]. Although that period also had many financial restrictions since resources were principally used for military purposes, this early period led to the appearance of the first vaccines against typhoid fever, shigellosis, tuberculosis, plague, diphtheria and tetanus.

The modern era of vaccine science began in about 1950 with the bacterial capsular polysaccharide vaccines such as *pneumococcus*, *meningococcus* and *Haemophilus influenzae*. Moreover, the Sabin oral polio vaccine in the early 1960s had an important role in the programme for the global eradication of polio and brought mucosal immunization to prominence [3]. The appearance of viral vaccines such as the inactivated poliovaccines and live vaccines for preventing pediatric diseases, measles, mumps, rubella and varicella vaccine also played important roles in the history of vaccines. More recently, the discovery of vaccines against hepatitis in the early 1960s was initiated with the purpose of discovering the etiological agent causing hepatitis A and B. Blumberg and colleagues in 1965 discovered the surface antigen of the hepatitis B virus present in the blood of human carriers of the infection [1]. This discovery opened the door to a hepatitis B vaccine, which has been considered among the most remarkable scientific achievements of the 20th century [4]. According to Hilleman [1], hepatitis vaccines represent the world's first subunit vaccine, the world's first licensed vaccine against human cancer and the world's first recombinantly expressed vaccine.

Detailed reviews focusing on the history of the hepatitis B vaccine can be found in the scientific literature [1, 4-7]. In brief, the plasma-derived hepatitis B vaccine was licensed in 1981, 16 years after Blumberg's discovery. The main reason for this delay was the inability to propagate HBV in tissue culture systems. The pioneering work of Krugman and colleagues [8] made feasible the production of a vaccine containing hepatitis B surface antigen obtained by purification of the serum of asymptomatic chronic HBV carriers and characterized elsewhere [9-12], which was decisive for the development of the vaccine.

The hepatitis plasma derived vaccines successfully immunized several million individuals world-wide over almost a decade. However, because these vaccines had a poor acceptance rate due to concerns regarding the safety of the plasma derived products and because the supplies of acceptable human carrier plasma were inadequate to meet market needs, recombinant DNA techniques were investigated as an alternative production method. Therefore, a yeast-derived hepatitis B vaccine based on recombinant

DNA technology was licensed in 1986, and its properties have been reviewed elsewhere [13-15].

According to Hilleman [1], contemporary vaccinology research is very complex, at least for viral vaccines, and is largely dedicated to the subunit vaccine approach. Moreover, subunit vaccines are built on the same base and may be considered to be an extension of recombinant subunit hepatitis B technology, one example being the investigation of a vaccine against AIDS. The discovery of new vaccines to control more than 20 diseases, especially malaria, tuberculosis, hepatitis C and AIDS, rely on the identification of appropriate antigens and epitopes, and progress towards this goal will benefit from the expansion of knowledge in the fields of immunology and molecular biology.

The major questions for current vaccine research seem to be “what to present to the immune system” and “how to present it” [1]. The answer to the last question will depend on the parallel development of new, safe and efficient adjuvants.

1.2 MUCOSAL VACCINATION

Mucosal vaccination has been the common generic name attributed to the oral, intranasal, pulmonary, rectal and vaginal routes of vaccine administration. However, the mucous membranes do not only cover the aerodigestive and urogenital tracts, but also the eye conjuntiva, the inner ear and the ducts of all exocrine glands, which have been less explored as routes of vaccine administration.

Mucosal surfaces, with a combined surface area of about 400 m^2 [2], are undoubtedly the major site of entry for most pathogens. Therefore, these vulnerable surfaces are associated with a large and highly specialized innate and adaptive mucosal immune system that protects the surfaces and the body against potential destructive agents and harmless substances from the environment. In a healthy human adult, this local immune system contributes almost 80% of all immune cells [16]. These immune cells accumulate in a particular mucosa or circulate between various mucosa-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system [2].

In theory, mucosal surfaces seem to be the more accessible lymphoid organ for the induction of an immune response such as that required for immunization. Nevertheless, one of the more important reasons for the development of mucosal vaccines is the increasing evidence that local mucosal immune responses are important for protection against disease, principally for diseases which start on mucosal surfaces such as the respiratory, gastrointestinal or urogenital mucosae. On the other hand, mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, while injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces [17]. However, even with the many attractive features of mucosal vaccination described below (see 2.1), it has often proven difficult (see 2.2) in practice to stimulate strong sIgA immune responses and protection by mucosal antigen administration [16]. As a consequence, no more than half a dozen mucosal vaccines are currently approved for human use (Table 1), and no subunit vaccines are listed among those approved.

Table 1 – Licensed mucosal vaccines (adapted from ref [16])

Infection	Vaccine	Route
Polio	Live attenuated vaccine (OPV)	Oral
Cholera	Cholera toxin B subunit + inactivated *V. cholerae* O1; whole cells	Oral
Cholera	CVD 103.HgR live attenuated *V. cholerae* 01 strain	Oral
Typhoid	Ty21a live attenuated vaccine	Oral
Rotavirus	Live attenuated monovalent human rotavirus strain	Oral
Influenza	Live attenuated cold-adapted influenza virus reassortant strains	Nasal

1.2.1 Additional advantages of oral and nasal vaccination

Oral and/or nasal vaccination has several attractive features that can be summarized as follows:

- Both vaccination routes do not require injection and are therefore less painful.
- Oral administration has a high patient compliance among infants and adults.

- Both vaccination routes do not require trained medical personnel for delivery and are thus more appropriate for mass vaccination programmes, especially in under developed countries. Moreover, this would be also a benefit in pandemic and bioterrorism situations.
- Oral administration is a more natural route of administration.
- Oral formulations can in theory allow vaccines with a higher stability.
- Both vaccination routes can generate mucosal antibodies.
- Nasal administration is the most effective route to elicit optimal protective immunity in both mucosal and systemic immune compartments.
- Nasal administration can avoid the degradation of the vaccine antigen caused by digestive enzymes, so it requires a smaller antigen dose than oral immunization.
- Nasal vaccination can generate cross-protective immunity in the gut through the common mucosal immune system.

1.2.2 Challenges in mucosal vaccine design:

Thorough knowledge of the principal obstacles to mucosal vaccination is essential to design vaccine delivery systems, and can be summarised in this way:

- Nasal vaccination may leads to a possible deposition of antigen in the central nervous system through the olfactory bulbs and olfactory nerves (this feature requires further investigation however should not be excluded).
- There is a low ability for the antigens to be taken up by the mucosal immune system.
- Gastrointestinal deactivation of the vaccines can occur, so high doses of the vaccine are required for oral vaccination.
- Oral vaccination has been associated with a high variability of the response and mixed clinical data.
- High clearance in the nasal mucosa is a cause of low absorption of biomacromolecules.
- Nasal tissue has significant enzymatic activity.
- Nasal administration has limited applicability in patients with upper respiratory-tract infections.
- Induction of Immunological tolerance can occur (this subject will be discussed below).

1.3 UNDERSTANDING THE ANATOMOPHYSIOLOGY OF THE MUCOSAL IMMUNE SYSTEM

Mucosal-associated lymphoid tissue (MALT) includes the gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), nasopharynx-associated lymphoid tissue (NALT), the mammary and salivary glands and the urogenital organs. The common mucosal immune system (CMIS) acts as an integrated pathway that establishes communication between the organized mucosa-associated lymphoid tissues (inductive sites) and the diffuse mucosal tissues (effector sites). However, there is some evidence supporting the theory that this CMIS is compartmentalized. For instance, stimulation at one mucosal site in MALT can induce an immune response at remote mucosal effector sites [18, 19]. However, the extent of the immune response at the effector sites depends on where the induction occurred [20]. Holmgrenn & Czerkinsky [16] recently summarized this phenomenon in this way: “Oral immunization may induce substantial antibody responses in the small intestine (strongest in the proximal segment), ascending colon and mammary and salivary glands and it is relatively inefficient at evoking an IgA antibody response in the distal segments of the large intestines, tonsils or female genital tract mucosa. Conversely, intranasal immunization in humans results in antibody responses in the upper airway and cervicovaginal mucosa, and regional secretions (saliva, nasal secretions) without inducing an immune response in the gut”. Kiyono [21] recently referred to important evidence that may explain, at least in part, the dependence of the mucosal site where the IgA is generated on the route of antigen administration. He cited a study [22] which showed that nasal immunization induces the expression of high levels of chemokine receptor 10 (CCR10) and $\alpha_4\beta_1$–integrin by IgA-committed B cells, allowing them to efficiently traffic to the respiratory and genito-urinary tracts, which express the corresponding ligands, chemokine ligand 28 (CCL28) and vascular cell adhesion molecule 1 (VCAM1). In contrast, orally induced IgA-committed B cells express CCR9 and CCR10 as well as $\alpha_4\beta_7$ and $\alpha_4\beta_1$-integrins, so the cells migrate to sites such as the small intestine, which express CCL25 and/or CCL28 together with mucosal addressin cell-adhesion molecule -1 (MADCAM1) and/or vascular cell adhesion molecule-1 (VCAM1). Therefore, despite the fact that NALT and Peyer’s patches are apparently colonized by similar immune cells, subtle differences like the example referred to above indicate that these two lymphoid structures may have somewhat

different biological functions which are most probably related to their anatomically and environmentally distinct locations [21].

This work will focus on the GALT and NALT. In particular, we will focus on Peyer's patches and NALT as the inductive sites, and the effector sites will be briefly described as well. Effector sites include the lamina propria of the intestinal and respiratory tracts responsible for the generation of antigen-specific T helper 2 (Th2)-cell-dependent IgA responses and Th1-cell and cytotoxic T lymphocyte (CTL)-dependent immune responses, which function as the first line of defence at mucosal surfaces.

1.3.1 Gut-associated lymphoid tissue (GALT)

The gut-associated lymphoid tissue described elsewhere [2] lines the digestive system and has two organizational levels to its structure: one with little organization, characterized by loose clusters of lymphoid cells in the lamina propria of the intestinal villi, and the other with a high level of organization called Peyer's patches.

The so-called intraepithelial lymphocytes (IELs) can be found in the outer mucosal epithelial layer, and the majority of these cells are CD8+ T-lymphocytes. Due to its localization, it is thought that this population of T cells may function to encounter antigens that enter through the intestinal mucous epithelium. Under the epithelial layer is the lamina propria, which contains large numbers of B cells, plasma cells, activated T_H cells and macrophages in loose clusters. It is interesting to note that in healthy children, histological sections of the lamina propria have revealed more than 15,000 lymphoid follicles in total (described in [2]).

Peyer's patches, located in the submucosal layer underneath the lamina propria, contain 30-40 lymphoid follicles organized as macroscopic nodules or aggregates. In a similar way to what happens with lymphoid follicles in other sites, those from mature Peyer's patches can develop into secondary follicles with germinal centers, supported or connected by follicular dendritic cells.

Parafollicular T-lymphocyte zones located between the large B-cell follicles present a large number of high endothelium venules, allowing cellular migration and lymphocytes recirculation.

Between the follicle-associated epithelium (FAE) and the organized lymphoid follicle aggregates, there is a more diffuse area known as the subepithelial dome (SED).

The FAE is the name given to the mucous membrane overlying the organized lymphoid follicles. The FAE is a small region characterized by the presence of specialized flattened epithelial cells called M-cells. Together, the FAE, lymphoid follicles and associated structures form the antigen sampling and inductive sites of the mucosal immune system [23].

The function and structural characteristics of microfold epithelial cells (M cells) have been described in several recent reviews [2, 23, 24]. It has been widely accepted that M cells are probably play a key role in mucosal infection and immunity. It is thought that the main role of M-cells is the sampling of antigens to transport them across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are generated. In addition, M-cells are a common, if not the only, route for complex antigens and pathogen invasion, for examples several invasive *Salmonella* species, *Vibrio cholerae, Yersinia* species, *Escherichia coli* and the polio virus [24].

M-cells have been identified in the epithelia of a variety of mucosal tissues and within the FAE of a wide variety of animal species, including laboratory animals (mice, rats, rabbits), domestic pets and man. In mice and men, M-cells reside in about 10% of the FAE in contrast with 50% in the rabbit. In the gut, M-cells are easily recognized by the lack of surface microvilli and the normal thick layer of mucus that characterizes the rest of the epithelial cells. Additionally, M-cells contain a deep invagination similar to a pocket in the basolateral cytoplasmic membrane that contains one or more lymphocytes and occasional macrophages [23]. The epithelium of the gut intestine provides an effective barrier to the entrance of most pathogens and particulates due to strong connections between epithelial cells called tight junctions. In contrast, the M-cells can be exploited by microorganisms as the port of entrance for two reasons; the organisms can adhere with less difficulty to the apical cell membrane, and after that these agents need only be transported a short distance before reaching the M cell pocket, where by interaction with lymphocytes, the antigens or the particulates gain rapid access to the organized MALT inductive site.

1.3.2 Nasopharynx-associated lymphoid tissue (NALT)

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered to be analogous to Waldeyer's ring in

humans (Pharyngeal lymphoid tissue that includes adenoid, tubal tonsil, palatine tonsil, lingual tonsil) [25]. In the rat, lymphoid aggregates are situated at the nasal entrance to the pharyngeal duct [26]. Detailed reviews of NALT and nasal vaccination can be found elsewhere [26-28]. NALT is a well organised structure consisting of B- and T-cell-enriched areas which are covered by an epithelial layer containing microfold M-cells, the so-called follicle-associated epithelium (FAE). The function of these antigen-sampling M cells seems to be similar to those found on the FAE of Peyer's patches [21]. Although NALT and Peyer's patches share certain similarities, the two differ markedly in morphology, lymphoid migration patterns and the binding properties of the [high] endothelial venules [25]. Additionally, intraepithelial lymphocytes and antigen-presenting cells including dendritic cells (DCs) and macrophages can also be found in NALT [29]. Therefore, according to Kiyono [21], NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune responses to antigens delivered to the nasal cavity.

1.4 NALT- AND PEYER'S-PATCH-INITIATED IMMUNE RESPONSES

Several evidences converge on the insight that the organized MALT plays an important role in antigen sampling and generation of lymphocytes, including specific IgA effector B cells, memory B cells and T cells. This implicates active lymphocyte proliferative activity, local production of cytokines and continuous cellular trafficking [30].

In stratified and pseudostratified epithelia (which lack tight junctions), antigen-processing dendritic cells move into the epithelium, internalise antigens from the lumen and migrate back to local or distant organized tissues. In the intestinal and airway epithelia, whose mucosal epithelial cells are sealed by tight junctions, antigen transport is carried out by the M cells. Luminal antigens are endocytosed into vesicles that are transported from the luminal membrane to the underlying M-cell pocket membrane. Vesicles and the pocket membrane experience fusion, and the antigens are delivered to the clusters of lymphocytes present within the pocket. It is not known whether M cells participate in antigen processing and presentation nor if they express MHC class II molecules [30, 31]. Simultaneously, it is believed that the intact antigens are processed by professional APCs such as macrophages and dendritic cells, either in the epithelium

or in the underlying dome region immediately below M cells which is thus ideally located to sample transported antigens. Moreover, chemokines secreted by the FAE result in an additional attraction of DCs to the FAE, resulting in a high density of phagocytic cells at sites of entry of foreign antigens and pathogens [17]. Phenotypically immature DCs are subsequently moved to the T-cell areas, where they upregulate the expression of maturation markers and MHC molecules [17].

In the follicle, B cells undergo immunoglobulin class switching from expression of IgM to IgA under the influence of several local factors, including transforming growth factor (TGF-β), IL-10 and cellular signals delivered by dendritic cells and T cells [31]. Furthermore, it is thought that because dendritic cells are migratory cells, they can transport microbes to the mesenteric lymph nodes and to the spleen for the induction of systemic responses [32]. Therefore, these cells also possibly transport antigens, especially those sampled directly from the luminal contents.

The lymphocytes primed in the Peyer's patches move through the draining lymphatics to the mesenteric lymph nodes (MLN) where they can reside for an undefined period for further differentiation before they migrate again to the mucosa. According to Kiyono [33], Peyer's patches contain all the cellular and microarchitectural environments (e.g., a B cell follicle including germinal centers, a dendritic cell network and an interfollicular T cell area) needed for the generation of IgA-committed B cells. Therefore, B cells primed in the Peyer's patches or in NALT and transported to the MLN migrate again to the diffuse mucosal effector tissues such as the lamina propria of the upper respiratory and intestinal tract where full maturation is achieved under the influence of IgA-enhancing cytokines IL-5, IL-6 and IL-10, and are transformed into immunoglobulin-secreting active plasma or blast cells [21, 33].

How the lymphocytes know where to return is an interesting and important aspect of the mucosal immune response. It seems to be well established already that following activation in organized mucosal lymphoid tissues, B and T cells are able to upregulate the expression of tissue-specific adhesion molecules and chemokine receptors that function as "homing receptors" to guide the lymphocytes back to the mucosa through the recognition of endothelial counter-receptors in the mucosal vasculature [17, 22, 34]. For example, the exit of the lymphocytes into the mucosa occurs because lymphocytes that are primed by antigen in the GALT lose expression of L-selectin and selectively upregulate the expression of $\alpha_1\beta_7$ integrin. This guides the emigration of lymphocytes from the bloodstream by interacting with mucosal addressin cell-adhesion molecule 1

(MADCAM1) [31]. Another example refers to the expression of the chemokine receptor-9 (CCR9), induced by gut-derived T cells that respond to the chemokine ligand-25 (CCL25), also known as TECK (thymus-expressed chemokine), which is expressed selectively by small bowel epithelial cells. On the contrary, T cells primed in peripheral lymphoid organs cannot migrate to mucosal surfaces because they do not express the same molecules.

IgA-secreting B cells that are activated in MALT express CCR10, the receptor for CCL28. Therefore, CCR10+IgA+ B cells can be attracted by all tissues containing CCL28-secretor epithelial cells, which include the small and large intestines, salivary glands, tonsils, respiractory tract and lactating mammary glands [22]. This mechanism explains why mucosal immunization at one site can result in the secretion of specific IgA antibodies in other mucosal or glandular tissues. On the other hand, there are also some receptor-mediated recognition systems that have a more selective function. For example, IgA+ B cells that are generated in the intestinal inductive sites express the homing receptor $\alpha_4\beta_7$-integrin that interacts strongly with MADCAM1, an addressin that is expressed by venules in the small and large intestines and in lactating mammary glands, but not in other mucosal tissues [22]. The reason that IgA+ B cells which are activated in the peripheral lymph nodes following systemic immunization do not migrate to mucosal sites seems to be related to their inability to express the CCR10, $\alpha_4\beta_7$-integrin and other mucosal “homing receptors”.

Mucosal antigen delivery can either up-regulate or down-regulate systemic immune responses. Therefore, the understanding of both mechanisms will provide better guidance on the technical aspects for mucosal vaccine design.

1.4.1 Production of immunoglobulin A (IgA)

Although IgA constitutes only 10%-15% of the total immunoglobulin in serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva, tears and mucus of the bronchial, genitourinary and digestive tracts [2]. In humans, more IgA is produced than all other immunoglobulin isotypes combined, and IgA is concentrated over 1 mg /ml in secretions associated with the mucosal surfaces [35]. The IgA of external secretions, called secretory IgA (sIgA), consists of a dimer or tetramer, a j-chain polypeptide and a polypeptide chain called the secretory component [2, 36]. This

sIgA is resistant to degradation in the protease-rich external environment of mucosal surfaces. The resistance is due to its dimerization and high degree of glycosylation during its synthesis in mucosal plasma cells, and by its association with a glycosylated fragment (the secretory component) [17].

The secretory immunoglobulin A has several functions in mucosal defence described elsewhere [16, 17, 21] . So-called “immune exclusion” is a mechanism that consists of the entrapment of antigens or microorganisms by the sIgA in mucus, preventing direct contact of the antigen with the mucosal surface [17, 37]. Additionally, specific sIgA might block or sterically hinder the microbial surface molecules that mediate epithelial attachment [38].

1.4.2 Immunological tolerance

Epithelial cells are active participants in the mucosal defence. They have been described as functioning as sensors detecting dangerous signals like microbial components through pattern recognition receptors such as Toll-like receptors (TLRs) [17]. The epithelial cells respond to the dangerous signals by producing cytokine and chemokine signals to underlying mucosal cells, such as dendritic cells (DCs) and macrophages, to trigger innate, non-specific defences and promote adaptive immune responses [17, 39].

In the intestine, the environment is extraordinarily rich in food antigens and microorganisms that constitute the normal flora. For this reason, there are mechanisms that reduce and modulate the cytokine and chemokine signals to avoid undesirable responses (reviewed in [40-42]) such as mucosal inflammation. The mucosal surfaces are in a permanent state of alert, but they adapt to the presence of foreign microorganisms. As a consequence, vaccines that produce a strong immune response if injected in sterile tissues such as muscle could be ignored when administered through mucosal surfaces [17]. This state of unresponsiveness or so-called immunological tolerance is dependent on the route of administration of the vaccine (see table 2) and has been appointed as one of the bigger challenges for mucosal vaccine development. Therefore, intended mucosal vaccination strategies should overcome mucosal tolerance mechanisms, and will require a more detailed understanding of the underlying mechanisms behind the phenomenon.

Table 2 – Route of antigen administration affects immunological response (adapted from ref. [43])

Route of antigen administration	Usual outcome
Subcutaneous	Immunization
Intramuscular	Immunization
Injury	Immunization
Intravenous	Tolerance
Mucosal (Oral, nasal and pulmonary)	Tolerance
Portal vein	Tolerance
Anterior chamber of the eye	Tolerance

Although the phenomenon of oral tolerance has been known for almost a century, the mechanistic basis is still not fully understood. For instance, the molecular mechanism by which the innate immune system distinguishes commensal from pathogenic bacteria is a topic of great interest which is so far not understood. Answers to this and others questions will provide vital information for the development of effective oral vaccines. Some review articles about the state of the art of this knowledge have been published recently [31, 43], therefore only a short summary concerning immunological tolerance is presented here.

Increasing evidence has shown that the induction of mucosal tolerance is related to the path for antigen internalization. One important pathway for tolerance might involve passing through intestinal epithelial cells, escaping capture by lamina-propria phagocytes and transport through blood capillaries to the liver [43]. Another important pathway for the entrance of the antigens from the lumen are via dendritic cells, which can intercalate between epithelial cells and sample antigens directly from the intestinal lumen [44]. It was recently demonstrated that the expansion of dendritic-cell populations mediates the enhancement of oral tolerance [45]. Moreover, these unprocessed antigens are carried through the lymphatics to the mesenteric lymph nodes, which have been implicated in oral tolerance [43, 46]. On the contrary, as demonstrated in more recent studies, Peyer's patches appear not to have an important role in the induction of tolerance [47-49], although the uptake of antigens via Peyer's patches is essential for the

induction of an immune response and determines the profile of the induced immune response when using particles as oral antigen carriers [50].

Another important approach for the induction of immunological tolerance is the administration of a single high dose of the antigen or a repeated exposure to lower doses. These two forms of tolerance, now the so-called high- and low-dose tolerance, are mediated by distinct mechanisms as described recently [43]. It is thought that T cells are the major cell type involved in the induction of mucosal tolerance. It is generally agreed that the status of oral tolerance can be explained by clonal anergy, clonal deletion of T cells or by active suppression by T regulatory cells through the secretion of inhibitory cytokines. The most controversial issue is how and where the antigen-specific T cells in the MLNs first encounter antigen, and Mowat [31] has reviewed several studies addressing this question. According to the same author, however, it seems more probable that presentation of the antigen to naïve T cells occurs in the MLNs themselves due to unprocessed antigen brought there by APCs that traffic to the MLNs after being loaded with antigen in the mucosa or Peyer's patches [31].

1.5 CHALLENGES IN ORAL AND NASAL VACCINE DESIGN:

Vaccines administered mucosally encounter the same host defence barriers as do microbial pathogens and other foreign macromolecules: they are diluted in mucosal secretions, detained in mucus gels, attacked by proteases and nucleases and barred by epithelial barriers [17]. Therefore, it is estimated that large doses of antigen would be required. Moreover, soluble non-adherent antigens are taken up at low levels if at all, and in the intestine, such antigens generally induce immune tolerance [43].

To circumvent or minimize these difficulties, vaccine formulations and delivery strategies have to be carefully designed in order to efficiently stimulate the innate and adaptive immune response appropriate for the target pathogen [17]. Following this idea, delivery strategies are likely to be most promising when they mimic pathogens. Therefore, particulate delivery systems that adhere to mucosal surfaces or even better that would be able to selectively target M-cells are likely to be the most effective [17]. Moreover, to be distinguished from commensal microorganisms, the vaccine formulations should also carry substances that activate innate signalling pathways in the

epithelial cells and/or in the underlying antigen-presenting cells. These substances which are included in vaccine formulations with the aim of enhancing its immunogenicity are termed adjuvants (*adjuvare*; latin, to help). Presently, there is no optimal adjuvant classification. Although the complete working mechanism of many adjuvants is not entirely known at the moment, classification based on their mode of action has been suggested [51, 52]. Increasing evidence has demonstrated that most non-particulate mucosal adjuvants act by binding to specific receptors, and this adjuvant-class is frequently named immunopotentiators. Particulate adjuvants mainly function to concentrate vaccine components and to target vaccines towards antigen presenting cells (APCs) or carry out a depot action (see table 3). Therefore, this section will briefly review existing mucosal adjuvants, mainly dealing with those which have been used in the present experimental work of this thesis. So, chitosan based particulate delivery systems and CpG oligodeoxynucleotides, described in the following chapters.

Table 3 – Classification of vaccine adjuvants (adapted from [52])

Antigen delivery systems	*Immunopotentiators*
Alum	MPL and synthetic derivatives
Calcium phosphate	MDP and derivatives
Tyrosine	CpG oligos
Liposomes	Alternative PAMPS - flagellin
Virosomes	Lipopeptides
Emulsions	Saponins
Microparticles	DsRNA
Iscoms	Small-molecule immunopotentiators
Virus-like particles	

1.5.1 Micro- and nanoparticles as polymeric vaccine delivery systems

The category of particulate carriers includes different particles which have been widely reviewed in the recent scientific literature, including microemulsions (such as MF59) [52,

53], iscoms [54, 55], liposomes [54], virosomes [56], virus-like particles and polymeric microparticles [52, 57-61]. These particles have a common feature, which is that their size should be similar to the size of a pathogen in order to be taken up by APCs [62, 63] and subsequently deliver the associated antigen into these cells. Therefore, the main role of the delivery systems is to concentrate the antigen in the lymphoid tissues responsible for immune response induction. However, the potency of these delivery systems can be significantly improved by the association of an immunopotentiator (see 5.2) [64]. This aspect is of particular importance for recombinant vaccines and other weak antigens.

Therefore, there is a huge amount of information about the interaction of immune cells with different compounds (immunopotentiators) and particulate delivery systems, which allow for vast combination possibilities to be used in adjuvant formulations. According to O'Hagan [52], we are entering an exciting and dynamic time in vaccine research in which the principles leading to the successful induction of potent and protective immune responses are becoming better understood. This explosion of knowledge is not only for the traditional parenteral routes of vaccine administration but also for mucosal vaccination. Regarding oral and nasal vaccination, the entrapment of vaccine antigens in delivery systems has two main purposes. The first goal is to protect the antigen against degradation on mucosal surfaces, and the other is the enhancement of their uptake in MALT. The most successful work in achieving these two goals has been done with nano- and microparticles. The interaction between particulates and the GALT has been a subject of several reviews [65-68], and a deep understanding of this interaction would be key in the design of successful nanoparticles. The uptake of inert particles has been shown to take place transcellularly through normal enterocytes and specialized M-cells, or to a lesser extent across paracellular pathways through the tight junctions between cells [66]. Although transport by the paracellular route has been shown for example with polyalkylcyanoacrylate nanocapsules in the jejunal mucosa of the rat [69], the probability of its incidence does not seem to be high since the opening diameter of the gap junctions between the cells is between 7 and 20 nm in diameter [66].

Regarding the transcellular transport, its occurrence via M-cells appears to be a very natural mechanism since M-cells are specialised for endocytosis and subsequently transport the particulates to the adjacent lymphoid tissue (Peyer's patches in the gut). Therefore, after the particle binds to the M-cell apical membranes, the particulates are rapidly internalized and offered to the continuous lymphoid tissue. Depending on their

size, the particles can be retained within the lymphoid tissue (>3μm) [65], or they can be internalized by phagocytic cells and subsequently transported to another lymphoid tissue through the lymphatic vessels that innervate the PP dome area. There is a broad consensus that M-cells, associated with Peyer's patches are the main target for vaccination purposes. However, several questions have arisen regarding this issue. One issue is related to the number of Peyer's patches in the gut and therefore the total area covered with M-cells. Mice and rats have between 6 and 10 discrete Peyer's patches, while a human being has many hundreds [70]. In this respect, the differences between mice and men mean that one must take extreme caution when extrapolating from animal models to humans. On the other hand, these uptake studies have been performed in a small target area in the animal models. Another question is related to the factors that may influence the particle uptake across the gastrointestinal tract epithelium. Some examples reviewed in [65, 71] are the particle size, polymer composition, particle hydrophobicity, particle surface charge, particle dose, administration vehicle, animal species and age, feeding state of the animals, use of penetration enhancers and use of targeting agents.

A number of polymeric delivery systems have been evaluated by mucosal routes, however most of the work in this area has focused on poly(lactide-co-glycolide) (PLGs) polymers (some examples in ref. [72-78]). These polymers are biodegradable and biocompatible, and there is already a long experience of their use in humans as a suture material [71]. Moreover, they have appropriate release characteristics for use in single-dose vaccines [79]. One of the limitations of this polymer is its insolubility in water, which makes the use of organic solvents necessary in particle preparation. Additionally, the antigen may also be exposed during the manufacturing process of the particles to high shear stress, aqueous/organic interfaces and elevated temperatures, which have been considered extreme conditions for working with proteins and antigens. More recently, a different approach was adopted with these nanoparticles as the antigen was adsorbed onto the cationic modified surface of PLG nanoparticles after their preparation [80, 81].

Although PLG have been successfully used for the entrapment of several antigens, the investigation of new polymeric delivery systems produced in a harmless environment has emerged over the last years. Some examples include the preparation of alginate microspheres for the entrapment of rotavirus [82], polyacryl starch for the entrapment of salmonella [83] and chitosan nanoparticles for the entrapment of diphtheria [84].

1.5.1.1 Chitosan

The term chitosan is applied to a family of deacetylated chitins and is the only largely available cationic polysaccharide. Chitosan is a copolymer of glucosamine and N-acetyl glucosamine linked by β-(1-4) linkages. It has been considered a non toxic, biodegradable and biocompatible polymer, so a lot of research has been directed toward its use in medical applications such as drug delivery. The properties of chitosan and its biomedical applications have been recently reviewed [85-91]. One major advantage of this polymer is its ability to easily produce nanoparticles under harmless conditions. This has been one of the main reasons for its wide applicability to the encapsulation of macromolecules such as DNA, proteins, vaccines and peptides. Additionally, the loading efficacy for these macromolecules is generally very high.

Another important issue is the finding of the capability of chitosan nanoparticles to be internalized in tissues or in cells. Recent studies using *in vitro* cell culture models demonstrated the uptake of chitosan nanoparticles by a number of different cell lines [92-97]. These studies emphasised that the polycationic, mucoadhesive chitosan nanoparticles association more strongly with mucus-secreting cells (MTX-E12) than with Caco-2 cell monolayers [92]. Moreover, an intense electrostatic interaction between positively charged chitosan nanoparticles and negatively charged mucins is regarded as the cause for the strong interaction of chitosan with mucus [92, 98].

Those nanoparticle formulations have been used as delivery platforms for different vaccines such as meningococcal C conjugate [99], diphtheria [84] and tetanus toxoid [100, 101]. Chitosan has also been used without any modification, suspending the bulk powder in a solution of the meningococcal C conjugate vaccine [102], using a soluble chitosan derivative with the influenza vaccine [103, 104] or finally by using chitosan to surface-modify PLGA microspheres containing hepatitis B vaccine for intranasal immunization [105].

1.5.2 Immunopotentiators

Non-microbial particles, macromolecules and protein-subunit antigens generally induce weak or undetectable adaptive immune responses when applied mucosally. The encapsulation of the antigen in a particulate delivery system can direct the antigen to the inductive site, ideally to the Peyer's patches, but may not be sufficient to evoke an

appropriate immune response, because it may not be recognized as a harmful particulate. To be distinguished from harmless substances and nutrients, therefore, mucosal vaccines should raise alarms in the mucosa by including substances that activate innate signalling pathways [17].

The best-known mucosal immunopotentiators are the secreted enterotoxins of *V. cholerae* and *E. coli*, cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT). However, this kind of adjuvants has been shown to be toxic for humans. Therefore, several genetically modified forms have been engineered to reduce or eliminate the toxicity associated with the enzymatic A subunits of these toxins [106, 107]. Furthermore, many live attenuated mucosal vaccine vectors, including poliovirus, adenovirus and enteric bacteria are currently under development and have been extensively reviewed [108, 109]. Although the superiority of these live attenuated pathogens as mucosal vaccines and vaccine vectors is due in part to their ability to activate multiple innate immune responses, some safety and acceptability issues will delay their use in humans.

Meanwhile, with the recent progress in this area, a number of immunopotentiators have become available for inclusion in vaccines (see table 3), which have been extensively reviewed elsewhere [52, 64, 110]. Moreover, in more recent years, new information about the functions of immunomodulatory cytokines and the discovery of Toll-like receptors (TLRs) have provided promising new alternatives. It has also been demonstrated that the vertebrate innate immune system uses pattern recognition receptors, including TLRs, specifically to detect pathogen-associated molecular patterns (PAMPs) present in infectious agents [111]. To date, at least ten different human TLRs have been identified, as well as a number of naturally occurring TLR ligands (some examples are described in table 4). For example, various TLR ligands including CpG-containing oligonucleotides [111], flagellin [112] and bacterial porins [113] have shown adjuvant activity when administered mucosally together with antigens. However, synthetic TLR ligands have also been identified, including imidazoquinoline compounds such as Imiquimod and Resiquimod (R-848), which activate human TLR7 and TLR8 [111] as well.

Table 4 - Toll-like receptors and naturally occurring ligands

Receptor	Ligand	References
TLR2	Lipoproteins and peptidoglycans	[114, 115]
TLR3	Double-stranded RNA of viral origin	[116]
TLR4	Lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acid from gram-positive bacteria	[117-120]
TLR5	Flagellin, a protein found in bacterial flagella	[121, 122]
TLR7/ 8	Single-stranded viral RNA	[123]
TLR 9	Unmethylated CpG motifs found in bacterial DNA	[124-126]

1.5.2.1 CpG ODNs

According to a definition of Krieg [127], CpG motifs are DNA oligodeoxynucleotide sequences that include an unmethylated cytosine-guanosine sequence and certain flanking nucleotides, which have been found to induce innate immune responses through interaction with the Toll-like receptor 9. The three major classes of CpG ODN that are structurally and phenotypically distinct are comprehensively described (A, B, C-class) elsewhere [111, 127-130]. B-class CpG ODN has been frequently used in animal studies due to their strong B cell activation and capacity to induce potent Th1-type immune response. The same B-class CpG have also been shown to be safe and efficacious vaccine adjuvants in humans [131, 132].

Although most cell types have the capacity to internalize CpG ODN via endocytosis [130], only those cells that express the TLR9 are activated. In humans, only B cells and plasmacytoid dendritic cells (pDCs) are able to express the TLR9, whereas in mice, TLR9 is also found on myeloid dendritic cells (mDCs), macrophages and monocytes [111]. Within minutes after exposure to CpG ODN, these cells take up the CpG ODN into an endosomal compartment where interaction with the TLR9 occurs [133]. This leads to the activation of cell signalling pathways comprehensively described by McCluskie [111].

CpG ODN has been shown to be an effective mucosal adjuvant after administration to different mucosal surfaces such as the respiratory tract [134-136], the genitourinary tract [137] and the gastrointestinal tract [138, 139] in combination with different antigens including the hepatitis B antigen [135, 140].

The combination of CpG with other adjuvants has been considered to be useful regarding several issues. One issue is CpG ODN, a strong Th1 profile inducer which has been shown to be able to dominate the Th2 bias associated with adjuvants such as alum or Freund's incomplete adjuvant (FIA) [135, 141]. Another important advantage is the depot effect offered by several adjuvants that may result in an extended release period during which both antigen and CpG ODN are available. Finally and not less important is the fact that the association of CpG ODN with nanoparticles may protect the CpG ODN from degradation on mucosal surfaces, particularly the ODN synthesized with the native phosphodiester (PO) backbone, which rapidly degrades *in vivo*.

1.6 THE PRESENT INVESTIGATION

Most pathogens gain access to their hosts through mucosal surfaces. The induction of helpful specific antigen mucosal antibodies is feasible only when the antigen is administered by one of the mucosal routes. On the other hand, a number of obstacles must be overcome in order to efficiently stimulate innate responses and evoke adaptive immune responses without disturbing mucosal homeostasis. This is why fewer mucosal vaccines have been developed. Therefore, the investigation of novel non-toxic adjuvants is urgently required. This thesis has as its main objective the development of a new nanoparticulate delivery system to be used as a mucosal adjuvant, and it will therefore contribute to the area of modern adjuvants.

1.6.1 Aim of the thesis:

The aim of this thesis is the design of a chitosan based nanoparticulate system as a novel antigen delivery system for mucosal surfaces. This delivery system is composed of a chitosan core with adsorbed antigen and a sodium alginate coating.

Coated nanoparticles should have an appropriate size (< 10μm) for internalization by M-Cells. Therefore, an appropriate coating method must be developed. Moreover, the use of polymers with mucoadhesive properties such as chitosan and alginate would confer optimized surface properties on the delivery system in order to have lower clearance rates in the NALT and in the GALT. A complete characterization of the delivery system was performed after the optimization of the preparation conditions.

The principal mode of action of this delivery system is to promote the internalization of the antigen into the Peyer's patches via M-cells of the FAE, and subsequently antigen uptake into APCs. Therefore, uptake studies in rat Peyer's patches were performed and are shown in chapter 3. Additionally, cytotoxicity and ovalbumin release studies from the particles are also shown in the same chapter.

The adjuvant capacity of the coated chitosan nanoparticles was evaluated using the recombinant hepatitis B antigen (HBsAg).

Hepatitis B vaccine represents the world's first subunit vaccine and the world's first recombinantly expressed vaccine, therefore the recombinant antigen was used as a model vaccine to evaluate the adjuvant properties of alginate coated chitosan nanoparticles. A second adjuvant was used, the CpG ODN 1826, with the goal of evaluating synergistic effects between the systems. At same time, the suitability of the new delivery system to encapsulate the CpG ODN was also evaluated. Therefore, several formulations have been tested. Chapters five, six and seven present the results of the *in vivo* studies with Balb/c mice for the oral, intranasal and subcutaneous routes of administration, respectively. Additionally, the mucosal adjuvants used with the hepatitis B antigen for each of the routes are reviewed in the introduction of each chapter.

Finally, although it is not a main concern in this thesis, the immunostimulatory properties of the polymers used in the construction of the present delivery system were evaluated. More precisely, mice spleen cells were cultured together with the polymers, and their stimulation status was estimated. Chapter four presents these results and performs a bibliographic review of the immunostimulatory properties of chitosan and alginate.

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CHAPTER

2

PREPARATION OF COATED NANOPARTICLES FOR A NEW MUCOSAL VACCINE DELIVERY[1]

[1] *Adapted from International Journal of Pharmaceutics 299 (2005) 155-166*

ABSTRACT

It has been found that the adsorption of antigens onto chitosan particles is an easy and unique mild loading process suitable to be used with vaccines. In order to increase the stability of this particles and to prevent an immediate desorption in gastrointestinal fluids, a coating process with sodium alginate was developed. One of the challenges of this developing process was to keep the particles in the nanosized range in order to be taken up by M-cells of the Peyer's patches. The observed inversion of the particles' zeta potential values after coating suggested the presence of an alginate coating layer. These results were confirmed by FTIR and DSC techniques. Additionally, in vitro release studies showed that the presence of the alginate layer around the particles was able to prevent a burst release of loaded ovalbumin and to improve the stability of the nanoparticles in simulated intestinal fluid at 37ºC. The optimisation of the coating process resulted in 35% (w/w) for the loading capacity of the coated particles. SEM investigations confirmed a suitable size of the coated nanoparticles for the uptake by M-cells.

2.1 INTRODUCTION

In recent years, mucosal vaccination is being considered as a subject of great interest due to its advantages above the i.m. or s.c. application. The presence of specific antibodies in mucosal surfaces has long been recognized as the first barrier against pathogens entrance. The most effective way to induce mucosal immunity (i.e., secretory IgA) is to administer a vaccine directly to the mucosal surface. Additionally, the existence of a common mucosal immune system allows successful targeting of vaccines to inductive compartments within mucosa-associated lymphoid tissues, inducing local humoral responses in lymphoid tissues at distant mucosal loci [1]. Both intranasal and oral routes have been used in several studies to achieve this goal. Particularly, the oral administration permits targeting of a suitable vaccine loaded delivery system to the ports of entry (so-called M-cells) of the largest inductive lymphoid tissue in the body, the intestine. The oral route is well accepted and easily allows the vaccination of large populations. However, the acidic environment of the stomach and the presence of

enzymes make the oral delivery of vaccines a challenge where is difficult to achieve high and reproducible effects. In order to solve these difficulties, a considerable number of polymeric microparticulate systems are under investigation to deliver vaccines to the intestine while protecting them from adverse conditions that could affect their bioactivity [2]. Another important aspect is that these delivery systems could act as imunostimulants or adjuvants, increasing the immunogenicity of poor immune response antigens [3, 4].

Nevertheless, from a pharmaceutical perspective, it became evident that further advances in the formulation of delivery platforms needs to be introduced in order to increase both the stability of the antigens in the gastro-intestinal tract and the uptake of antigen-containing particles by the M-cells. One of the parameters that should be addressed is the size of the particles. It is well known that the size of the particles should be below 10 □m in order to be taken up by M-cells of the Peyer's patches in the gut [5, 6]. Moreover, the preservation of antigen stability during encapsulation is also essential for the development of a successful controlled release vaccine delivery platform.

Chitosan microparticles as an oral and intranasal vaccine delivery system were already used in our group showing promising capabilities [7-9]. In these studies the vaccine was loaded by a mild and simple but effective adsorption method. By this method, deleterious preparation conditions, like elevated temperatures, high shear rates or the presence of organic solvents were avoided. This method has also been described by other groups that reported good adsorption capacities for different substances [10, 11]. In the case that the chitosan particles are not very porous, the antigen will be preferentially adsorbed to the particle surface. This can cause stability problems because processes like desorption or the attack of the antigens by enzymes or acidic substances from the body fluids may occur. These obstacles may be overcome by coating those particles with an acid resistant polymer, like sodium alginate.

The two chosen polymers chitosan and sodium alginate, for this novel delivery platform are naturally occurring polysaccharides. They are polyelectrolyte polymers of opposite charges, biocompatible and biodegradable, and show a good safety profile. Furthermore they have been reported as pharmaceutical excipients (PhEur 2002). Chitosan is the deacetylated form of chitin comprising copolymers of glucosamine and N-acetyl glucosamine linked by □-(1-4) linkages. The primary amino groups lead to special properties that make chitosan very interesting for pharmaceutical applications. Sodium alginate is also a hydrophilic polymer and comprises D-mannuronic (M) and L-guluronic acid (G) residues joined linearly by 1,4-glycosidic linkages [12]. The wide pharmaceutical

applicability of alginates is, to a large extent, associated with their gel-forming capacity. Di- or polyvalent cations (calcium being the most widely studied example) can induce the gelation by cross-linking of the guluronic acid units [12, 13]. Sodium alginate has been used for preparing nanoparticles [13, 14], microspheres [15-20], microcapsules [21] and beads [15], for oral delivery. In particularly the use of alginate microparticles as an antigen delivery system has been described in several publications and there are some indications that they are able to induce a mucosal and systemic immune response in a variety of animal species by both oral and intranasal administration [22-24].

Over the last years, sodium alginate has also been used as a coating material for cells with some advantages. It seems that the coating acts as a barrier to microbial contamination and thus improved survival prospects of the coated cells [25]. In another study the coating is performed to protect donor mammalian cells against antibodies and cytotoxic cells of the host immune system, allowing the transplantation of cells in the absence of immunosuppression [26].

This manuscript describes the development of a true nanocoating procedure, whereas other publications describing the entrapment of cells, liposomes [27] or microspheres [10, 28] in an alginate gel matrix. This is, as far as we know, the first time that the construction of a nanosized alginate coated chitosan delivery system is described with the particularly aspect that the antigen is adsorbed to the chitosan particles surface.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Chitosan was purchased from Primex BioChemicals AS (ChitoClearTM, Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation is 95% (titration method) and the viscosity is 8 cP (1% solutions in 1% acetic acid). Low viscosity sodium alginate was kindly donated by ISP Technologies Inc (MANUCOL LB□, Surrey, UK). Ovalbumin (grade V; minimum 98%) was purchased from Sigma Chemicals (St.

Louis, USA) and all the others reagents used were of analytical grade. All solutions were prepared in Millipore water.

2.2.2 Preparation of chitosan particles

Chitosan particles were prepared by the precipitation/coacervation method described previously [29]. Shortly, chitosan was dissolved at a concentration of 0.25% w/v in a solution with 2% (v/v) of acetic acid and 1% (w/v) of Tween 80. The formation of the particles was achieved after the addition of 3.5 ml of sodium sulfate solution (10% w/v) to 200 ml of the chitosan solution. The addition was made at a rate of 1 ml/minute under mild agitation (< 50 rpm) and continuous sonication (vibracell sonicator; 600-Watt model (output control "1"); Sonics & Materials, INC, Danbury; USA). Sonication was maintained for additional 15 minutes and the agitation for 60 minutes at room temperature (RT). The suspension was centrifuged for 30 minutes at 3500 rpm (2800 x g) and the supernatant was discarded. The particles were re-suspended twice in Millipore water, centrifuged again for 30 minutes and the supernatants were discarded. The particles were frozen in liquid nitrogen and freeze-dried overnight using a Christ freeze-dryer (Osterode am Harz, Germany). The dry powder was kept frozen until further use.

2.2.3 Loading of the particles with ovalbumin

The first step of the loading procedure was the suspension of the freeze dried particles in a phosphate buffer (pH =7.4) placed in an ultrasound bath for 30 minutes in order to disaggregate the particles. The loading was done by incubating a solution of ovalbumin with chitosan particles under mild agitation at room temperature. The various concentrations used are presented in table 1.

The loading efficacy and the loading capacity of the uncoated particles were calculated by an indirect way, quantifying the protein that remained in solution. After incubation, an aliquot of the particle suspension was centrifuged at 14,000 rpm for 30 minutes and the protein in supernatant was quantified by BCA-protein assay (PIERCE, Rockford, USA) using a microplate reader with a 590 nm filter (BIO-RAD model 550, Veenendaal, The Netherlands). The absorbance reading value was corrected subtracting the average absorbance reading obtained in the BCA-protein assay from that one of the

supernatants of unloaded nanoparticles prepared exactly in the same conditions. The corrected OD value was then used to calculate the concentration using the standard curve prepared at same time from individual ovoalbumin standards.

The drug loading capacity (LC) and loading efficacy (LE) were calculated from the following equations:

LC (%w/w) = (total amount of ovalbumin – non-bound ovalbumin)/weight of the particles *100

LE (%) = (total amount of ovalbumin – non-bound ovalbumin)/total amount of ovalbumin *100

Table1 - Composition of the different systems used for the development of the alginate coating method of the chitosan nanoparticles.

	A	B	C	D	E
Loading	1% particles 0.5% ovalbumin (PB; pH=7.4)	0.5% particles 0.25% ovalbumin (PB; pH=7.4)	0.375% particles 0.19% ovalbumin (PB; pH=7.4)	0.4% particles 0.25% ovalbumin (PB; pH=7.4)	0.5% particles 0.25% ovalbumin (PB; pH=7.4)
Coating	No	0.125% particles 0.5% alginate (water)	0.188 % particles 0.5% alginate (phosphate buffer pH=7.4)	0.2 % particles 0.5% alginate (phosphate buffer pH=7.4)	0.25 % particles 1% alginate (phosphate buffer pH=7.4)

2.2.4 Coating of the nanoparticles with alginate

Various amounts of the ovalbumin loaded particle suspension were added under agitation to various solutions of sodium alginate (table 1). The suspension of the particles was maintained under agitation with a magnetic stirrer for 20 minutes at RT. The suspension was then centrifuged for 10 minutes at 1600 rpm and the supernatant was discarded. To chemically cross-link the alginate at the particle's surface, the particles were re-suspended in 0.524 mM CaCl2 solution and kept under agitation for another 10 minutes. For the characterization of the nanoparticles (section 2.5.) the optimised formulation batch was used as given in table 1 (system D).

2.2.5 Evaluation of the desorption during the coating procedure

During the incubation of the particles with sodium alginate, aliquots of the particles suspension were collected, centrifuged at 14,000 rpm for 30 minutes and the protein in the supernatant was assayed with a BCA-protein assay as described in section 2.3.

Statistical methods used in this section include descriptive statistics (arithmetic mean and standard deviation) and Student's t-test.

2.2.6 Characterization of the nanoparticles

2.2.6.1 Morphology

The morphology and surface appearance of the particles were examined by scanning electron microscopy (SEM). One drop of the particles suspension was placed on a gold-coated plate and maintained at least 12 hours at room temperature in a desiccator for complete dryness of the sample. The dry samples were coated with a thin gold layer using Emitech K650X large sample coater (Emitech, Kent, UK) and observed with a Jeol JSM-6700F field emission scanning electron microscope (JEOL BV, Schiphol-Rijk, The Netherlands).

2.2.6.2 Size and zeta potential measurments

The particle size and zeta potential were evaluated by a dynamic light scattering technique with a Zetasizer 3000HSA (Malvern Instruments, Bergen op Zoom, The Netherlands). Zeta potential determinations were based on electrophoretic mobility of the nanoparticles in diluted aqueous suspensions. These measurements were performed at least in triplicate with independent particle batches.

2.2.6.3 FT/IR studies

The coated particles were washed with Millipore water, centrifuged and the sediment was freeze-dried overnight (Labconco, Kansas City, USA). The coated and uncoated particles were kept in desiccator at room temperature until analysis. The IR spectra of

the samples were recorded using a Fourier-transformed infrared spectrophotometer instrument FT/IR – 420 Jasco (Jasco Inc, Tokyo, Japan) with attenuated total reflection (ATR).

2.2.6.4 Differential scanning calorimetry (DSC)

DSC scans were recorded using a differential scanning calorimeter (DSC-50, Shimadzu Co, Kyoto, Japan). Two to 4 mg of the dry particles were accurately weighed into aluminium pans without seals and heated from 25 to 350ºC at a heating rate of 10ºC/min under a nitrogen flow of 20 ml/min.

2.2.6.5 Invitro release studies

The ovalbumin release from the coated and uncoated particles was performed in simulated intestinal fluid (SIF) as described in USP XXIV. The nanoparticles suspensions were added (1:4) to individual tubes containing the release medium previously equilibrated at 37ºC and placed in a shaker bath adjusted to 50 rpm. At appropriate time intervals, samples from each tube were filtered with a low protein-binding filter (MILLEX®GV - 0.22 µm; durapore PVDF membrane; MILLIPORE, Malsheim, France) followed by centrifugation for 20 minutes at 14,000 rpm and the ovalbumin in supernatant assayed with a BCA-protein assay. Simultaneously coated and uncoated blank nanoparticles suspensions were submitted to the same conditions and the filtered samples were used as blanks for correction of the BCA-protein assay as described in section 2.3.

For the determination of the total protein content, 0.5 ml of the suspensions of the coated and uncoated particles were incubated with 1.5 ml of 0.085N hydrochloric acid solution (pH=1.2) in an ultrasound bath for 30 minutes, followed by 3 hours in a water bath at 37ºC. The samples were filtered and the protein was assayed by the BCA-protein assay. Suspensions of unloaded particles were analysed under the same conditions and were used as a blank for the correction of the OD value of the samples analysed with BCA-protein assay as described in section 2.3. Additionally, the unbound ovalbumin in the particle's suspension was also determined in order to calculate the amount of the ovalbumin encapsulated in the beginning of the assay.

All experiments were performed at least in triplicate.

2.2.6.6 SDS-polyacrylamide gel electrophoretic (PAGE) analysis of released ovalbumin (OVA)

Samples from the loading and coating particle suspensions were centrifuged at 14000 r.p.m, the supernatants were collected and the concentration was adjusted in order to have the same theoretical concentration (i.e. assumption of 0% of loading efficiency) of the protein. Alginate coated chitosan nanoparticles loaded with ovalbumin were diluted (1:5) in buffer phosphate pH= 7.5 at 37ºC and incubated overnight at 50 r.p.m. An aliquot was collected and centrifuged. The ovoalbumin samples were then suspended in the loading buffer and heated for 5 min at 100ºC just before the run.

The SDS-PAGE was performed with gels composed of 12% acrylamide, cast and run in tris-glycine buffer. Gels were stained with 0.1% Coomassie Brilliant blue in 10% of acetic acid in a solution of methanol:water (1:1).

2.3 RESULTS AND DISCUSSION

2.3.1 Preparation of the vaccine delivery system

The preparation of the delivery system contains three main steps, the manufacturing of the chitosan particles, their ovalbumin loading by adsorption and finally the coating with sodium alginate. The formation of the chitosan particles by a precipitation process with sodium sulphate has been described by several authors [29-31]. They all describe an existing correlation between the necessary amount of sulphate ions, the molecular weight and deacetylation degree of the polymer.

The loading of the cationic particles with the protein is a very mild process, which can be achieved by suspending the particles in a solution of the protein in an appropriate buffer. The adsorption of the protein onto chitosan particles is mainly caused by ionic interaction of the chitosan amino groups with the carboxyl groups of protein substrate in a buffer phosphate solution of pH = 7.4 with high buffer capacity. According to previous work done in our laboratory [30], the loading capacity of ovalbumin at a chitosan particles concentration of 1% (w/v) is not substantially influenced in the range of 0.5% to 2% (w/v) ovalbumin. Therefore, an ovalbumin concentration of 0.5% (w/v) was used as loading

solution resulting in a sufficient high loading capacity. For instance, for the system A, as shown in figures 1and 2 the loading capacity and the loading efficacy were about 40% and 80%, respectively. It was also found from the measurements of the unbound protein at different time points during the loading process that the adsorption equilibrium is rapidly reached. This means that the protein is bound to the surface of the particles and that the adsorption process of the ovalbumin to the particles surface occurs immediately after the addition of the protein solution to the particle suspension. Better loading capacity results ($p<0.00001$) of the uncoated particles were found when the ratio particles/ovalbumin in the loading suspension was decreased from 2 (system A, B and E) to 1.6 (system D) while the loading efficacy (uncoated particles) was not different ($p=0.15$) between the systems.

The third step of the preparation was the coating of the chitosan nanoparticles with alginate solution. To our knowledge this coating process has not been described in literature earlier. Consequently, the first part of this work is focused on the selection of the appropriate conditions and experimental methodology for the coating process with sodium alginate. For that purpose several systems with different ratios sodium alginate/chitosan particles have been investigated with the main objective to obtain a stable particle suspension. In the majority of the first trials an immediate flocculation was observed, particularly in systems with higher particles concentration. The formation of these agglomerates was easily observed because of the formation of a precipitate and a clear supernatant. Systems with a ratio alginate/particles > 2 have shown to be stable. Thus, in the following optimization steps of the coating methodology always ratios higher than 2 were used.

Another important parameter studied was the desorption of ovalbumin from the particles during the coating process. The addition of the sodium alginate solution to the suspension of the loaded particles resulted in a new adsorption equilibrium characterized by the different concentration of protein and by the presence of alginate polymer that can compete with the interaction of the charges at the particle's surface. A significant decrease of the loading capacity of the coated particles was observed in all the systems ($p< 0.05$ for systems B, C, D, E). Furthermore, we have observed that modifications in the pH of the coating medium can also modify the adsorption equilibrium of the protein. In our first experiments, the coating process was carried out at pH 5.5. This pH value seems to be the most favourable for the interaction between chitosan and alginate as the pKa of the chitosan is around 6.5 and the pKa of the sodium alginate is between 3.4 and

4.4. However at this pH value we have observed more than 60% of ovalbumin desorption (data not shown). For that reason a pH =7.4 was adopted as it was observed to have the better compromised between loading capacity and efficiency of the coating process.

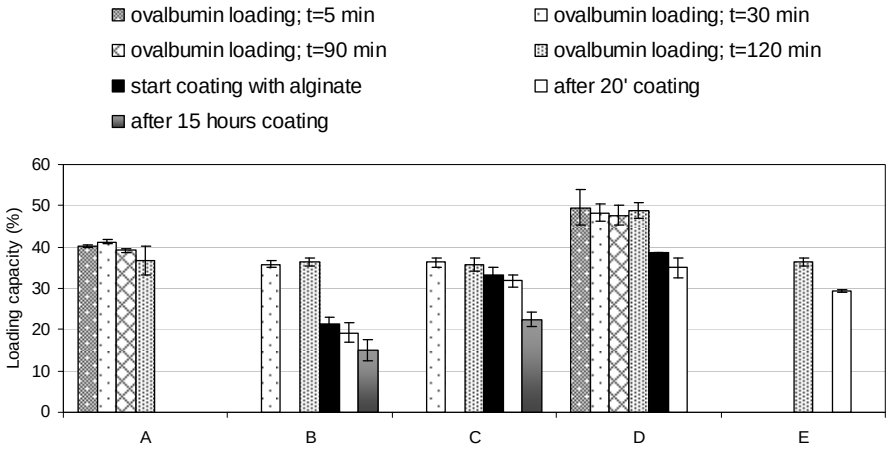

FIGURE 1. Loading capacity of the particles during the different stages of the coated particles preparation. Results for the different systems (A, B, C, D, and E)

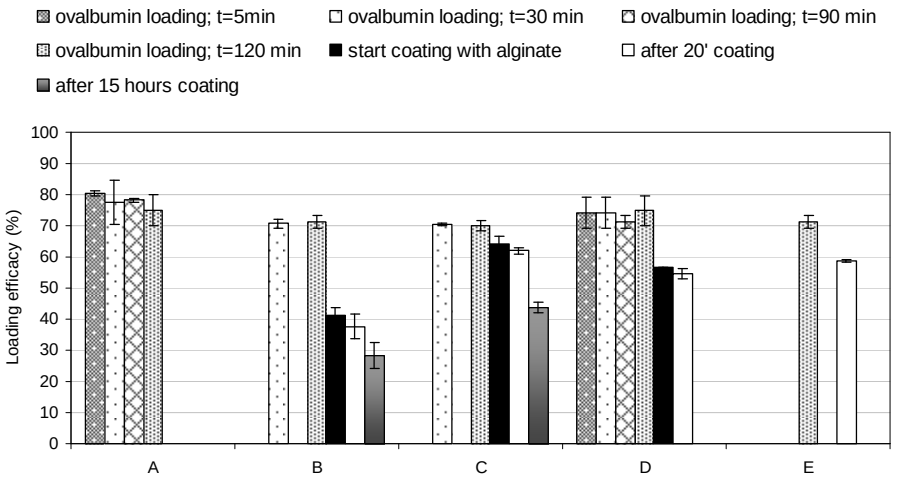

FIGURE 2 Loading efficacy of the particles during the different stages of the alginate coated particles preparation. Results for the different systems (A, B, C, D, and E)

The highest loading capacity for the alginate coated nanoparticles was achieved with system D (p=0.086 when comparing with system B and p=0.032 comparing with

system E). On the other hand the comparison with system B (p=0.001) and E (p=0.004) showed, that this option (system D) was achieved on the expenses of a slight decrease of the loading efficacy (figure 2).

2.3.2 Characterization of the nanoparticles

2.3.2.1 Morphology, size and zeta potential measurements

The precipitation of chitosan with sodium sulphate using ultrasounds for homogenisation led to the formation of particles in the nanoscale size (table 2). One of the major currently described drawbacks of this technique is the high polydispersity of the obtained nanoparticles [32]. Sizes between 100 nm and 1000 nm were found by SEM technique. The mean hydrodynamic diameter of the obtained particles after the precipitation process was 684 nm and the size increased after the freeze-drying process (table 2). This is a direct consequence of particle aggregation during the drying process. To overcome particle aggregation the use of trehalose as a lyoprotectant was tried at concentrations of 3.3%, 5% and 7% (w/v).

A complete redispersibility of the freeze-dried nanoparticles in all trehalose concentrations and the maintenance of the particle size could be observed by light microscopy and dynamic light scattering technique. However, we optimised the duration of the freeze-drying process in order to avoid the use of cryo-protectants as they would interfere with the coating process.

Table 2 - Size of the chitosan nanoparticles in different stages of the preparation process.

	Cumulant Z Ave (nm) **Mean ± STDEV**	**Polydispersity) Mean ± STDEV**
After the precipitation process	**643.2 ± 171.7 (16 batches)**	**0.379 ± 0.168 (16 batches)**
After lyophilization process	**955.6 ± 161.0 (3 batches)**	**0.387 ± 0.129 (3 batches)**

SEM observations (figure 3) indicated that the size range of the particles remained unchanged. This result indicates the feasibility of coating of ovalbumin loaded chitosan nanoparticles with a thin layer of alginate. SEM images (figure 3) show in first picture some small particles (< 100 nm) and a particle's agglomerate. However it is not clear if this is a consequence of the freeze-dry process or if it is a consequence of the sample preparation (particle's concentration of the sample) for the SEM (figure 3b).

The stability of many colloidal systems is directly related to the magnitude of their zeta potential. In general, if the value of the particle zeta potential is large, the colloidal system will be stable. Conversely, if the particle zeta potential is relatively small, the colloid system will agglomerate. The surface charge of the particles is of substantial importance in all the production steps of these coated particles because the efficiency of the different steps is directly related to the establishment of electrostatic interactions. Chitosan particles showed a positive value of about 37 mV, which explains the stability of the particle suspensions in water and in several buffer systems. After adsorption of ovalbumin, the particles are still positively charged with values higher than 30 mV. During the coating procedure an inversion of the surface charge of the particles to negative values was observed. This zeta potential inversion explains the difficulties that were found in preventing the formation of particles agglomerates during the coating process. After complete adsorption of the alginate to the particles, a surface charge of the particles of about –35 mV was found (table 3). This zeta potential inversion is a strong indication of the presence of an alginate coating on the surface of the particles.

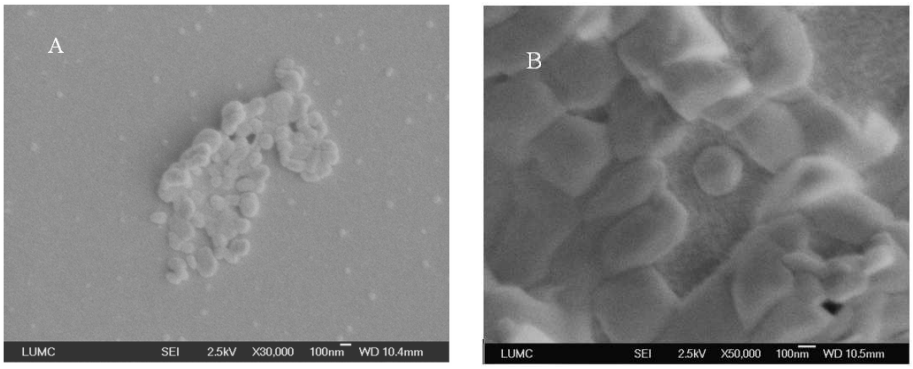

FIGURE 3. Scanning electron micrographs of chitosan nanoparticles. A) After freeze-dry and ressuspending in water. B) After the coating procedure with sodium alginate.

Table 3 - Zeta (□) potential of unloaded, ovalbumin loaded chitosan nanoparticles and alginate coated chitosan nanoparticles.

	Empty chitosan nanoparticles	Ovalbumin loaded chitosan particles	Alginate coated chitosan particles
Zeta potential (mV) mean average ± STDEV	+ 37.0 ± 3.6 (7 batches)	+ 41.3 ± 6.4 (5 batches)	- 34.9 ± 8.3 (5 batches)

2.3.2.2 Differential scanning calorimetry

As shown in Figure 4, the DSC scans of the chitosan polymer exhibited an endothermic peak at about 66ºC that has been attributed to the evaporation of absorbed water. The exothermic baseline deviation beginning around 250 ºC indicates the onset of chitosan degradation [33]. The analysis of the DSC curves for chitosan particles showed two additional endothermic peaks at about 237ºC and 275ºC. The peak at 237ºC is probably related to the breakdown of weak unspecific electrostatic interactions. The second peak is probably related to the cleavage of the electrostatic interactions between the polymer and the sulphate ions.

After the coating process with sodium alginate, the particles exhibited a completely different behaviour as shown in figure 5, which means that an alteration occurred in the composition of the particles. In the DSC scans the two endothermic peaks disappeared and no endothermic peak was found in the temperature range studied. In fact, a gradual appearance of an exothermic behaviour was detected starting around 200ºC that coincides with the exothermic behaviour of the sodium alginate as referred to in several publications [34] as the decomposition of the polymer.

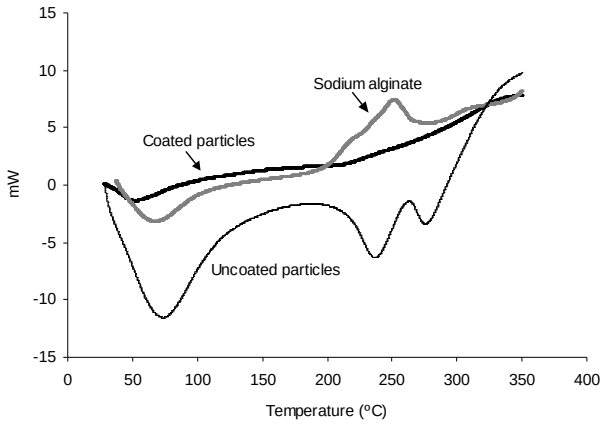

FIGURE 4. Differential scanning calorimetry curves of chitosan, unloaded chitosan particles and sodium sulphate

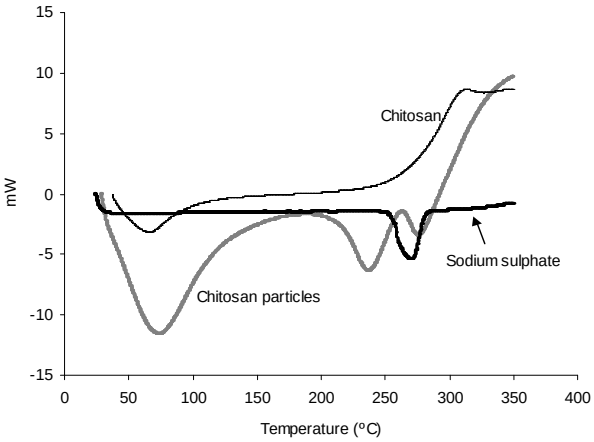

FIGURE 5. Differential scanning calorimetry curves of sodium alginate, uncoated chitosan particles and alginate coated particles. The DSC investigation was conducted using the unloaded particles.

These observations strongly support the presence of alginate molecules linked to the surface of the chitosan particles: when the temperature of the sample reaches values around 200ºC, the two phenomena, the exothermic contribution from the alginate and the endothermic contribution from the chitosan particles determine the shape of the curve.

2.3.2.3 FTIR characterization

The FTIR spectra of chitosan and the chitosan particles, sodium alginate, and the coated particles are shown in Figure 6 and 7, respectively.

In the chitosan spectra the strong and broad peaks in the 3400-3200 cm-1 ranges correspond to combined peaks of O-H stretching and intermolecular hydrogen bonding. The N-H stretching from primary amines are overlapped in the same region. The C=O stretching (amide) peak near 1633 cm-1 and N-H bending (amide and amine) peak near 1542 cm-1 was observed as well. The intense peak at 1414 cm-1 belongs to the N-H stretching of the amide and ether bonds give the peaks in the fingerprint region of the spectra, where the symmetric stretch of C-O-C is found around wave numbers of 1065-1027 cm-1. In the chitosan particles the peak of 1558 cm-1 is shifted to 1535 cm–1 and the relative intensity of this peak is reduced. In addition the peak related with C-N stretch (1414 cm–1) has almost disappeared in the chitosan particles. Similar observations are reported for chitosan particles prepared with tripolyphosphate [35]. These observations are in agreement with the fact that the sulphate ions interact with the primary amino groups of the chitosan, resulting in the formation of crosslinked chitosan particles (reticulation process).

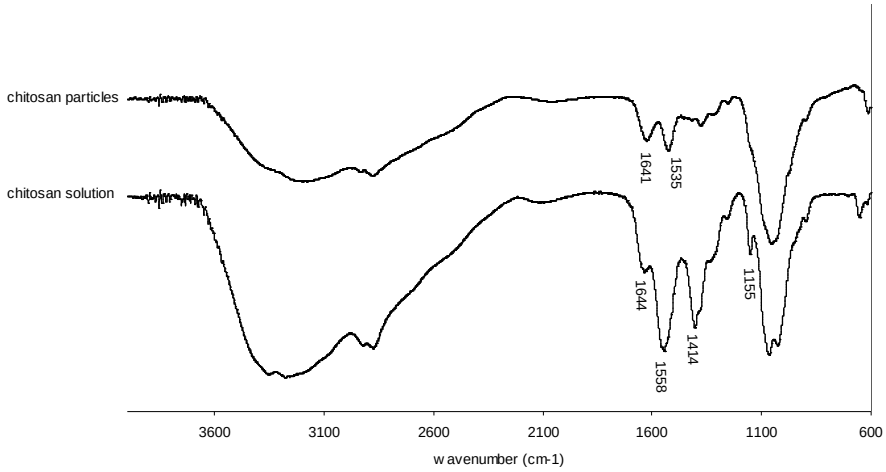

FIGURE 6. FTIR spectra of chitosan and unloaded chitosan particles

Sodium alginate as a carboxylate salt showed a strong asymmetric stretch at 1605 cm-1. The frequency of carbonyl absorption is lowered compared to the value

found for the parent carboxylic acid due to a resonance phenomenon. The carboxyl and carboxylate groups are present at wave number of about 1000-1400 cm-1. From the FTIR analysis spectra of the coated particles we were able to distinguish the presence of these three peaks that are different from those of the chitosan uncoated particles. As conclusion of these findings, the results clearly show the existence of alginate coating layer around the chitosan particles.

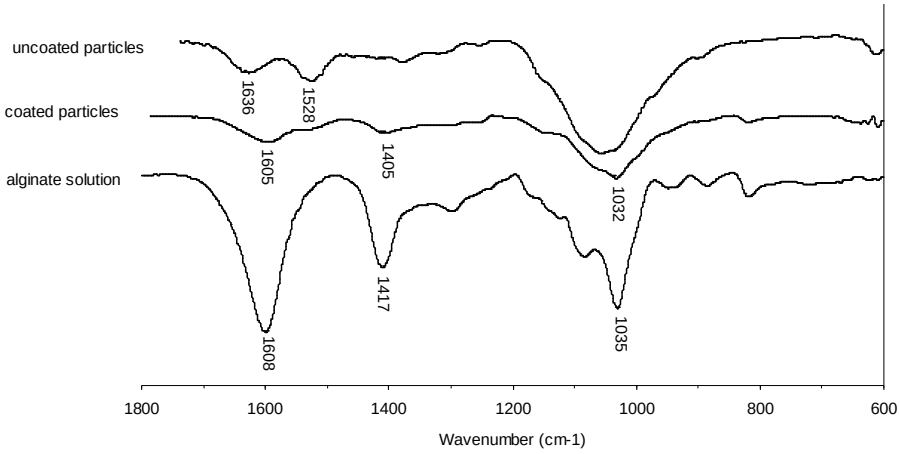

FIGURE 7. FTIR spectra of sodium alginate, uncoated chitosan particles and alginate coated chitosan particles. The FTIR investigation was conducted using the unloaded chitosan particles.

2.3.3 Release studies

In a preliminary study we observed that the temperature is an important determinant for the integrity of the chitosan particles placed in different pH buffers. The particle integrity was monitored by turbidity transmission measurements at 500 nm. In fact at room temperature we were able to obtain stable suspensions of the particles in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF). In contrast when the chitosan particles were added to the same solutions (SGF and SIF) at 37ºC, a loss of integrity was observed translated with high values of transmission measurements. Thus, the complete release of the ovalbumin from the chitosan particles in SIF at 37ºC (Fig. 8) is directly related to the loss of integrity of the particles and less with a simple desorption phenomenon. In contrast, the coating of the particles with alginate increased the stability

of the particles (lower values of transmission measurements) in SIF at 37ºC resulting in a slower release rate of ovalbumin. After 7 hours more than 60% of ovalbumin was still found in the alginate coated particles. These release studies also suggest that the formation of an alginate layer increases the stability of the chitosan particles and reduces the release of the adsorbed protein from these particles.

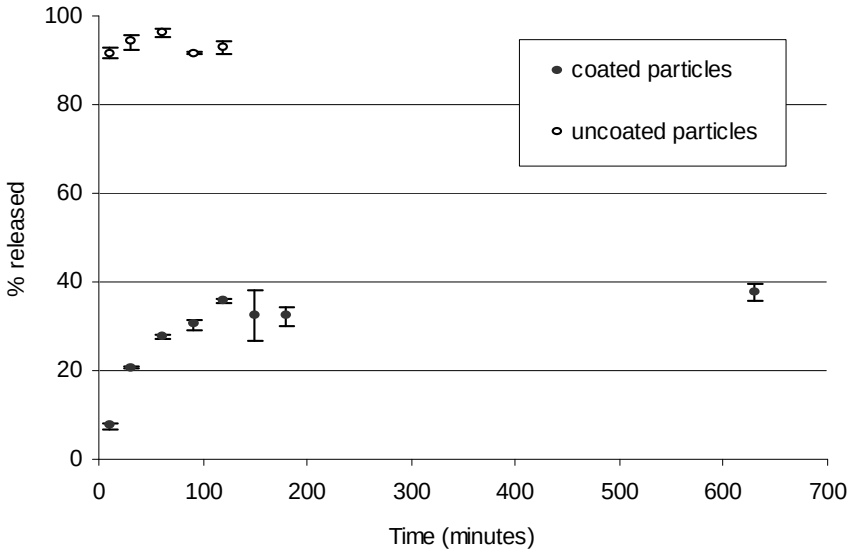

FIGURE 8. Release behaviour of ovalbumin from alginate coated and uncoated chitosan particles in SIF at 37ºC. Individual points represent the mean averages from 3 assays

2.3.4 SDS-PAGE

The main task of this work was the development of an appropriate method for the adsorption of antigens to an optimised delivery platform. With this method the protein antigen was never exposed to potentially harsh conditions, such as contact with organic solvents or mechanical agitation or sonication. The SDS-PAGE (figure 9) of the released ovoalbumin from the particles showed identical bands for the entrapped (lane 4, 5 and 6) and native ovoalbumin (lane 1) and there were no additional bands to indicate the presence of molecular weight aggregates or fragments greater or less than 45K (the molecular weight of ovalbumin). Hence, the data suggest that the structural integrity of ovalbumin was not significantly affected by the entrapment procedure.

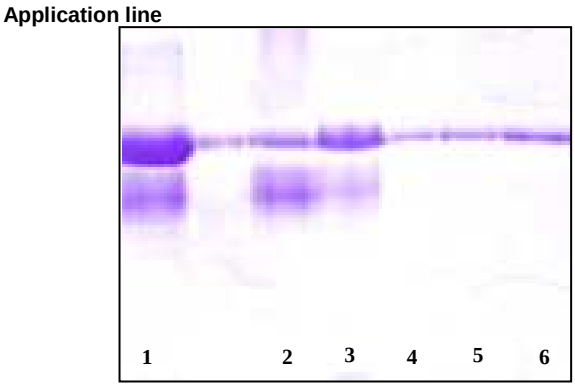

FIGURE 9. Electrophoretic analysis on SDS-12% PAGE with Coomassie brilliant blue staining. Shown are solution of ovalbumin before conjugation to the nanoparticles (lane1), ovoalbumin from the supernatant remaining after the conjugation (lane 2), ovoalbumin from the supernatant remaining after the coating with alginate (lane 3), ovoalbumin released from alginate coated chitosan nanoparticles (lanes 4, 5 and 6).

2.4 CONCLUSION

This work describes a new nano-sized mucosal vaccine delivery system, consisting of chitosan particles that are prepared by crosslinking with sodium sulphate. The chitosan nanoparticles are loaded under very mild conditions with a model antigen (ovalbumin), which was negatively charged in the used buffer systems. The validity of an easy and economic loading process was shown. In order to increase the stability of the loaded chitosan particles at physiological temperature in SGF and SIF a coating process with sodium alginate was developed. This coating process was optimized in such ways that only small amounts of ovalbumin were desorbed during the coating process and the antigen release from the coated nanopaticles was strongly reduced in comparison to the uncoated chitosan nanoparticles. Hence a nano-sized delivery platform is described with improved features of antigen stability in simulated gastrointestinal fluids. In vivo studies are under way to show the efficacy of these systems for mucosal vaccination.

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CHAPTER

3

UPTAKE STUDIES IN RAT PEYER'S PATCHES, CYTOTOXICITY AND RELEASE STUDIES OF ALGINATE COATED CHITOSAN NANOPARTICLES FOR MUCOSAL VACCINATION[2]

[2] *Adapted from Journal of Controlled Release 114 (2006) 348-358*

ABSTRACT

The design of particulate vaccine delivery systems, particularly for mucosal surfaces, has been a focus of interest in recent years. In this context, we have previously described the development and the characterization of a new nanosized delivery system, consisting of a model antigen adsorbed to chitosan particles and coated with sodium alginate. In the present work the ovalbumin release profiles from these coated nanoparticles in different pH buffers were investigated and compared to those of the uncoated particles. Cytotoxicity of the polymers and nanoparticles was assessed using the MTT assay. Finally, particle uptake studies in rat Peyer's patches were performed. It was demonstrated that the coating of the nanoparticles with sodium alginate not only avoided a burst release observed with uncoated particles but also increased the stability of the particles at pH 6.8 and 7.4 at 37ºC. At neutral pH, the release was lower than 5% after 3.5 hours incubation in a low ionic strength buffer. For both, chitosan and alginate polymers, and for the nanoparticles, comparable cell viability data close to 100%, were obtained. Additionally, based on confocal laser scanning microscopy observations, it was shown that alginate coated nanoparticles were able to be taken up by rat Peyer's patches, rendering them suitable carriers for intestinal mucosal vaccination.

3.1 INTRODUCTION

The primary reason for using the mucosal route of vaccination is that it is the most effective route to induce a local protective immune response, resulting in the release of sIgA, against infections originating at a mucosal surfaces [1, 2]. However, only few vaccines currently approved for human use are administered mucosally, most of which are live-attenuated ones. Mucosal vaccines, comprising soluble protein antigens, in general yielded rather disappointing results. Many factors have been described causing this problem [2] and different strategies have been applied to meet this challenge [2, 3]. One such strategy is the development of polymeric nanoparticulate delivery systems. In general, these systems are thought to promote entrapment and retention of antigens in local lymph nodes [4]. On the other hand, they might protect the antigens from the

adverse environment in the presence of hydrolytic enzymes or low pH at the gastrointestinal mucosal surface.

In a recent publication we have described the development and the characterization of a new nanosized delivery system, consisting of chitosan nanoparticles with ovalbumin adsorbed at the surface and coated with sodium alginate [5]. These particles were designed for crossing mucosal barriers and releasing the antigen into lymphoid tissue, in particular in the ileal Peyer's patches. In the present manuscript, successive studies related to this new delivery system are presented.

One of the objectives of the present study was to assess the model vaccine release profiles from coated nanoparticles in order to examine the nature of the interactions between the chitosan core and the alginate coating. Furthermore, these studies were performed to obtain information about the in vivo antigen released from the particles.

The second objective was to evaluate the potential of these coated particles for being taken up by Peyer's patches. Mucosal uptake of microparticulates has been a matter of several reviews [6, 7]. Particularly, a number of studies, not only in rodent animal models [8, 9] but also in porcine gut [10] showed the uptake of chitosan nanoparticles by Peyer's patches [8, 9] or by gut villous cells [10]. Likewise, studies using in vitro cell culture models demonstrated the uptake of chitosan nanoparticles by a number of different cell lines [9, 11-15] and it was emphasised that the polycationic, mucoadhesive chitosan nanoparticles revealed a stronger association with mucus-secreting cells MTX-E12 than with Caco-2 cell monolayers [9]. An intense electrostatic interaction between positively charged chitosan nanoparticles and negatively charged mucins is regarded as the cause for the strong interaction of chitosan with mucus [9, 16]. In one of our own recent studies the coating of the chitosan nanoparticles with sodium alginate has inverted the surface charge of the particles from positive to negative values [5], however, the hydrophilicity of these mucoadhesive [17] nanoparticles remained unaltered. The present coated nanoparticles presumably have the same surface properties as alginate nanoparticles and recent studies [18-20] have shown that alginate particles, despite of their negative surface charge, are also able to be taken up by Peyer's patches. Thus, we examined the internalization of these coated particles by Peyer's patches.

Finally, the third objective was to evaluate the cytotoxicity of the coated and uncoated nanoparticles in comparison to the polymers used to prepare these particles.

The in vitro evaluation of the cytotoxicity of chitosan has been performed in a number of previous studies [11, 21-24] with different cell lines and its low cytotoxicity has already been demonstrated. However, different results were found in the literature, which may possibly be related to the presence of impurities on the different sources of bulk polymer. On the other hand, the differences observed can also be associated with the concentration of the polymer in the cell culture or to different incubation times of the polymer with the cells or finally to the cell line used. For instance for A549 cells, a cell viability near 100% (MTT assay) was demonstrated [11] with concentrations lower than 0.74 mg/ml, and a mean IC_{50} value of 1.1 mg/ml and 1.2 mg/ml for chitosan and chitosan nanoparticles, respectively. With Calu-3 cells a concentration of 1.5% of chitosan in culture medium decreased the cell viability to 30% [23]. Similarly, sodium alginate has been widely used because of its biodegradability, biocompatibility [25] and low toxicity [26]. Examples of its utilization include the immobilization of living cells [27] and the production of antigen delivery systems [28].

The MTT assay is considered an easy and rapid test for the evaluation of cytotoxicity and was used in this work to assess the cytotoxicity of the polymers, chitosan or sodium alginate and the alginate coated or uncoated chitosan nanoparticles in mice spleen cells.

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 Polymers

Chitosan (ChitoClear™) was purchased from Primex BioChemicals AS (Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation was 95% (titration method) and the viscosity was 8 cP (measured in 1% solutions in 1% acetic acid). Sodium alginate (MANUCOL LB®) was kindly donated by ISP Technologies Inc. (Surrey, UK).

3.2.1.2 Reagents

Ovalbumin (grade V; minimum 98%), [3-(4,5-dimethylthiazol-2-yl)] 2,5-diphenyl tetrazolium bromide (MTT), trypan blue and concanavalin A (con A) were purchased from Sigma Chemicals (St. Louis, USA), certified fetal bovine serum (fbs), L-glutamine (200 mM) and gentamicin were from Gibco (Invitrogen Co, Paisley, Scotland, UK), 1M HEPES buffer (0.85% NaCl) and RPMI 1640 without L-glutamine were from Biowhitaker (Cambrex Bio Science, Verviers, Belgium), ovalbumin fluorescein conjugate (3.9 moles dye/mole protein) and BODIPY® 665/676 from Molecular Probes (Leiden, The Netherlands). All other reagents used were of analytical grade. All solutions were prepared in Millipore water.

3.2.2 Preparation of alginate coated chitosan nanoparticles

Alginate coated chitosan nanoparticles were prepared by the method described previously [5]. Chitosan was dissolved at a concentration of 0.25% (w/v) in an acetic acid solution and the formation of the particles was achieved after the addition of 3.5 ml of sodium sulfate solution (10% w/v) to 200 ml of the chitosan solution. The resultant suspension of particles was centrifuged for 30 minutes at 3500 rpm (2800 x g) and the supernatant was discarded. The particles were suspended in Millipore water and centrifuged twice more and then were frozen in liquid nitrogen and freeze-dried overnight using a Christ freeze-dryer (Osterode am Harz, Germany). The dry powder was kept frozen until further use.

The loading with ovalbumin was performed by incubating a solution of ovalbumin with a suspension of chitosan particles at pH 7.4 (phosphate buffer) under mild agitation at room temperature during 120 minutes. The resulting suspension of 0.25% (w/v) ovalbumin and 0.4% (w/v) nanoparticles was used in successive release studies as uncoated loaded nanoparticles and was used in the subsequent coating step. Alginate coated nanoparticles were obtained by mixing of equal volumes of nanoparticles suspension and a buffer phosphate solution of sodium alginate (1% w/v) under magnetic stirring. The agitation was maintained during a 20 minute period. The suspension was then centrifuged for 10 minutes at 1600 rpm and the supernatant was discarded. The particles were re-suspended in 0.524 mM $CaCl_2$ in 50 mM HEPES buffer solution and kept under agitation for another 10 minutes.

3.2.3 In vitro release studies

Ovalbumin release from the coated and uncoated particles was performed by incubation in several buffers: 8 mM phosphate buffer pH 7.4, 100 mM phosphate buffer pH 5.5, 5 mM HEPES buffer pH 7.4, simulated intestinal fluid (SIF) and in simulated gastric fluid (SGF), as described in USP XXIV. The nanoparticle suspensions were added (1:4) to individual tubes containing the release medium previously equilibrated at 37ºC and placed in a shaker bath adjusted to 50 rpm. At appropriate time intervals, samples were taken from each tube and filtered through a low protein-binding filter (MILLEX®GV - 0.22 µm; Durapore PVDF membrane; MILLIPORE, Molsheim, France) followed by centrifugation for 20 minutes at 14,000 rpm. The concentration of ovalbumin in the supernatant was assayed by BCA-protein assay. Simultaneously coated and uncoated blank (without ovalbumin) nanoparticle suspensions were subjected to the same conditions and the filtered samples were used as blanks in the BCA-protein assay. For the determination of the total protein content in the particle suspension, 0.5 ml of the suspensions of the coated and uncoated particles were incubated with 1.5 ml of 0.085N hydrochloric acid solution (pH=1.2) in an ultrasound bath for 30 minutes, followed by 3 hours in a water bath at 37ºC. The samples were filtered and the protein was assayed by BCA-protein assay. Suspensions of unloaded particles were analysed under the same conditions and were used as a blank for the correction of the OD value of the samples analyzed with BCA-protein assay. In order to calculate the amount of the ovalbumin encapsulated, the concentration of unbound ovalbumin in the supernatant of particle suspensions was also determined and subtracted from the total concentration of the ovalbumin in suspension. All experiments were performed at least in triplicate.

The data of ovalbumin release from the coated nanoparticles in the phosphate buffers at different pH's (5.5, 6.8 and 7.4), were analyzed according to zero-order kinetics, first-order kinetics, Higuchi, Korsmeyer-Peppas, Hopfenberg and Weibull as described in [29]. All kinetic models were fit to the release date (graph M_t/M_0 versus time) using the SigmaPlot software (version 8.0, SPSS Inc.)

The drug loading capacity (LC) and loading efficacy (LE) of the nanoparticles were calculated using the following equations:

Eq. 1: LC (w/w) = (total amount of ovalbumin – non-bound ovalbumin)/weight of the nanoparticles

Eq. 2: LE (%) = (total amount of ovalbumin – non-bound ovalbumin)/total amount of ovalbumin *100

3.2.4 Cytotoxicity studies

3.2.4.1 Preparation of single cell suspensions of spleen cells

Three 8-week old female BALB/c mice (Harlan Iberica, Barcelona, Spain) were sacrificed by cervical dislocation and their spleens were aseptically removed. Individual spleen cell suspensions were prepared in a Petri dish using curved needles and washed twice with RPMI 1640. The final suspension was adjusted to a final concentration of $1x10^7$ cells per ml in complete RPMI 1640 medium (supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) glutamine, 1% (v/v) gentamicin and 2% (v/v) 1M HEPES buffer).

3.2.4.2 Solutions of the polymers

The 0.25% chitosan test solution was prepared with complete RPMI and the 0.25% sodium alginate was prepared in 50 mM HEPES buffer (pH=7.4). The solutions were heated and sonicated in order to completely dissolve the polymers. Immediately prior to incubation with the cells, the solutions were aseptically filtered

3.2.4.3 In vitro stimulation of spleen cells with polymer solutions and nanoparticle suspensions and MTT assay

Using sterile 96-well tissue culture plates (TPP, Switzerland), 25 μl of splenocyte suspension from three mice were plated individually, in triplicate along with 20 μl of a concanavalin A solution (50 μg/ml in complete RPMI solution) and 135 μl of a complete RPMI solution of the polymer or suspension of the nanoparticles and incubated (95% relative humidity, 5% CO_2) for 20 h. Assayed were 138.8 μg/ml and 208.3 μg/ml of the polymers (chitosan and sodium alginate), respectively, and 277.6 μg/ml and 416.6 μg/ml of the coated and uncoated nanoparticles, respectively.

The solvents used for the preparation of the polymer solutions (see 2.4.2) were also assayed as a control of the experiment. In the same way, the solutions of the

polymers and the suspension of the particles were assayed without cells in order to evaluate possible interferences of the polymers or the particles in the MTT assay.

Cytotoxicity was evaluated by measuring the reduction of MTT by the mitochondrial dehydrogenase of living cells as an indication of cell viability. 18 μl of MTT solution (5 mg/ml in a phosphate buffered saline pH 7.4) were added to each well following 2.5 hours of additional incubation at 37ºC. To ensure solubilization of the formazan crystals, 100 μl of 0.1N isopropanol-HCl was added to each well and the optical density values were measured at 570 nm using a microplate reader. The relative cell viability (%) related to control wells containing spleen cells in culture medium without polymer was calculated by equation:

Eq. 3: % cell viability = [A] test/ [A] control x 100.

In order to analyse and compare the results for the control group with the polymer and nanoparticle groups, a one-way ANOVA analysis followed by Dunnett's post test was performed using the GraphPad Prism 4 software. Significant differences were considered for $p<0.05$.

3.2.4.4 Trypan blue and propidium iodide stain

The viability of the splenocytes was also evaluated by the capability of these cells with uncompromised membrane integrity to exclude the dye. The in vitro stimulation of the cells with the polymers or the nanoparticles was done as described above in the absence of concanavalin A and for a period of 15 hours. Three samples of the same animal were mixed with the a PBS solution of trypan blue and the resultant suspensions (0.2% trypan blue) were visualized using a standard light microscope. Stained and unstained cells were counted manually using a hemocytometer. Additionally, the pooled culture cells from the same animal and the same formulation test were also stained with propidium iodide (PI)(0.25 μg/ml) and the fluorescence immediately measured with a fluorescence activated cell sorter (FACS Calibur) (BD, Biosciences, Madrid, Spain). Data were analysed by Cell Quest software (BD, Biosciences, Madrid, Spain).

Data were presented as means ± S.E.M. for three experiments and statistical significance was assessed using repeated measured one-way ANOVA followed by Dunnett's post test using the GraphPad Prism 4 software. Significant differences were considered for $p<0.05$.

3.2.5 Uptake studies in Rat Peyer's patches

The studies with Wistar rats (Charles River, Someren, The Netherlands) were performed according to the guidelines of the Ethical Committee of Leiden University. Male rats weighing 250-340 g were housed for acclimatization one week before the experiments with free access to food and water with 12h light/dark cycle. On the day before the experiment they were starved overnight, allowing only free access to water.

The rats were anaesthetized by IM administration of 0.5 ml/kg of Hypnorm® (fentanyl citrate 0,315 mg/ml and fluanisone 10 mg/ml) and 0.5 ml/kg of Dormicum® (midazolam 5 mg/lm). The animals remained anaesthetized throughout the experiment and were placed on electrical heating mats. A small incision in the lower stomach was made and a teflon tube (Ø: 0.5 mm I.D. x 1.0 mm O.D.) was introduced through the pylorus approximately 3 to 5 cm into the duodenum. The coated particle suspensions loaded with FITC-ovalbumin or the 0.05% FITC-ovalbumin PBS solutions were administered (500 µl) and the incision was closed after the removal of the tube from the stomach. The rats were sacrificed after 2 hours by cervical dislocation. The whole intestine was removed and flushed with 20 ml of phosphate buffered saline. Between 4 and 5 Peyer's patches were excised from each intestine and fixed with 2% paraformaldehyde, rinsed again with PBS (4ºC) and the tissue was permeabilized by immersion in 0.1% Triton X-100 (PBS) for 20 minutes. The tissue was rinsed again and stained with a 0.0617% solution of BODIPY® 665/676 (Pierce, Leiden, The Netherlands) in methanol for 60 minutes. Finally, the Peyer's patches were mounted on glass slides and observed using a confocal laser scanning microscope (CLSM) (Bio-Rad, Alphen a/d Rijn, The Netherlands). The confocal images were obtained by scanning the tissue samples in the x,y plane with a z-step of 500 nm.

Fluorescein label (FITC) coated nanoparticles were prepared according to the procedure described above, using a 0.05% FITC-ovalbumin solution in phosphate buffered saline.

Three rats for the group control (solution of the FITC-ovalbumin) and four rats for the group treated with the suspension of the FITC-coated nanoparticles were included in this study.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of the nanoparticles

In a recent publication [5] of our group, the development of alginate coated chitosan nanoparticles was described and the characterization of this new delivery system reported. Before coating with sodium alginate, chitosan nanoparticles had a mean diameter of 643 nm (dynamic light scattering technique) and were positively charged (+37 mV). Scanning electron microscopy (SEM) images of uncoated particles also revealed the presence of small nanoparticles (around 100 nm). After the coating process, however, SEM micrographs allowed us to verify that most of the particles were in a range between 300 and 600 nm. The presence of the alginate coating layer was confirmed by FTIR and DSC studies and by the observation of the inversion of the zeta potential (-34 mV) of coated nanoparticles. The loading of the nanoparticles was done by an adsorption process based on electrostatic interaction [30-32] between the negatively charged ovalbumin at pH 7.4 and the positively charged chitosan nanoparticles. The excellent properties of the chitosan delivery systems to adsorb at its surface proteins [33, 34] and vaccines [30, 35], renders them a promising carrier systems. In the present work, the ovalbumin loading efficacy (see eq. 2) of uncoated particles prepared under the conditions described before was 75%, and the loading capacity (see eq. 1) was 0.49 mg ovalbumin/mg of nanoparticles which are close to those results found in literature [34]. Smaller values of those quantities, of loading efficacy and loading capacity, 57% and 0.39 mg ovalb./mg particles respectively, were observed for the alginate coated particles.

Moreover, physical degradation, including aggregation of the ovalbumin, released from the coated nanoparticles, was not observed as was previously demonstrated by SDS-PAGE [5]. This result was not a surprise because the ovalbumin loading process, as well as the following step, the coating of the nanoparticles with sodium alginate, were performed using a phosphate buffer (pH 7.4), considered as mild entrapment process, having no deleterious effect on the conformation of the protein [36].

3.3.2 In vitro release studies

3.3.2.1 Ovalbumin release profiles from chitosan nanoparticles

In vitro release studies in various aqueous media were performed in order to evaluate the differences of ovalbumin release profiles from uncoated and coated nanoparticles and as an attempt to understand the physicochemical structure of these two delivery systems. The results are illustrated in figure 1 for the uncoated chitosan nanoparticles and in figure 2 for the alginate coated chitosan nanoparticles.

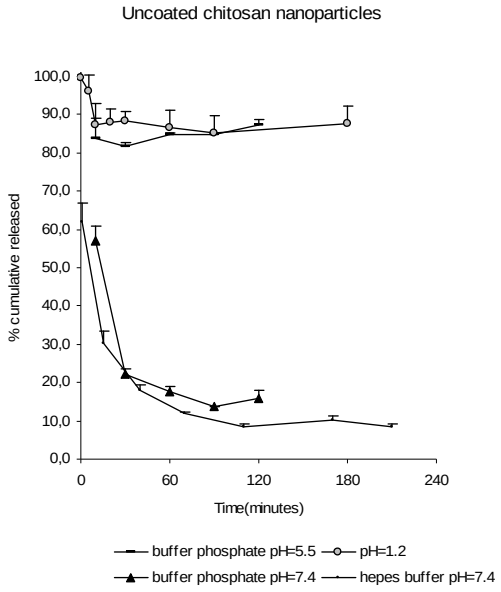

Figure 1 - *In vitro* release profiles of ovalbumin from uncoated chitosan nanoparticles into various aqueous dissolution media at 37ºC. Mean ± standard deviation, n=3

An almost complete ovalbumin desorption from uncoated particles was observed immediately after the addition of the suspension of the particles to the equilibrated release medium at pH 1.2 (simulated gastric fluid) and pH 5.5 (100 mM buffer phosphate). At low pH values, chitosan nanoparticles displayed probably the highest positive surface charge due to the protonation of the free amine groups of chitosan. Additionally, ovalbumin has also predominantly positive charges below its isoelectric point (ovalbumin isoforms: pI between 4.8 and 5.0) [37], resulting from the protonation of

the amine group. The combination of these two factors can explain the immediate desorption of ovalbumin. Such behaviour, the burst release, also allowed us to suggest that ovalbumin was mostly adsorbed to the surface of the particles and not inside the pores.

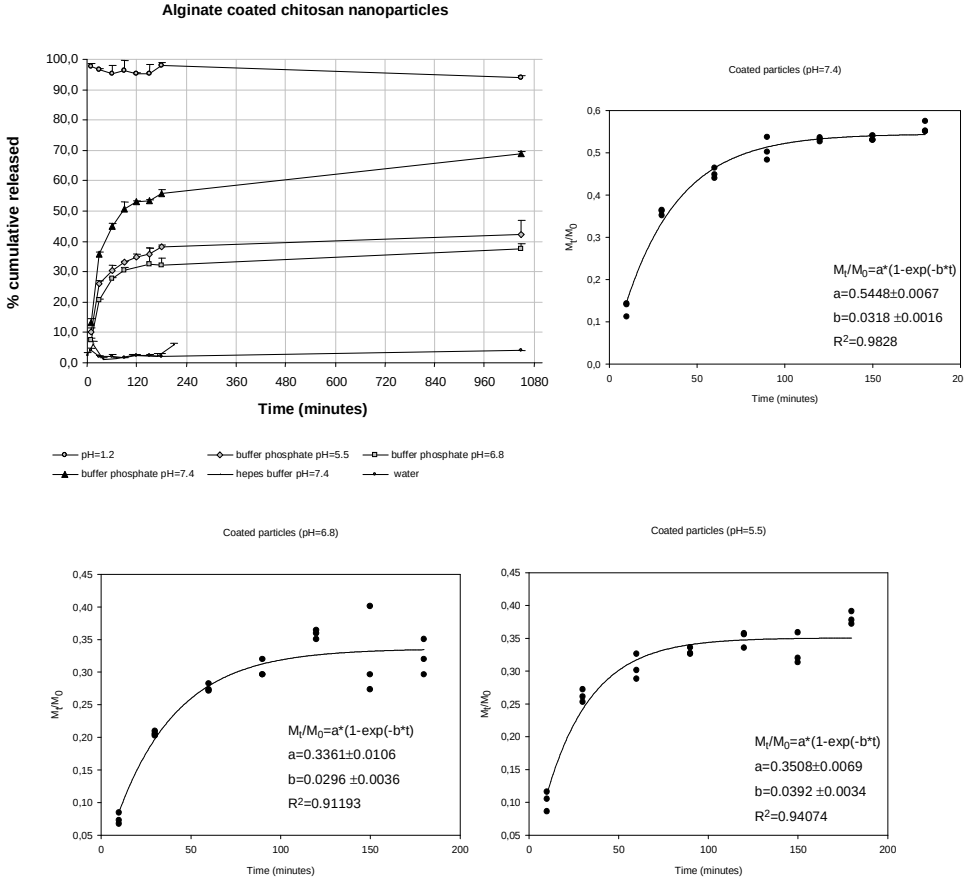

Figure 2 – a) *In vitro* release profiles of ovalbumin from alginate coated chitosan nanoparticles into various aqueous dissolution media at 37ºC. Mean ± standard deviation, n=3; b, c and d) Release-time profile (M_t/M_0 versus time) from alginate coated particles up to 180 minutes with the first order mass balance function. M_t/M_0 is the fraction of drug released under time t.

On the other hand, when the release studies of uncoated particles were conducted at pH 7.4 (phosphate buffer), the initial white colour of the nanoparticle suspension disappeared, immediately after its addition to the release medium, reappearing, in a progressive way after a few seconds. This phenomenon was believed

to be related to the destruction of the initial structure of the nanoparticles as a consequence of its instability at 37º C in phosphate buffer (pH 7.4), followed by the formation of new nanoparticles (precipitates), indicated by turbidity [32] examined by transmission measurements at 500 nm (data non shown). As a consequence, in the first moment after the addition of the particles suspension to the release medium, an immediate desorption of the ovalbumin from the particles occurred, to which followed a progressive re-association of the protein to the recently formed particles and the establishment of a new adsorption equilibrium. This new equilibrium was then stable, under the given conditions of buffer strength and pH. This occurrence was reflected by the atypical release profile shown in figure 1. Similar results were observed when the study was repeated at 37º C with HEPES buffer (pH 7.4) (figure 1). Once the ovalbumin loading of the particles was conducted with the same buffer (phosphate buffer) at room temperature, it was assumed that the observed intermediate instability of the particles was probably related, not only to the pH of the release medium, but also to the temperature. Furthermore, the small size of the particles is also an important factor contributing to the instability of the delivery system and consequently to the initial fast release [38] of ovalbumin. This instability was also described in our own previous studies [5] in simulated intestinal fluid (SIF). However, this behaviour was not observed by others which could mean that despite of the apparently similar formulation and preparation procedures, a diversity of characteristics of the chitosan particles exist [33, 34, 39-43]. The cause of different release behaviours from chitosan nanoparticles may be related with its process of preparation. Recently, the importance of controlling the pH of the formulation during chitosan nanoparticles preparation was demonstrated [14]. Other parameters, such as deacetylation degree and molecular weight of the polymer also has an impact on the properties of the final nanoparticles [33]. Nevertheless, it should be emphasised that a correct procedure during the release studies must be followed, including the rigorous verification of the temperature of the release medium, 37ºC, before the start of the experiment.

3.3.2.2 Ovalbumin release profiles from alginate coated chitosan nanoparticles

In contrast to uncoated chitosan nanoparticles, the release profile of ovalbumin from nanoparticles was significantly modified after coating with sodium alginate (figure 2a). In a pH 7.4 (phosphate buffer) more than 40% of the ovalbumin was released within the

first 60 minutes and during the following 16 hours not more than an additional 25% was released. On the other hand, when using HEPES buffer of the same pH or plain water, not more than 5.8 % of the ovalbumin was released within the first 3 hours and only 4% of ovalbumin was released in water after 17.5 hours from the alginate-coated particles. The observed differences at the same pH, are believed to be related, not only to the ionic strength of the buffers used, but also to the composition of the buffer. The high affinity of phosphate ions to the calcium ions is well known. Therefore, when pH phosphate buffers are used, the destabilizing of calcium-crosslinked alginate matrix has to be taken into account. This effect may cause a faster opening or breakdown of crosslinking compared to the results in HEPES buffer or to plain water where this reaction does not occur. The loss of the calcium ions from the alginate coating, has as a consequence, the increase of the permeability of the coating and consequently an increased diffusion of the ovalbumin into the release medium. Furthermore, the highest ionic strength of the phosphate buffer, compared with HEPES buffer and water should have an important impact on the weakening of the interactions, between the chitosan core and the alginate coating. This dependence on the ionic strength (pH 7.4) is typical of electrostatic interaction, and consequently these observations reinforce our previous considerations about the electrostatic nature of the interactions between the alginate coating and the chitosan core [5]

Finally, when the ovalbumin release study was conducted using a pH 5.5 phosphate buffer, the differences observed when compared to the same phosphate buffer of pH 7.4, should be attributed to the pH of the buffer. The pH (5.5) seemed to be favourable to maintain strong interactions between alginate and chitosan and this was probably the cause for the maintenance of the entrapment of ovalbumin at an elevated percentage. A comparable result was observed (figure 2a) at pH 6.8 in SIF.

As evident from what was referred before, the release of ovalbumin from the coated particles is actually a result of a combination of different processes which made the application of a kinetic model a difficult task [38]. In particular, the electrostatic interactions between the chitosan core and the ovalbumin should have a determinant role in the release mechanism and should be impeditive to observe a good fit with a diffusion-controlled release mechanism. In fact among several models referred, including the ones of Higuchi, Korsmeyer-Peppas, Hopfenberg and Weibull [29], the M_t/M_0 versus time graph, from the release studies performed in the phosphate buffers at different pH's

(5.5, 6.8 and 7.4), showed to have the best fit for the first order model (figure 2b,c,d), described by following equation:

Eq. 4 $M_t/M_0 = a*[1- \exp(-bt)]$

Where M_t/M_0 is the fraction of drug released at time t. The two parameters of eq. 4, the pre-exponential term a and the first-order constant b were estimated by this fitting process and their values are shown in figure 2b, c and d. The first-order model release mechanism has been associated with particular characteristics of the delivery system, they reflected to some extent a reservoir-type delivery system [44]. Moreover, it was suggested that the first order kinetics could describe the release profile, from the pharmaceutical dosage forms such as those containing water-soluble drugs (that is the case of ovalbumin in the present study), where drugs would be released at the rates proportional to the amounts of drug remaining inside the dosage form [45].

Similar to our results, it was demonstrated in a recent study [46], that the 5-Flu release data, from both lipossomes and PLGA or PLA microspheres, were modelled as a first-order process, with excellent reproducibility of the release kinetics.

The alginate coating of the nanoparticles not only improved the stability of the chitosan particles at 37ºC in 6.8 and 7.4 pH buffers, but also allowed a significant reduction of the ovalbumin burst release observed for the uncoated particles with the different buffers, with an exception at pH 1.2 (SGF). The release characteristics of the present nanoparticles may be altered by the increase in complexity (presence of surfactants, enzymes, salts) of the selected release buffer. Thus in order to obtain a better knowledge of the situation in vivo, more complete in vitro models would be required.

3.3.3 In vitro cell viability studies

Splenocytes have been used for the evaluation of the toxicological profiles of several compounds and delivery systems. For example, in a recent study, the evaluation of the cytotoxicity of tetanus toxoid loaded poly(lactide-co-glycolide) nanospheres was done with splenocytes [47]. The principal reason for the choice of spleen cells in this study, was related to the fact that they are obtained and cultured easier, compared to other lymphoid organs, like Peyer's patches, and represent a very good and sensitive representative of the different cells of mucosal immune system. The spleen is a lymphoid

organ, bridging the innate and adaptive immune system in a uniquely organized way (reviewed in ref. [48]). A primary culture of mixed spleen cells normally contains (FACS analysis performed in our own lab) more than 50% of B lymphocytes, about 30% of T lymphocytes (18% CD4+; 12% CD8+) and less than 20% erythrocytes, fibroblast, macrophages, dendritic cells, and granulocytes. Similar to the spleen, Peyer's patches also contain a large number of B cells, T cells, macrophages and dendritic cells [15]. Thus, a primary culture of spleen cells should have the requisites to mimic possible toxic effects of the nanoparticles, when the nanoparticles, internalized by Peyer's patches, remain in a close contact with those immune cells during a certain period of time.

In this study, the viability of primary culture of splenocytes in the presence of the polymers sodium alginate and chitosan, at two different concentrations, was evaluated by different methods, and in order to estimate whether the preparation process of the particles would introduce any cytotoxicity, uncoated and alginate coated chitosan particles were also studied.

The MTT test was used to evaluate the effects of the polymers and the particles on the metabolic activity of mice spleen cells. No evidence of cytotoxicity was observed for the polymer solutions (figure 3) or the particle suspensions (figure 4) and a cell viability of around 100% was observed in all test groups. Moreover, the statistical analysis of the results underlined the higher mean values of cell viability (% of control) in the groups treated with higher concentrations of polymers, chitosan or alginate and with particles coated or uncoated (in both concentrations) when compared with the control group. Considering that proliferating cells are metabolically more active than non-proliferating (resting) cells, these results can also be interpreted as the possible impact of polymers and particles on cell proliferation. This is probably an indication that the polymers may favourably influence lysosomal and mitochondrial activity of the cells [22], nevertheless, this observation needs to be confirmed by specific experiments.

On the other hand, the cytotoxicity results as measured by the reduction of the MTT substrate could, in theory, be "masked" by cellular proliferation. Therefore, it was decided to conduct cytoxicity studies with a different methodology using trypan blue or propidium Iodide (PI) dyes for staining the cells whose membrane integrity may have been compromised. The results of this study are summarized in figures 5, 6 and 7.

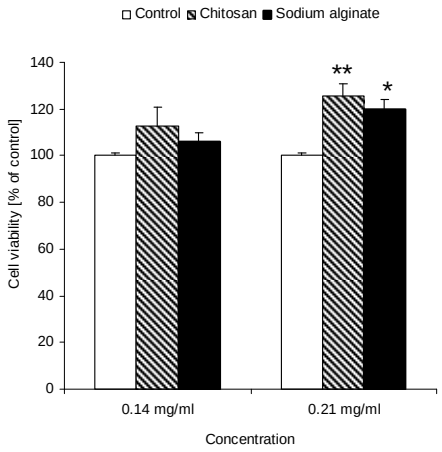

Figure 3 - Viability of splenocytes measured by the reduction of MTT. The cells were incubated for 20 hours in the presence of two different concentrations of the polymers, chitosan and sodium alginate. The control group corresponds to the assay just with cells in culture medium. Results represent the mean ± standard error of the mean for 3 different mice, each performed in triplicate (n=9). Statistical differences between control group and formulations are reported as: ∗ $p<0.05$, ∗∗ $p<0.01$. Cell viability (% of the control) = [A] test/ [A] control x 100

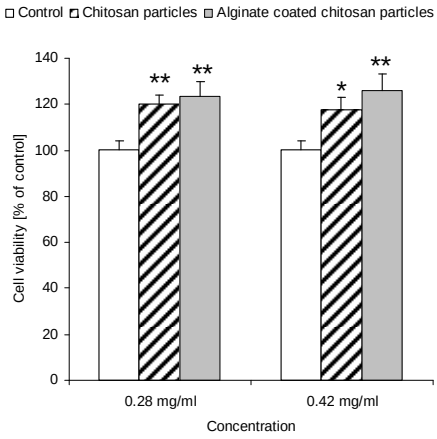

Figure 4 – Viability of splenocytes measured by the reduction of MTT. The cells were incubated for 20 hours in the presence of two different concentrations of the nanoparticles, alginate coated chitosan particles and uncoated chitosan particles. The control group corresponds to the assay just with cells in culture medium. Results represent the mean ± standard error of the mean for 3 different mice, each performed in triplicate (n=9). Statistical differences between control group and formulations are reported as: ∗ $p<0.05$, ∗∗ $p<0.01$. ll viability (% of the control) = [A] test/ [A] control x 100

The count of non-viable splenocytes stained with trypan blue (figure 5) in comparison with the non-stained cells revealed that the presence of the polymers during 15 hours did not decrease the percentage of viable cells and are in close agreement with the results obtained from the MTT assay. FACS sorting and analysis (figure 6) of lymphocytes previously treated with PI strengthened these observations and the proportion of dead (M1) cells in all the samples was always lower than 3%. The statistical analysis of these results showed that the values obtained in each treatment group were not different from the control group. However, a more pronounced difference between the location of FL3 histograms from control groups (figure 7, left and right picture) and groups treated with a solution of the polymers chitosan or sodium alginate (figure 7, right picture) was observed. These results possibly indicate that in the presence of the polymers, a slight perturbation of the plasma membrane occurred and these lymphocytes are more permeable to the fluorescent nuclear probe, PI. Nevertheless, this event did not induce cell death, as was demonstrated by trypan blue dye exclusion and MTT assays. The present results correlate with those found in the literature [21] where it was demonstrated that chitosan increased the Caco-2 cell permeability in a reversible and dose-dependent way and was not accompanied by cell extrusion.

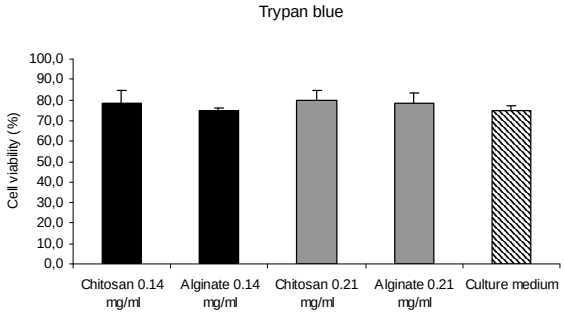

Figure 5 – Viability of splenocytes measured by counting trypan blue stained and unstained cells with a hemocytometer. The cells were incubated for 15 hours in the presence of two different concentrations of the polymers, chitosan and sodium alginate. The control group corresponds to the assay just with cells in culture medium. Results represent the mean ± standard error of the mean for 3 mice (n=3) and each count was the resulted of pooled samples from the same mice. No statistical significant differences were found between the control and the polymer groups.

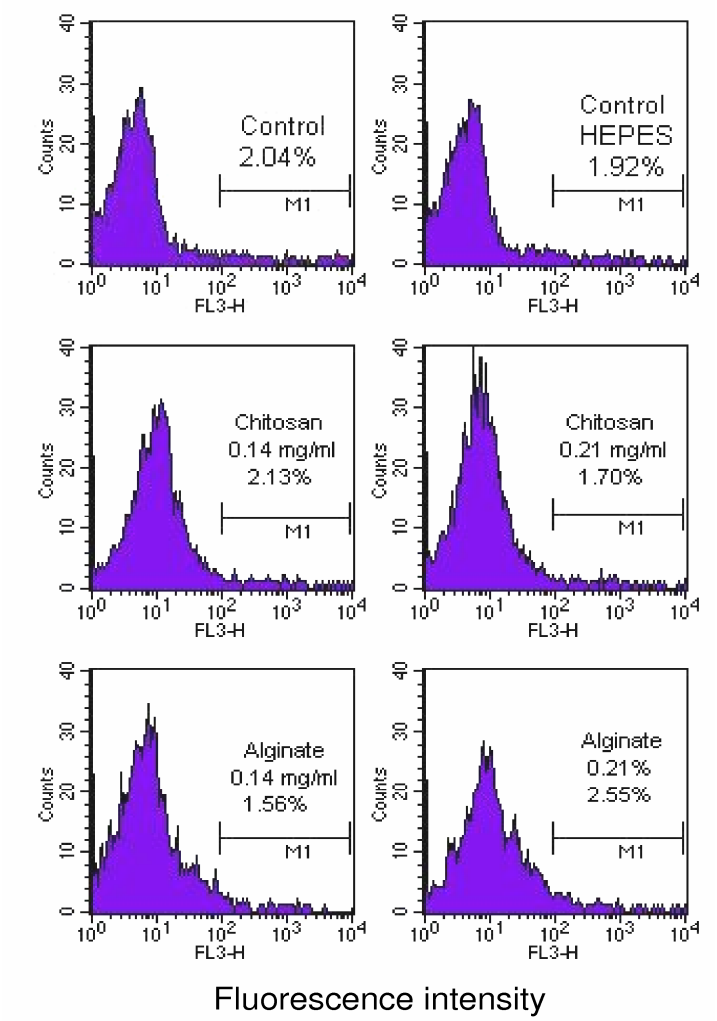

Figure 6 - Flow-cytometric analysis of mice splenocytes previously incubated in the presence of two different concentrations of the polymers chitosan and sodium alginate during 15 hours. The FL3 histograms show the proportion of PI stained non-viable (M1) and viable cells from pooled samples of one mouse. Each histogram is representative of three animals studied individually. No statistical significant differences were found when the control group was compared with the others treatment groups.

The combination of these three different methods adds reliability to the final evaluation of the cytotoxicity profiles of these polymers and nanoparticulate formulations.

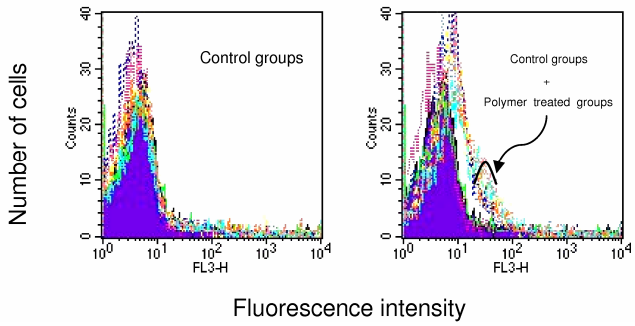

Figure 7 - Flow-cytometric FL3 histogram overlay of control groups on left picture and control plus polymer treatment groups (sodium alginate and chitosan) on right picture.

3.3.4 Uptake studies of alginate coated chitosan nanoparticles into rat Peyer's patches after intra-duodenal administration.

The uptake of alginate coated chitosan nanoparticles into the rat Peyer's patches through M-cells was investigated. Chitosan particles were loaded with ovalbumin labelled with fluorescein as a model vaccine and then coated with sodium alginate. To better visualise the contour of the cells, the Peyer's patches were stained with Bodipy®, which is a nonpolar lipophilic dye used to stain the cell membrane. The same methodology was recently used successfully to visualise the uptake of N-trimethyl chitosan nanoparticles by nasal epithelia and NALT cells [49] or to visualize chitosan nanoparticles within Peyer's patches [8]. In the present work, CLSM images of Peyer's patches from the rats treated with the suspension of fluorescent coated nanoparticles, showed the presence of fluorescent nanoparticles (green spots) inside the Peyer's patch (upper pictures in figure 8). The nanoparticles were visualized, not only in the region underneath (0.5 μm) the follicle-associated epithelium (FAE) (right upper picture in figure 8), the subepithelial dome region (SED), but also in deeper regions of the secondary lymphoid organ (left upper picture in figure 8). Z-scan images (21 images), in successive steps (0.5 μm distance between steps) and starting 6 μm deep from the surface of the Peyer patches, also indicated that the nanoparticles were also transported to deeper regions (data not shown). On the contrary, the photos from the control group (lower pictures in figure 8), treated with a solution of ovalbumin-FITC did not show any green fluorescence except a

slight shade green (lower left picture) that could mean that a small amount of ovalbumin-FITC could be absorbed by the tissue.

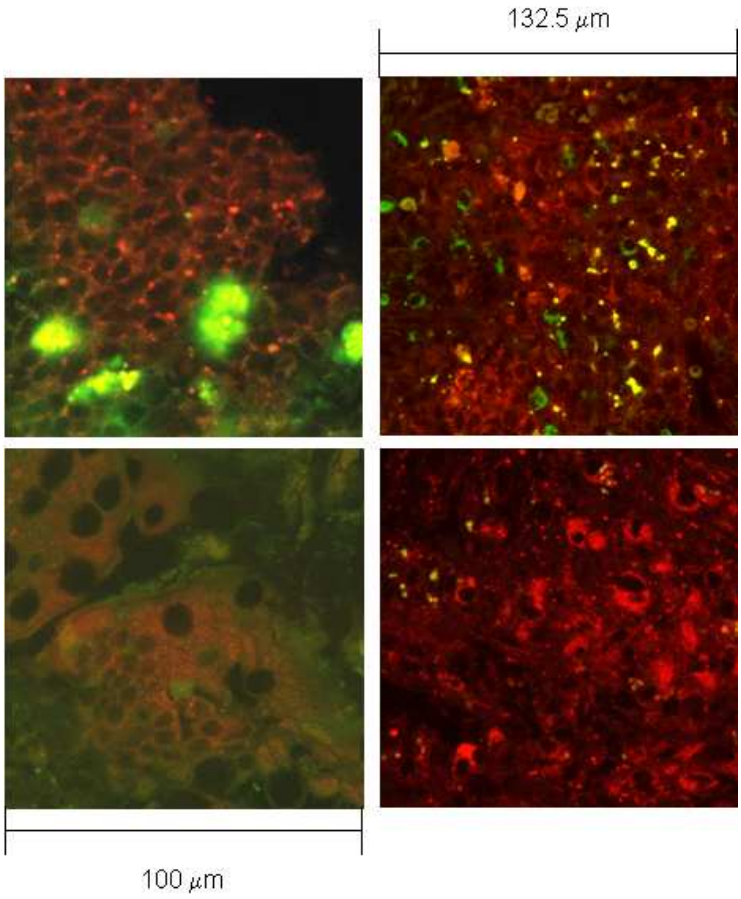

Figure 8 - CLSM visualization of rat Peyer's patches. Upper images belong to two different animals fed with FITC-ovalbumin loaded alginate coated chitosan nanoparticles. Left image was taken from the transversal cross section of the intestinal epithelial barrier and the right image was taken from the villi side of the intestinal epithelial barrier. In all the four rats fed with the nanoparticles similar CLSM images could be made. ower images belong to two different animals fed with FITC-ovalbumin in PBS solution. Right and left images were taken from the villi side of the intestinal epithelial barrier.

Some of the green dots visualized in the Peyer's patches seemed not to be individual particles, but agglomerates of the nanoparticles taken up into lysosomal compartments. Focusing of sections at high power suggested that microparticle clusters

were localised intracellularly (left upper picture in figure 8), an observation consistent with a recently published study [50] in which CLSM images were presented, showing clusters of polystyrene nanoparticles present in the subepithelial dome region underlying the Peyer's patch. Moreover, in the same study, dendritic cells labelled by immunostaining were shown to efficiently phagocytose microparticles and when the microparticles were associated to enterotoxin adjuvants, they were transported from the SED region into underlying B-cell follicles and adjacent parafollicular T-cell zones.

The SED region underlying the FAE are rich in phagocytic cells (dendritic cells and macrophages) [50]. Recent published studies, showed that alginate nanoparticles are able to be taken up not only in vivo by Peyer's patches [18-20], but also in vitro by phagocytic cells [19, 51]. Following this idea, alginate coated chitosan nanoparticles were most likely internalized by phagocytic cells, most probably dendritic cells, present in Peyer patches. In future work it will be important to determine the phenotype and maturation state of the microparticle-loaded cells as it can be important to follow and to know the mechanism of phagocytic cells migration.

As an important finding, this study, provided an answer to our initial question related to the capability of this new delivery system, formed by hydrophilic negatively charged particles, to be internalized by Peyer´s patches. In spite of the argument that positive or a more hydrophobic surface increases cellular uptake due to its better interaction with lipophilic cell membranes, this study showed that also negatively charged hydrophilic particles are being internalised. Moreover in a recent study [51], the increase of the hydrophobicity of alginate particle surface did not improve its uptake by mouse macrophages and consequently, cellular uptake seems to be the result of a complex combination of size, hydrophobicity, as well as specific interactions between surface functional groups of alginate microspheres and the cell membrane.

3.4 CONCLUSIONS

The adsorption of therapeutic proteins and model antigens onto chitosan nanoparticles has proven to be a very mild process resulting in a very high loading efficacy. In this study, it was shown that coating of this delivery system with sodium alginate yielded coated particles, in the nanosize range, with a much better stability and controlled

release properties for vaccine delivery, than the chitosan loaded cores themselves. It was also demonstrated that the preparation process of the nanoparticles did not introduce any toxic compound on the particles. On the contrary, a slight stimulation of the splenocytes co-cultured with the nanoparticles was observed. Finally it was shown that these hydrophilic coated nanoparticles even with a negative surface charge were taken up by rat Peyer's patches, which made them promising carriers for mucosal vaccination. In vivo studies with a real vaccine are underway to investigate the efficacy of this new mucosal delivery system.

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CHAPTER

4

INDUCTION OF LYMPHOCYTES ACTIVATED MARKER CD69 FOLLOWING EXPOSURE TO CHITOSAN AND ALGINATE BIOPOLYMERS [3]

[3] *Adapted from International Journal of Pharmaceutics (in press)*

ABSTRACT

CD69 is a very early cell activation antigen expressed on the surface of activated immune cells. It can appear within 1 to 2 hours of activation and exhibits maximal expression levels between 18 and 24 hours after stimulation. In this work, the expression profile of CD69 in mice splenocytes was evaluated following exposure to the biopolymers, chitosan or alginate and the immunostimulatory factors, CpG ODN 1826 or concanavalin A. We have shown that both polymers are able to upregulate expression of CD69 on B cells and CD4+ T-lymphocytes, with alginate as the least potent stimulus. Moreover the expression of the CD69 molecule on CD8+ T-lymphocytes was observed only in splenocytes cultured with chitosan. However, activation of lymphocytes did not result in cell proliferation. On the other hand, CpG ODN proved to be a potent B cell stimulator, as evidenced by the upregulation of CD69, but had less effect on T-cells. These results, together with previous discoveries reported in scientific literature, may contribute to the clarification of the adjuvant effect, which has been attributed to chitosan and alginate formulations or to the biopolymers itself.

4.1 INTRODUCTION

The combination of biomaterials with proteins, DNA, vaccines, and cells has been a matter of study in several groups. Two well-known examples are the development of polymeric carriers of vaccines and tissue engineered constructs in which cells are delivered with a polymer component for the renovation or replacement of damaged tissue or organ function. In these two examples, different potential immune responses, due to the possible adjuvant effect of the biomaterial would be required. Clearly, from a tissue engineering point of view, immune responses are to be minimized or altogether avoided while an adjuvant effect of the polymeric vaccine delivery system would be desirable and even necessary, particularly for weak antigens [1]. Among the natural biomaterials, chitosan and alginate have been used for both of the previously stated purposes. In particularly the use of alginate [2-4] and chitosan [5-8] particles as an antigen delivery system were used with success in a number of vaccination studies. The

mechanism, by which particulates showing adjuvant effect is not completely understood. In general, are thought that particulate delivery systems promote trapping and retention of antigens in local lymph nodes and protecting them from degradation, which seems to be important in triggering protective T cell responses [9]. However in a recent study [7], a soluble derivative of the chitosan was administered in solution with influenza vaccine. An adjuvant effect of the chitosan was observed; therefore a different mechanism in this case may have to be considered and investigated.

We also have been particularly interested in the use of chitosan and alginate for the design of vaccine carriers. In a recent publication [10], we described the development of a new vaccine delivery system of alginate coated chitosan nanoparticles, specially designed to deliver vaccines into mucosal surfaces. This delivery system is now being used in mucosal vaccination studies with the recombinant hepatitis B surface antigen associated with the adjuvant, the CpG ODN 1826. By this reason, the study of the effect of the polymers, used in our group for the preparation of the nanoparticles, on the cells from the immune system are of extreme importance.

Both polymers are naturally occurring polysaccharides. Chitosan, a copolymer of D-glucosamine and N-acetyl-D-glucosamine is a derivative of chitin, one of the most abundant polysaccharides in nature. Alginate is composed of alternating blocks of 1-4 linked □-L-guluronic (G) and β-D mannuronic (M) acid residues. The sources of these polymers as well as their physical and chemical properties have been extensively reviewed in several publications [11-13].

The immunostimulatory properties, found in certain alginates, in same cases have been associated with the presence of impurities, like endotoxins or mitogenic contaminants in alginate raw materials [14-16]. In a recent work, the production of the proinflammatory cytokine TNF-α was approximately 100 times higher in the case of non-biomedical grade alginate in comparison to the purified one [14]. According to the same authors, this feature has implications on the utilization of impure alginates, for instance as carriers for cell immobilization. In fact, TNF-α is considered to be a potent cytokine that is known to activate leukocytes, stimulate fibroblast proliferation, promote migration of inflammatory cells into the intercellular matrix, and trigger local secretion of other proinflammatory cytokines. In addition to the considerations above, a vaccine nanocarrier should also be made with purified alginates given that stimulation of TNF-α would lead to an overgrowth of collagen-secreting fibroblasts and activated macrophages secreting growth regulatory cytokines over the nanocarrier and may have

as a consequence, an alteration of the vaccine release from the nanocarrier [14]. On other hand a highly purified alginate, with low polyphenol and protein content, prepared from Laminaria pallida, was evaluated in vitro using mice lymphocytes and no evidence of significant mitogenic activity was observed [16]. However there are also some studies that showed that the mannuronic acid rich alginates, have itself immunostimulating properties, stimulating monocytes to cytokine production [17] in where the TLR2 and TLR4 seemed to be involved [18]. In a more recent study [1], the effect of biomaterials on dendritic cell maturation was evaluated and it was demonstrated that the expression levels of CD86, CD40 and HLA-DQ (MHC class II) molecules, indicative of dendritic cell maturation, were decreased in the presence of alginate. On the contrary, the presence of chitosan in the same in vitro study caused a high expression level of the same co-stimulatory molecules.

The expression of CD69 was never investigated in lymphocytes cultured with chitosan; however the activation of lymphocytes following the administration of chitosan has been reported in some recent publications. For instance it was shown that rats fed orally with a purified low molecular weight chitosan triggered the release of IL-10 as well as the expression of IL-4 and TGF-β mRNA at the gut mucosa local microenvironment and stimulated CD3+ T-lymphocytes in the spleen [19]. The presence of chitosan also caused the enhancement of the natural killer (NK) cell activity in intestinal intraepithelial lymphocytes and splenic lymphocytes [20]. Furthermore, it has been demonstrated that chitosan [21, 22], and chitosan microparticles [23, 24] are able to up-regulate, to some extent, a number of macrophage functions. For instance, a low molecular weight water soluble chitosan induced the activation of macrophages through the production of cytokines such as IFN-γ, IL-12, and IL-18 from the intestinal intraepithelial lymphocytes [20]. Another example found in Scientific literature based on in vitro studies, shown that chitosan nanoparticles (1 a 10 μm) that can be phagocytised, stimulated alveolar macrophages, but at a significantly lower level than that elicited by oxidative responses observed with phagocytized chitin [24]. In a more recent study [21], using peritoneal macrophages, different results among the biomaterials chitosan, low molecular weight chitosan (LMW), and chitin were obtained. Therefore chitosan treatment induced activation markers, such as the major histocompatibility complex (MHC) class I, class II, Fc receptors, transferrin receptor, mannose receptor, Fas, and macrophage inflammatory protein (MIP)-2, whereas chitin and LMW chitosan induced only the expression of MHC class I and II molecules. Thus, small variations in molecular weight

or degree of deacetylation on chitosan molecule might lead to different degrees of activation in cells from the immune system [25, 26].

The adjuvant effects of CpG ODN have been studied intensively in the last few years. Briefly, CpG ODN directly stimulates immune cell types that express Toll-like receptor 9 (TLR9), with human B cells and plasmocytoid dendritic cells (pDCs) being the dominant cell types expressing TLR9 [27]. Activation of these cells promotes the production of T helper 1 (Th1) and pro-inflammatory cytokines and the maturation/activation of professional antigen-presenting cells (APC) [27]. These actions enable CpG ODNs to act as immune adjuvants which can accelerate and boost antigen-specific immune responses. The maintenance of close physical contact between the CpG and the antigen has been reported to be the ideal situation for achieving optimal results [28]. This can be realized by encapsulating the CpG and the antigen together in the same delivery system. Moreover, if the polymers used to construct the delivery system, for instance chitosan and alginate, have themselves immune stimulating properties, then the formulation, may have optimized adjuvant properties.

Therefore, the main goal of this work was to evaluate the capacity of each component of our coated nanoparticles, and compare them with the adjuvant, CpGODN 1826, in order to determine which method resulted in the optimal activation of B and T-lymphocytes. With this purpose, the CD69 receptor, which is considered a very early cell activation antigen, expressed on the surface of activated immune cells, was examined for the first time on splenocytes cultured with the raw materials chitosan or alginate, which are used in the preparation of the coated nanoparticles. This cell surface antigen can appear within 1 to 2 hours of activation and exhibits maximal expression levels between 18 and 24 hours after stimulation, making it a useful tool, albeit one that has not been fully investigated, for early evaluation of the effect of biomaterials in the stimulation of immune cells.

4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 Animals

In this study, 6 to 7 week-old male BALB/c mice were used (Harlan Iberica, Barcelona, Spain). Animals were housed and cared for at the animal resource facilities of the Faculty of Pharmacy of the University of Porto, in accordance with institutional guidelines.

4.2.1.2 Polymers

Fine grade pure chitosan was purchased from Primex BioChemicals AS (Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation is 95% (titration method) and the viscosity is 8 cP (measured in 1% solutions in 1% acetic acid) that corresponds to a low molecular weight chitosan. (According to the provider's specifications a chitosan with a molecular of 40 kDa and another with 60 kDa have a viscosity of 25 cP and 35 cP respectively).

A low molecular weight sodium alginate (MANUCOL LB®) was kindly donated by ISP Technologies Inc. (Surrey, UK). According to the provider's specifications, the typical values for the percentage of mannuronic and guluronic acid for Manucol LB is 61% and 39%, respectively, and an estimated molecular weight of 18 kDa.

Both polymers meet the requisites described in the European Pharmacopoeia for use in pharmaceutical formulations. In this work a 22 µm filtered solutions of the polymers were used.

4.2.1.3 Reagents

Class B, CpG ODN 1826 (5'-TCC ATG ACG TTC CTG ACG TT-3') was purchased from Coley Pharmaceutical Group (Ottawa, Canada), concanavalin A (Con A) was purchased from Sigma Chemicals (St. Louis, USA), certified fetal bovine serum (FBS) and L-glutamine (200 mM) were purchased from Gibco (Invitrogen Co, Paisley, Scotland, UK),

1M HEPES buffer (0.85% NaCl), RPMI 1640 medium without L-glutamine and Pen-Strep (10 000 U penicillin/ml; 10 000 µg streptomycin/ml) were purchased from Biowhitaker (Cambrex Bio Science, Verviers, Belgium). [methyl-^{3}H] thymidine (1.0 mCi/ml) was obtained from Amersham Biosciences (UK), R-Phycoerythrin (PE)-conjugated hamster anti-mouse CD69, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8 were obtained from BD Biosciences (Madrid, Spain). The FITC-conjugated goat anti-mouse IgM (anti-µ) was purchased from PharMingen (San Diego, CA, USA). All others reagents used were of analytical grade. All solutions were prepared in Millipore water.

4.2.2 Methods

4.2.2.1 Preparation of spleen cell suspensions.

Three mice were euthanized by cervical dislocation and their spleens were aseptically removed. Individual spleen cell suspensions were prepared in a Petri dish using curved needles and washed twice with RPMI 1640. The final suspension was adjusted to a final concentration of 1x10^{7} cells per ml in complete RPMI 1640 medium (supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) glutamine, 1% (v/v) Pen-Strep and 2% (v/v) 1M HEPES buffer).

4.2.2.2 Polymer solutions

The 0.25% (w/v), 0.125% (v/v), and 0.01% (v/v) chitosan solutions were prepared in complete RPMI medium acidified with acetic acid. Sodium alginate was first dissolved in HEPES buffer (0.5% w/v) and then diluted in complete RPMI (0.25% (v/v), 0.125% (v/v), and 0.01% (v/v)). Immediately following, the polymer solutions were aseptically filtered and incubated with the cells. The solvents used for the preparation of the polymer solutions (RPMI acidified with acetic acid and HEPES) were also used as controls for the experiment.

4.2.2.3 Cell culture in the presence of the polymers and mitogens

Using sterile 96-well flat-bottomed tissue culture plates, 25 µl of splenocyte suspension ($1x10^7$ cells/ ml) from three individual mice were plated in triplicate along with 25 µl of complete RPMI solution with the mitogen [Con A (50 µg/ml) or CpG ODN (50 µg/ml) or without mitogen (control)] and 25 µl of a complete RPMI solution of the polymer [chitosan; or sodium alginate; or without polymer (control)]. Finally the volume of the well was diluted to 200 µl with complete RPMI and incubated at 37ºC with 95% relative humidity and in the presence of 5% CO_2.

4.2.2.4 Immunofluorescence labelling

After 20 h of incubation, cells were washed and resuspended in cold phosphate buffered saline (PBS) supplemented with 2% FBS, resulting in a concentration of 0.5 x 10^6 cells/ well. The cells were then incubated in the dark for 30 minutes at 4ºC with saturating concentrations of PE-conjugated hamster anti-mouse CD69 and FITC-conjugated rat anti-mouse CD4 or FITC-conjugated rat anti-mouse CD8 or FITC-conjugated goat anti-mouse IGM (anti-µ). After incubation, cells were washed three times with PBS-FBS and then resuspended with 500 µl of PBS-FBS. To exclude dead cells, 2.5 µl propidium iodide (50 µl/ml) was added just before data acquisition. At least 10,000 events were analysed by flow cytometric acquisition, performed in a fluorescent activated cell sorter (FACS Calibur) (BD Biosciences, Madrid, Spain). Data were analysed by CellQuest software (BD Biosciences, Madrid, Spain). Viable lymphocytes were selected on the basis of FSC/SSC values.

The concentration of the polymers studied was kept at 0.156 mg/ml. Data were presented as the mean ± S.E.M. for three experiments and statistical significance was assessed using repeated measured one-way ANOVA followed by Dunnett's post test using the GraphPad Prism 4 software. Differences were considered significant when $p<0.05$.

4.2.2.5 Lymphocytes proliferation assay

Splenocytes were obtained and cultured in a flat-bottomed 96-well plate with and without the mitogens and polymers as described above. The cells were cultured for 96 hours at

37ºC and on the last 8 h of incubation, each well was pulsed with 1µCi of [methyl-^{3}H] thymidine. 96 well plates with the cells were frozen for further analysis. The cells were later thawed and harvested onto a Fiberglass filter (filter mats, molecular devices, Skatron, Lier, Norway) using a semiautomatic cell harvester (Scatron Instruments, USA) and thymidine incorporation was counted by standard liquid scintillation techniques with a Beckman LS 6500 scintillation counter (Beckman Coulter Inc, Fullerton, USA). Thymidine incorporation was expressed as counts per minute (cpm).

The concentration in the well of the polymers studied were: conc1 = 0.312 mg/ml and conc2 = 0.0125 mg/ml. Data were presented as a mean ± S.E.M. for the three experiments and statistical significance was assessed using a nonparametric ANOVA with Kruskal-Wallis analysis using the GraphPad Prism 4 software. Differences were considered significant when $p<0.05$.

4.3 RESULTS

4.3.1 Effect of alginate or chitosan polymers or the immunostimulant CpG ODN on the size and granularity of spleen mouse lymphocytes

The spleen is a lymphoid organ combining the innate and adaptive immune system in a unique and organized way [29]. The principal reason for the choice of spleen cells in this study was related to the fact that they are obtained and cultured easily and that they are a very sensitive representative of different lymphocytes. According to FACS analysis performed in our own laboratory, a primary culture of mixed spleen cells normally contain more than 50% of B lymphocytes, more than 30% of T lymphocytes (20% CD4+; 12% CD8+) and of the remaining cells, less than 10% were erythrocytes, fibroblasts, macrophages, dendritic cells, and granulocytes.

Lymphocytic morphological alterations, as a consequence of the engorgement of the rough endoplasmic reticulum, which normally accompany cell activation, can be observed by flow cytometry analysis, combining forward light scatter (FSC) and side light scatter (SSC). In general, activated cells exhibited a dramatic increase in both forward and side scatter.

As a positive control, cells from the mouse spleen were cultured with concanavalin A (con A), a classical lymphocyte mitogen. As was expected and shown in figure 1, an increase of the forward scatter counts (FSC) of the stimulated lymphocytes was observed, suggesting augmentation of cell size. The size change was accompanied by an increase in cellular granularity (SSC) in all lymphocyte subtypes (CD4+, CD8+, and B cells) after 20 h culture (figure 2).

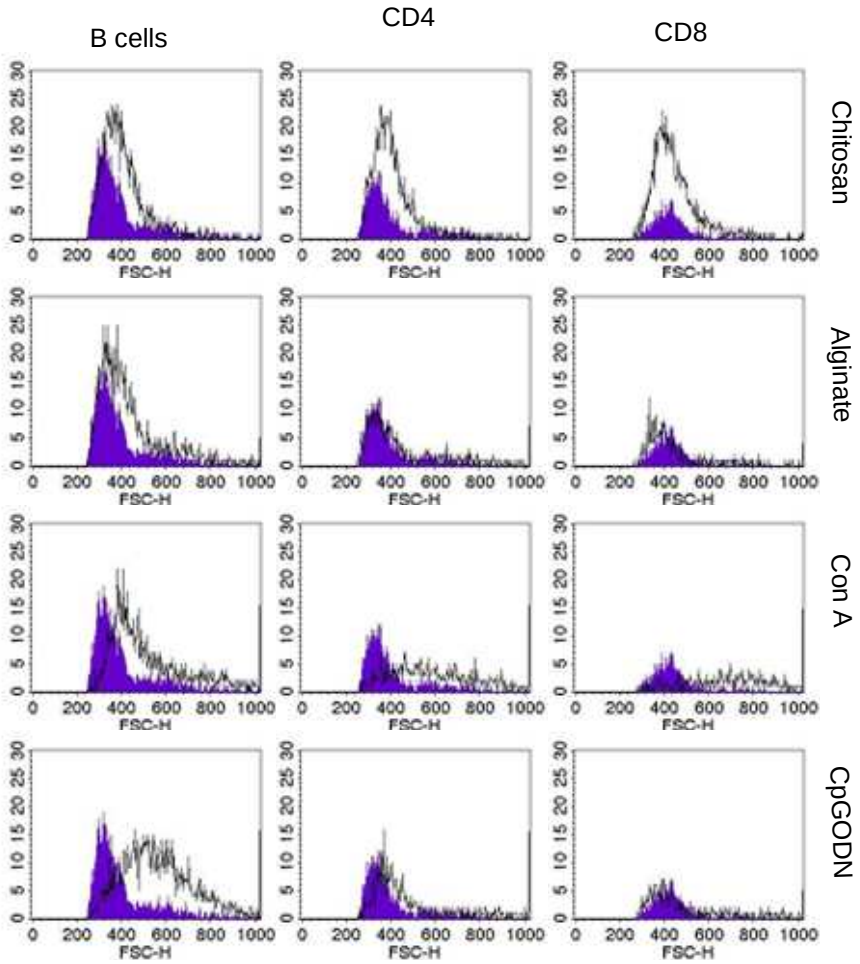

Figure 1 - Forward scatter counts–histograms (FSC-H) of B and T lymphocytes (CD4+ and CD8+) from Balb/c mice spleen stimulated with alginate or chitosan or CpG ODN for 20 h. The filled histograms represent the group control (splenocytes without stimulation) and the open histograms the stimulated (treatment) group. The results are representative of two separate experiments using three animals per experiment and each treatment.

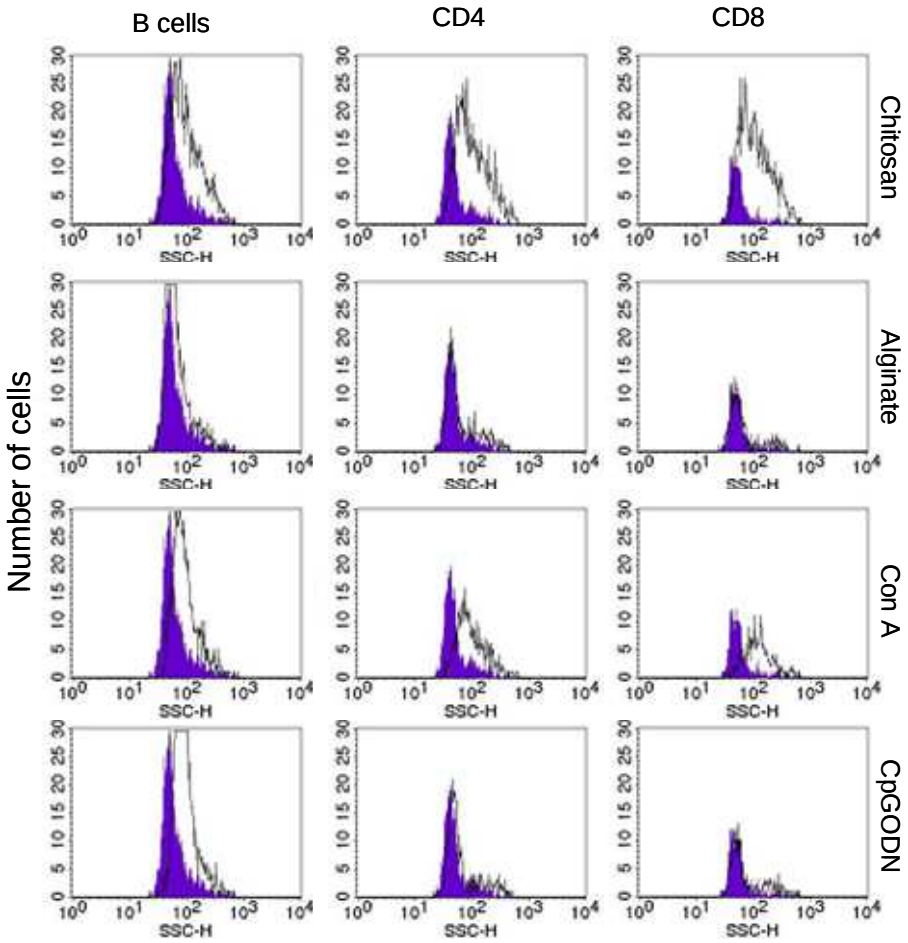

Figure 2 – Side light scatter counts–histograms (SSC-H) of B and T lymphocytes (CD4+ and CD8+) from Balb/c mice spleen stimulated with alginate or chitosan or CpG ODN for 20 h. The filled histograms represent the group control (splenocytes without stimulation) and the open histograms the stimulated (treatment) group. The results are representative of two separate experiments using three animals per experiment and each treatment.

In contrast, the presence of CpG ODN in the splenocytes culture, subjected to the same conditions did not induce a size (figure 1) and granularity (figure 2) augmentation in CD4+ and CD8+T-cells, however, a remarkable increase of these two parameters was observed in B cells. These results support the hypothesis that CpG ODN is predominately a B lymphocyte activator.

In the same way, to assess the differential effect of the alginate and chitosan biopolymer contact on spleen cell morphology, the cells were cultured with the polymers for 20 hours. Preliminary results [30] have shown cell viability near 100% with splenocytes cultured with 0.21 mg/ml of the polymers for 15 hours. In order to obtain non-toxic concentrations of polymers, we decided to decrease the concentration of polymers in the cell culture to 0.156mg/ml because we increased the incubation time to 20 hours.

As shown in figures 1 and 2, both polymers caused an increase in FSC and SSC of B cells. Chitosan was the polymer that showed a stronger effect, particularly on cell granularity (SSC). Moreover, CD4+ and CD8+ T-lymphocytes treated with chitosan also showed a strong modification of their morphology (SSC and FSC). T-lymphocytes treated with alginate did not show morphological differences (SSC and FSC) when compared to the appropriate control (filled overlap histogram in figures 1 and 2).

4.3.2 Evaluation of the expression of CD69 by T and B mouse spleen lymphocytes

As a positive control, we used Con A to induce the expression of CD69 in B and T lymphocytes and the results are shown in figures 3, 4, and 5. The CD69 expression results in spleen lymphocytes exposed to equal concentrations of polymers, alginate or chitosan (0,156mg/ml), or CpG ODN (6 μg/ml) are also shown in figures 3, 4, and 5. The percentage of CD4+ T-lymphocytes that naturally express CD69 is very low (3%). The addition of alginate to cell cultures result in a four fold increase (13%) of CD69+ T-lymphocytes (figure 3). A similar result (18%) was obtained with CpG ODN 1826, which correlates well with the above results of size and granularity. In contrast, CD4+ T-lymphocytes responded with elevated CD69 expression levels to the presence of chitosan (90%), similar to those observed with the positive control (Con A). The same robust effect of chitosan was also observed in CD8+ T lymphocytes (85%) (figure 4), contrasting with alginate, which has been shown not to induce CD69 expression on CD8+ cells. Therefore, these results are again, more closely related to the granularity and size results shown above. Following CpG ODN 1826 stimulation, we observed about 50% of CD8+ T-lymphocytes that upregulated CD69; however there was considerable variation in expression (figure 4).

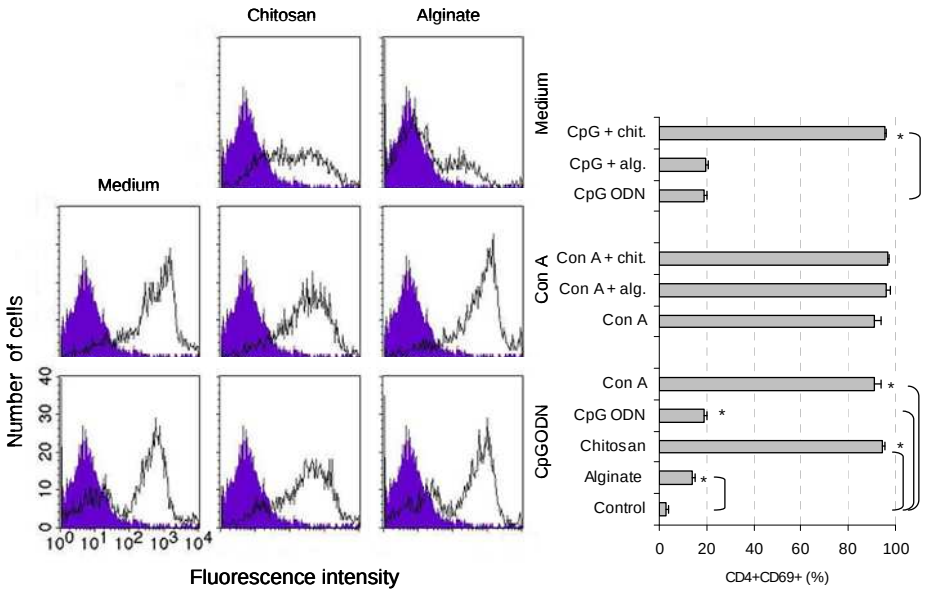

Figure 3 – Effect of the different compounds on the expression of the CD69 receptor on CD4+ splenocytes. The compounds used were the immunostimulators, CpGODN and Con A and the biopolymers chitosan and alginate. A combination between the biopolymers and the immunostimulators were also used. The filled histograms represent the group control (untreated) and the open histograms the stimulated (pretreated) group. The results are representative of two separate experiments using three animals per experiment and each treatment. The right picture corresponds to the entire data of one experiment. Similar histograms were obtained with the second experiment. The values are mean ± SEM of the percentage of the positive CD4 cells that express the CD69 antigen, obtained from three different mice.* $p<0.01$

The effect of CpG ODN or the polymers chitosan or alginate on B lymphocyte CD69 activation was also studied. The results presented in figure 5 show a strong effect of all the assayed biopolymers, with alginate showing the smallest effect (about 40%) and chitosan the strongest effect (85%). More than 95% of the B cells expressed the CD69 antigen when cultured with CpG ODN, which is in close agreement with a recent published study [31]. This study was done with two sets of ODN with similar base compositions and it was demonstrated that ODN 1826 is a strong stimulator driving B cells to express CD69 in a dose-dependent fashion. Considering that our results were obtained with lower concentrations of the ODN's and shorter culture times, these results may reflect saturation of the CD69 expression on B cells.

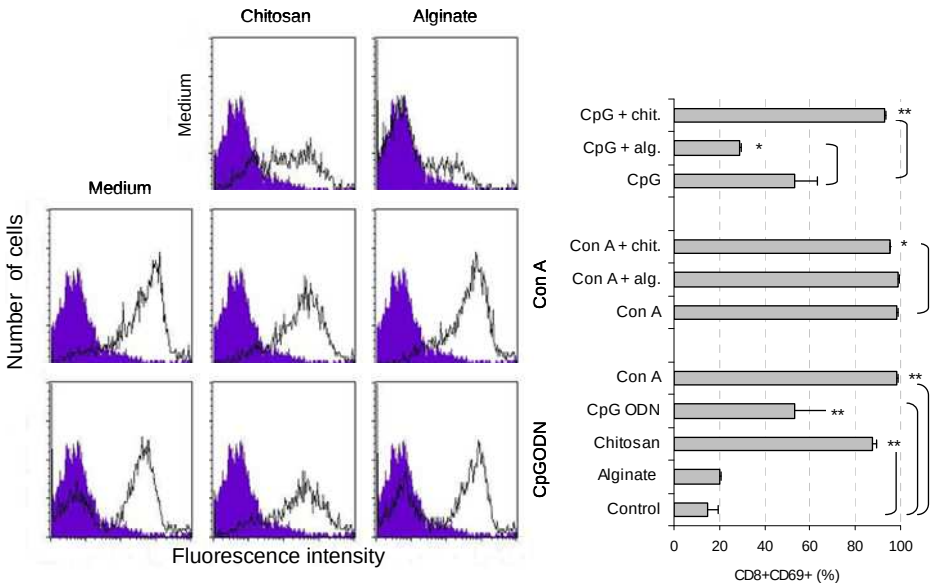

Figure 4 – Effect of the different compounds on the expression of the CD69 receptor on CD8+ splenocytes. The compounds experienced were the immunostimulators, CpGODN and Con A and the biopolymers chitosan and alginate. A combination of the biopolymers and the immunostimulators were also used. The filled histograms represent the group control (untreated) and the open histograms the stimulated (pretreated) group. The results are representative of two separate experiments using three animals per experiment and each treatment. The right picture corresponds to the entire data of one experiment and similar histograms were obtained with the second experiment. The values are mean ± SEM of the percentage of the positive CD8 cells that express the CD69 antigen, obtained from three different mice.*p<0.05; **p<0.01

To examine possible synergistic effects using a combination of the polymers chitosan or alginate and the immunostimulator, CpG ODN 1826, the cells were incubated in the presence of both, CpG and polymers (chitosan or alginate). Chitosan, surprisingly, showed to be a strong CD69 expression inducer for both B and T-lymphocytes, such that CD69 expression could not further be upregulated by CpG. However, a statistically significant difference was observed for the CD8+ T-lymphocyte subpopulation incubated only with chitosan or only with the positive control, Con A. With respect to alginate (figure 3 and 4), the results clearly showed that a synergy with CpG does not occur in T-lymphocyte populations. Furthermore, the presence of alginate in the lymphocyte culture together with CpG dowregulated the expression of CD69 antigen on CD8+ lymphocytes

(figure 4) when compared to the cells incubated only with CpG. Potentially, as a result of the saturation of B-lymphocyte CD69 expression induced by CpG, the additional presence of alginate in cell culture did not produce any statistically significant results (figure 5).

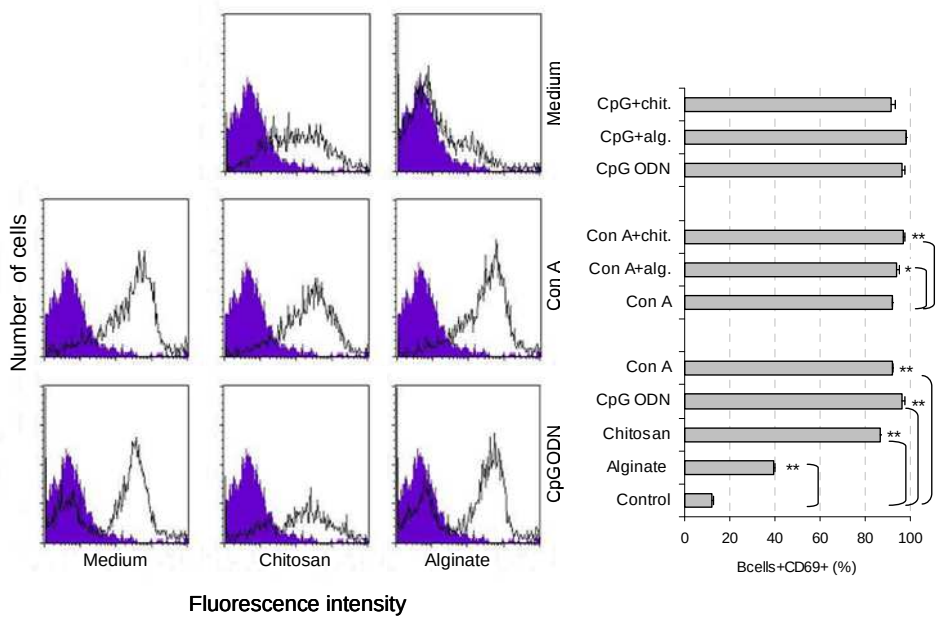

Figure 5 – Effect of the different compounds on the expression of the CD69 receptor on B cells. The compounds used were the immunostimulators, CpGODN and Con A and the biopolymers chitosan and alginate. Experiments with the combination between the biopolymers and the immunostimulators were also performed. The filled histograms represent the group control (untreated) and the open histograms the stimulated (pretreated) group. The results are representative of two separate experiments using three animals per experiment and each treatment. The right picture corresponds to the entire data of one experiment and similar histograms were obtained with the second experiment. The values are mean ± SEM of the percentage of the positive B cells that express the CD69 antigen, obtained from three different mice.*p<0.05; **p<0.01

4.3.3 Study of the effect of chitosan and alginate polymers on Lymphocyte proliferation

To study whether the polymers, chitosan or alginate, or the CpG ODN have a proliferative effect on lymphocytes and whether CpG activates the lymphocytes in

synergy with the polymers, mouse spleen lymphocytes were cultured in the presence of the polymers during 96 hours. Cells from the spleen are normally resting cells and, when cultured in the absence of a mitogen, do not proliferate (figure 6c). In the presence of a mitogen, Con A, the activated cells exhibited the highest values of [^{3}H] thymidine uptake. These values are used as the intended positive control (figure 6a). On the other hand, as shown in figure 6, while CpG ODN 1826 alone at concentrations of at least 6 µg/ ml stimulated splenocytes to proliferate, chitosan and alginate showed no significant stimulation in both concentrations used. However, with higher concentrations of alginate, a large variability of the results was observed (figure 6c).

The concomitant presence of the polymers chitosan or alginate and the CpG or the Con A in the cell culture did not lead to an increase of [^{3}H] thymidine uptake by the cells (figure 6a and b). These results may possibly indicate that no synergy between CpG or Con A and each of polymers occurred.

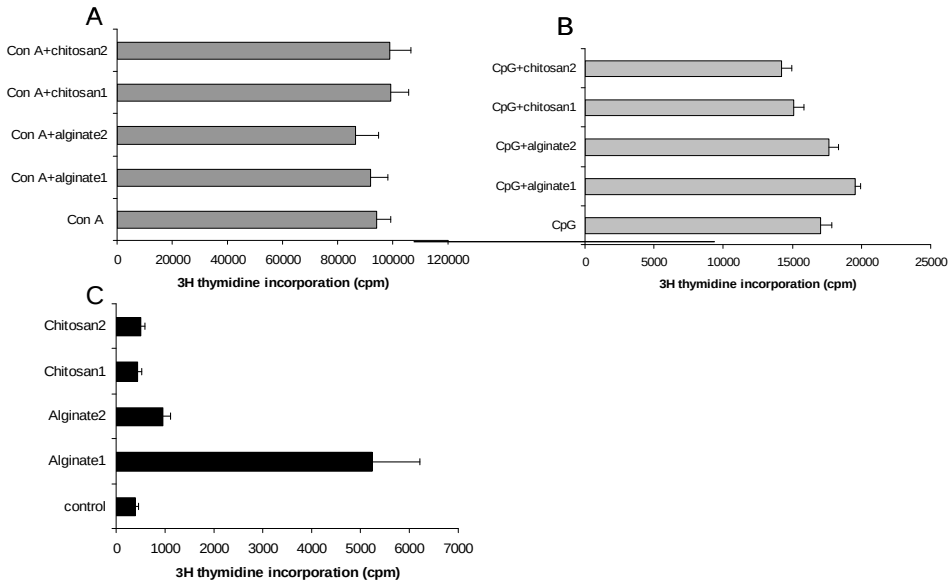

Figure 6 - [^{3}H] Thymidine incorporation, expressed in cpm (counts per minute), by the proliferating splenocytes cultured with different compounds for 96 hours. The compounds experienced were the immunostimulators, CpGODN and Con A and two concentrations of the polymers chitosan and alginate. Conc. 1= 0.312 mg/ml and conc. 2= 0.0125 mg/ml. Experiments with the combinations between the biopolymers and the immunostimulators were also performed. Each bar is the mean ± SEM from three spleen mice cultured in triplicate (n=9). Similar histograms were obtained with the second experiment.

4.4 DISCUSSION

Stimulation of lymphocytes leads to upregulation of various cell surface markers at various stages of cellular activation: CD69 (very early), CD71 (early), CD25 (late), and HLA-Dr (very late) [32]. Lymphocytes have little basal expression of CD69, but following activation, CD69 is rapidly expressed (2-3h) in all bone marrow-derived cells except erythrocytes, as reviewed in [33, 34]. The precise role of CD69 in immunity has not been elucidated owing to the absence of a known ligand and adequate in vivo models to study its physiological function [34]. It was recently suggested that a transient activation-induced CD69 surface expression may be important for regulating T cell trafficking [35]. Moreover, CD69 might affect the immune response during T-cell differentiation, involving immunoregulatory cytokines that include, but might not be limited to, TGF-β, which controls T-cell differentiation. Recent in vivo results indicate that this receptor acts as a regulatory molecule, modulating the inflammatory response [34]. Based on several studies, it was reported [36] that CD69 functions as a signal-generating receptor, possibly regulating the activity of the transcription factor AP-1 and IL-2 gene expression through both AP-1 and NF-AT complexes. The results presented by [36] also suggested that CD69 participates in a variety of processes within the overall activation cascade.

Therefore, in vitro manipulation of early activation marker CD69 is an approach used in many laboratories to explore potential pathways of cellular activation and can be used to measure the immunomodulatory effects of pharmaceutical agents and vaccine antigens [32].

Synthetic oligodeoxynucleotides (CpG ODNs) and bacterial DNA containing unmethylated CpG dinucleotides in the context of particular base sequences (CpG motifs) are known to mediate several immune responses. In the present study it was shown that CpG ODN 1826 is a potent in vitro CD69 stimulator. Its presence in a culture of mice splenocytes was shown to increase the size and granularity of B lymphocytes, which was accompanied by a strong expression of the CD69 receptor. Furthermore, a mild but statistically significant increase of the expression of CD69 in T-lymphocytes was also observed and was accompanied by high lymphocyte proliferation. These conclusions, however, can not be generalized to include other CpG ODN, even if the concept of the immunostimulatory CpG motif has become widely accepted. Recently, it was demonstrated that not all DNA/ODN containing CpG motifs were equally stimulatory

[31] and CpG 1982 showed itself to be weaker than CpG 1826 in promoting cell activation. CpG ODN 1826 at concentrations of at least 0.3 µg/ml stimulated Ig-Tg B cells to proliferate and drove the same cells to express CD69 in a dose dependent fashion [31]. Related to T-cell CD69 activation caused by CpG, the data are relatively scarce and in a recent publication [37], using human T cells, the addition of CpG ODN 2006 to peripheral blood mononuclear cells (PBMC) had only a small variable effect on the CD69 expression by CD4+ or CD8+ T cells. In contrast, the same CpG ODN sequence specifically caused increased expression of CD69 on CD4+ and CD8+ T cells when PBMC were stimulated via α-CD3 [37]. The study of possible synergistic effects between the CpG and other compounds, which could possibly explain some unexpected in vivo results, like broadening of the spectrum of CpG that primarily stimulate B cells with potential implications for the initiation and regulation of normal and pathologic immune responses, is an important aspect for a better understanding of the mechanisms involved [31]. Following this idea, the present study failed to show any synergistic effects between CpG and the polymers chitosan and alginate, respectively, in stimulating lymphocytes to proliferate or to express CD69 antigen. On the other hand, an unexpected result, to our knowledge described for the first time, was obtained with the polymers alone. The polymers proved to be potent CD69 activators, not only for B cells but in case of chitosan, also for T-cells.

In fact, concluding from comparable studies described in the literature with other compounds [36], it is not known whether the expression was stress-induced or rather activation-induced because the observed CD69 expression was not followed by lymphocyte proliferation. On the other hand, it is not known whether CD69 activation is always linked with T-lymphocyte proliferation and incomplete activation might be indicative of anergy [34]. Recently we have reported [30] an increase in cell permeability for propidium iodide, observed in lymphocytes treated with polymers chitosan and alginate, and in the present study the same effect was confirmed (data not shown). Moreover, in an in vitro study, chitosan was indicated as the cause of a slightly reversible plasma membrane perturbation in Caco-2 cell monolayers [38]. This feature is most likely an indication of cellular stress. Nonetheless, chitosan and alginate, in the concentrations studied, did not show a suppressive effect on the lymphocyte proliferation, induced by the mitogens, Con A or CpG nor had any cytotoxic effects on the cells. These observations are important and point out that the presence of these polymers, for instance in an antigen nanocarrier delivery system, might not be able to

induce a suppressive lymphocyte proliferative effect, when internalized by a lymphoid organ. On the contrary, and giving as example a recent study [39], where it was shown that chitosan based formulation administered intraperitoneally, together with a weak antigen, elicited antibody responses that were 100 times higher than those observed for the antigen in the absence of the adjuvant.

The second biopolymer in the present work investigated was a low molecular weight alginate, appropriate for pharmaceutical applications, like tissue engineering and vaccine delivery. Unexpectedly, lymphocytes upregulated CD69 expression when the immune cells were maintained in contact with the polymer. Similar to chitosan, our results showed for the first time that alginate can upregulate CD69 expression on B cells and to a lesser extent on CD4+ T cells and had no effect on CD8+ T lymphocytes. Moreover, alginate did not stimulate lymphocytes to proliferate, which is in accordance with other studies found in literature, and the highest individual variability observed has been attributed to their possible content in polyphenolic-like compounds [14].

In the present study, chitosan and alginate were shown to activate lymphocytes and induce expression of the CD69, being the chitosan the one that showed the strongest effect. This feature together with the immune properties mentioned above in the introduction section, led us to hypothesize that the adjuvant properties, suggested several times for both polymers, could not only be related to their capacity for transport and delivery of the antigen to the lymphoid tissues, but perhaps may be related to their capacity to stimulate immune cells to initiate or simply amplify an immune response specific to the antigen.

4.5 CONCLUSIONS

In this study lymphocytes underwent strong activation and rapid CD69 upregulation upon contact with the biopolymers chitosan or alginate. More controlled studies are needed to understand this activation mechanism and the possible consequences for the initiation of an immune response. Moreover, as these are in vitro studies employing mice spleen cells it remains unknown whether the same effects would be observed with human cells or other peripheral lymphoid organs, such as Peyer's patches, following in vivo oral administration.

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CHAPTER

5

EVALUATION OF THE IMMUNE RESPONSE FOLLOWING A SHORT ORAL VACCINATION SCHEDULE WITH HEPATITIS B ANTIGEN ENCAPSULATED INTO ALGINATE COATED CHITOSAN NANOPARTICLES [4]

[4] *In preparation for publication*

ABSTRACT

The purpose of this work was to assess the ability of recombinant hepatitis B vaccine, encapsulated in alginate coated chitosan nanoparticles, to induce local and systemic immune responses following oral vaccination. The antigen was administered either alone or in combination with the synthetic oligodeoxynucleotide containing immunostimulatory CpG motif (CpG ODN) as adjuvant, associated or not with the alginate coated chitosan nanoparticles. After two immunizations only the group I (HBsAg associated with nanoparticles) and the group VI (HBsAg and CpG, both associated with nanoparticles) induced an immune response. Both groups showed significant higher values of the CD69 expression in CD4+ and CD8+ T-lymphocytes and lower values of this marker in B lymphocytes. Moreover, the strongest proliferative response of the splenocytes, ex vivo stimulated with concanavalin A, was observed in the same groups. Once more, only mice of the groups I and VI elicit the generation of anti-HBsAg antibodies. Both IgA obtained from intestine washings and IgG from serum, (with IgG2a being the predominant isotype in the case of group VI), corresponded to a humoral Th1 profile of response. These data demonstrate some potential of the coated chitosan nanoparticles for their use as a carrier adjuvant for oral vaccination with the recombinant hepatitis B surface antigen.

5.1 INTRODUCTION

According to a report of the World Health Organization (WHO/UNICEF, 2005) [1], the estimated number of deaths in the world in all age groups from diseases preventable by vaccines in 2002 was 2.1 million, 600 000 deaths being due to hepatitis B. The global coverage of infants with three doses of hepatitis B vaccine in 2004 was 48%, contrasting with 3% in 1992 [1]. In the last few years, the majority of industrialized countries have introduced hepatitis B vaccination campaigns. Therefore, the above statistics of deaths due hepatitis B are representative predominantly for developing countries, where mass vaccination has not been implemented yet (18% of the 192 WHO member states). Its implementation is highly dependent on the development of more stable and cheaper

vaccines for which the intervention of specialised human resources for the administration would not be required.

Oral administration has been appointed as the only economically feasible approach to mass vaccination. Impressive logistical advantages of orally administered vaccines were exemplified by two national vaccination days in 1996, when 121 million Indian children were vaccinated against polio at 650,000 centres [2]. However, it has been shown that is very difficult to obtain a protective immune response following oral vaccination, the live-attenuated polio vaccines are being one of the few exceptions [3]. For this reason, only few vaccines currently approved for human use are being administered orally.

Unfortunately, a simple oral formulation is not easily achieved for the new generation of subunit vaccines, which hold the greatest promise for disease prevention in the 21st century [4]. Several explanations have been appointed to justify the disappointing results found for oral administration of subunit vaccines, being almost exclusively biotechnological products. One of the most important reasons is related with the adverse environment of the gastrointestinal tract (GIT), rich in acids and enzymes, which are able to destroy the antigen. Equally important is the mechanism of oral tolerance, the vital physiological role for dietary antigens in preventing hypersensitivity reactions to food or to commensal bacteria [5, 6]. This hyporesponsivity to antigens orally administered is not yet fully understood, but it is thought that the mucosal immune system has involved a variety of mechanisms to achieve and maintain tolerance against self-antigens and against the overabundance of environmental antigens present in the microflora and food. Among them, activation-induced cell death, anergy and especially the induction of regulatory T cells are frequently reported in the literature [3]. In a recent study [7] the induction of oral tolerance to hepatitis B virus proteins was achieved by the administration of five low oral doses of hepatitis B virus proteins, followed by two inoculations with a commercial vaccine.

In the case of the development of an oral hepatitis B vaccine, this mechanism has to be circumvented and the antigen must be protected from physical degradation and enzymatic digestion [3]. For this purpose, several strategies have been described in literature. Those approaches include the encapsulation of immunogenic peptide representing residues 127-145 of the immunodominant B-cell epitope of hepatitis B surface antigen (HBsAg) in poly(D,L-lactide co-glycolide) [8]. Another strategies are the encapsulation of the plasmid DNA encoding hepatitis B virus protein in poly(DL-lactide-

co-glycolic acid) (PLGA) [9] or in Salmonella typhimurium [10-13] or the genetic modification of edible plants for the production and delivery of the hepatitis B vaccine, within e.g. potato tubers [4, 14], cherry tomatillo [13] and lettuce [15, 16]. A very recent clinical study [4] with previously vaccinated volunteers showed that the ingestion of doses of 100 g of uncooked potato tubes (8.5 μg/g) induced the increase of the serum anti-HBsAg titers in about 60% of the volunteers, who ate three doses of the potatoes. Approximately 40% of the volunteers were non-responders to the HBsAg. The necessity of finding a good mucosal adjuvant in order to elicit an increase of the number of responders was emphasised by the authors of this study [4].

Chitosan, a copolymer of D-glucosamine and N-acetyl-D-glucosamine is a derivative of chitin, one of the polysaccharides most abundant in nature. In the last few years, the properties of this biodegradable biopolymer have been intensively investigated. In particular, its ability to stimulate cells from the immune system has been shown in several studies. For instance, the presence of chitosan in a dendritic cell culture induced the expression levels of the co-stimulatory molecules CD86, CD40 and HLA-DQ [17], indicative of dendritic cell maturation. Likewise, chitosan has also shown to be able to up-regulate, in some extent, a number of macrophage functions [18, 19].

The polymer has also been used in the nanoparticle formulation for loading and delivering different vaccines, like meningococcal C conjugate [20], diphtheria [21] and tetanus toxoid [22, 23] or used without any modification, suspending the bulk powder in a solution of the meningococcal C conjugate vaccine [24] or using a soluble chitosan derivative with the influenza vaccine [25, 26] or finally using chitosan to surface-modified PLGA microspheres containing hepatitis B vaccine for intranasal immunization [27].

In a previous study, we have formulated and characterised alginate coated chitosan nanoparticles [28]. They consist of a chitosan core (chitosan nanoparticles) to which the hepatitis B vaccine was adsorbed and in a second step, the sodium alginate. The alginate coating is afterwards cross linked with calcium ions. This delivery system has the particular advantage of being constructed under very mild conditions, which is a great benefit for the encapsulation of proteins, peptides and antigens. Moreover, in a very recent publication [29] we have demonstrated that these coated nanoparticles were able to be taken up by rat Peyer's patches which is one of the essential features to internalise, deliver and target the intact antigen to specialised immune cells from the GALT (gut associated lymphoid tissue) [30]. This property makes these new

nanoparticles a promising delivery system especially for oral vaccination in combination with a suitable potent adjuvant.

In the present study the feasibility of using the recombinant surface hepatitis B protein (HBsAg) encapsulated into the above mentioned alginate coated chitosan nanoparticles for the induction of local and systemic immune responses after oral vaccination was evaluated. Moreover, to improve the immune response, synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN), were also incorporated into the delivery system. CpG ODN acts as a potent adjuvant and has shown in a number of studies to induce a Th1 type immune response, not only when administered parenterally [31, 32] but also for mucosal vaccination [33-35].

5.2 MATERIAL AND METHODS

5.2.1 Materials

5.2.1.1 Polymers

Ultra pure chitosan was purchased from Primex BioChemicals AS (Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation is 95% (titration method) and the viscosity is 8 cP (measured in 1% solutions in 1% acetic acid). A low molecular weight pharmaceutical grade sodium alginate (MANUCOL LB®) was kindly donated by ISP Technologies Inc. (Surrey, UK). According to the provider's specifications, the typical values for the percentage of mannuronic and guluronic acid for Manucol LB are 61% and 39%, respectively, with an estimated molecular weight of 18 kDa .

5.2.1.2 Antigen, adjuvant and reagents

The hepatitis B surface antigen (HBsAg), (subtype ADW2) was kindly offered by GSK Biologicals (Rixensart, Belgium). Class B, CpG ODN (1826) (5'-TCC ATG ACG TTC CTG ACG TT-3') was purchased from Coley Pharmaceutical Group (Ottawa, Canada).

Concanavalin A (Con A), phenylmethanesulfonyl fluoride (PMSF), avidin peroxidise conjugate and the BCIP/NBT- purple liquid substrate system for membrane were from Sigma Chemicals (St. Louis, USA). Certified fetal bovine serum (FBS) and L-glutamine (200 mM) were from Gibco (Invitrogen Co, Paisley, Scotland, UK), 1M HEPES buffer (0.85% NaCl), RPMI 1640 without L-glutamine and Pen-Strep (10 000 U penicillin/ml; 10 000 μg streptomycin/ml) were from Biowhitaker (Cambrex Bio Science, Verviers, Belgium). [methyl-^{3}H] thymidine (1.0 mCi/ml) was obtained from Amersham Biosciences (UK), R-Phycoerythrin (PE)-conjugated hamster anti-mouse CD69, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8 were obtained from BD Biosciences (Madrid, Spain). The FITC-conjugated goat anti-mouse IgM (anti-μ), the anti-mouse IFN-γ and biotin rat anti-mouse IFN-γ was purchased from PharMingen (San Diego, CA, USA). The mouse IgA ELISA quantitation kit was obtained from Bethyl Laboratories, (Montgomery, USA). All others reagents used were analytic grade. All solutions were prepared in ultrapure water.

5.2.2 Methods

5.2.2.1 Preparation of the coated nanoparticles

The preparation of the alginate coated chitosan nanoparticles was performed according to the method previously described by us [28]. In brief, chitosan was dissolved at a concentration of 0.25% (w/v) in diluted acetic acid solution. The formation of the particles was achieved after the addition of 3.5 ml of sodium sulfate solution (10% w/v) to 200 ml of the chitosan solution. The resulting suspension was centrifuged for 30 minutes at 3500 rpm (2800 x g) and the supernatant was discarded. The particles were re-suspended in Millipore water and centrifuged twice. Finally they were frozen in liquid nitrogen and freeze-dried overnight using a Labconco freeze dry system (Labconco Corporation, Kansas, USA). The dry powder was kept frozen until further use.

The loading of the nanoparticles with HBsAg or with CpG ODN was performed by incubating a solution of HBsAg or the solution of CpG with a suspension of chitosan particles at pH 7.4 (phosphate buffer) under mild agitation at room temperature during 120 minutes. The resulting suspensions with the composition of 0.015% (w/v) HBsAg and 0.5% (w/v) nanoparticles and the second with 0.015% (w/v) CpG and 0.5% (w/v)

nanoparticles were used in the subsequent coating step. Alginate coated nanoparticles were obtained by mixing equal volumes of nanoparticles suspension and a buffer phosphate solution of sodium alginate (1% w/v) under magnetic stirring. The agitation was maintained for 20 min. The suspension was then centrifuged for 10 min at 1600 rpm and the supernatant was discarded. The particles were re-suspended in 0.524 mM $CaCl_2$ in 50 mM HEPES buffer solution, kept under agitation for another 10 minutes and immediately administered to the mice.

5.2.2.2 Evaluation of the loading efficacy of HBsAg and CpG ODN in coated nanoparticles.

The loading efficacy of the coated particles was calculated by an indirect way, quantifying the antigen that remained in solution as described before [29]. After the coating with alginate, an aliquot of the particle suspension was centrifuged at 14,000 rpm for 15 minutes and the protein in supernatant was quantified by micro-BCA-protein assay (PIERCE, Rockford, USA) using a microplate reader with a 570 nm filter.

For the CpGODN the same procedure was followed and the oligodeoxynucleotides was quantified, measuring the OD of the supernatants at 260 nm. To eliminate background interference, the supernatant of unloaded particles were treated the same way. The results refer to the nanoparticle batches used in the vaccination studies (4 batches for each immunization).

The loading efficacy (LE) and the loading capacity (LC) were calculated from the following equations:

Eq. 1 - LE (%) = (Total amount of HBsAg or CpG – free HBsAg or CpG)/Total amount of HBsAg or CpG*100

Eq.2 - LC (μg of HBsAg or CpG/mg chitosan nanoparticles dry weight) = (Total amount of HBsAg or CpG– free HBsAg or CpG)/ mg chitosan nanoparticles dry weight

5.2.2.3 Evaluation of the structural integrity of the vaccine

The integrity of hepatitis B antigen was confirmed using SDS-Polyacrylamide gel electrophoretic (SDS-PAGE) analysis of the hepatitis B vaccine released overnight from the nanoparticles (7.4 pH buffer phosphate; 37ºC; 50 rpm). The samples were centrifuged at 14000 rpm in order to separate the released antigen from the particles and an aliquot was then solubilised with the loading buffer and treated (5 minutes at 100ºC). The SDS-PAGE was performed in accordance with standard protocols [36] with 12% resolving gel, cast and run in tris-glycine buffer at 25 mA and finally stained with silver nitrate.

The antigenicity of the entrapped hepatitis B vaccine was assessed by Western blotting using a mouse antiserum raised against the native antigen. The hepatitis B vaccine samples were transferred from the unstained gel onto a nitrocellulose membrane, using semi-dry electroblotting system (115 mA; 1hour) and the membrane was blocked overnight at 4ºC with PBS-T (containing 0.05% of Tween 20 and 5% of milk). After washing with PBS-T, the membrane was incubated for 2 hours at room temperature with the positive anti-HBsAg IgG mouse antiserum, diluted 1:500 in PBS-T with 5% of low fat milk. After washing with PBS-T, the membrane was incubated with anti-mouse IgG conjugated to alkaline phosphatase, diluted 1:750. The ability of the mouse antiserum to recognize hepatitis B vaccine released from the nanoparticles was demonstrated colorimetrically using 5 ml of phosphatase buffer with 33 µl NBT (50 µg/ml) and 16.7 µl BCIP (50 µg/ml). The reaction was stopped by washing the membrane with water.

5.2.2.4 Immunization studies

5.2.2.4.1 Animals:

Six week-old female BALB/c AnNHsd mice were used (Harlan Iberica, Barcelona, Spain) with four or six mice per group. Animals were housed for acclimatization one week before the experiments at the animal resource facilities of the Faculty in accordance with institutional ethical guidelines. They had free access to food and water, with 12h light/dark cycle. Two hours before the oral vaccination and overnight before the end of the experiment, the mice were starved allowing only free access to water.

5.2.2.4.2 Treatment groups

Group I - suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg
Group II - suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg plus 10 μg of the adjuvant (CpG ODN) in solution.
Group III - untreated - control
Group IV - solution with 10 μg HBsAg
Group V - solution with 10 μg HBsAg and 10 μg CpG ODN
Group VI - suspension of the mixture of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg and the same particles loaded with 10 μg CpG ODN

5.2.2.4.3 Immunization schedule

Each animal received immediately before immunization 100 μl of a 7.5 % sodium bicarbonate solution in order to neutralise the acid environment of the stomach. The different formulations, corresponding to each treatment group (see 2.2.4.2.) were administered orally with a gavage-feeding needle. The primary immunization was followed by one boost three weeks later with 150 μl volume containing 10 μg of the vaccine. The animals were sacrificed 10 days post boost and a collection of blood from the heart, vaginal secretions, small intestinal washes and the spleen was carried out for analysis as described in the following sections.

5.2.2.4.4 Collection of samples

Blood samples were taken from the orbital sinus before the boost and by cardiac puncture at the end of the experiment and the sera were prepared by centrifugation and stored at -20ºC until analysis.

Vaginal and gut washes were obtained by rinsing with (75 μl x 3) 225 μl and 600 μl cold PBS (containing 0.1% sodium azide, 0.1% bovine serum albumin (BSA) and 1 mM PMSF) through the vagina or the intestine, respectively. The extract was vortexed, allowed to stay at room temperature for 15 minutes and then centrifuged (6000 rpm/15 min/4ºC). The clear supernatants were stored at –80ºC until analysis.

5.2.2.4.5 *Enzyme-linked immunosorbent assays (ELISA) for HBsAg specific immunoglobulins*

Ninety-six-well flat-bottomed microtiter plates (Nunc immunoplate maxisorb) were previously coated (incubated overnight at 4ºC) with the recombinant HBsAg (1 μg/well) in coating buffer (50 mM sodium carbonate, pH 9.6). The plates were washed 5 times with PBS-T (PBS containing 0.05% Tween-20) and blocked with 3% BSA in PBS-T (200μl/well) during 1 hour at 37ºC. The plates were then washed 5 times with PBS-T and the serial dilutions of each serum (100 μl/well) from the individual mice were tested in triplicate, starting from a dilution 1:100 in PBS-T. The serum was incubated during 2 hours at 37ºC and after washing the plates with PBS-T, the plates were incubated for additional 30 min at 37ºC with peroxidase-labeled goat anti-mouse immunoglobulin G and isotypes (anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG3). The bound antibodies were revealed adding 100 μl/well of 0.5 mg/ml of o-phenylenediamine dihydrochloride (OPD) (Sigma, Spain) in 10 ml of citrate buffer with 10 μl of H_2O_2. The reaction was stopped after 10 minutes with 50 μl of 3M HCl to each well. The absorbance was read out at 492 nm in an automatic ELISA reader (Easy Reader 400, SLT-LABINSTRUMENTS). Elisa titers were expressed as mIU/ml and 1 mIU is the OD mean of the pre-immune serum plus 2 times the standard deviation.

The measurement of IgA was carried out using a mouse IgA ELISA quantification kit (Bethyl Laboratories, Montgomery, Texas, U.S.) as described by the manufacturer. In order to make a correct evaluation of the sIgA in the gut and vaginal extracts, total sIgA and the specific anti-HBs sIgA were determined in the extracts. The results are presented as the anti-HBsAg IgA/total IgA. By this way differences between samples related with the extraction process or stability of the sIgA were minimized.

The IgA standard was diluted to appropriate concentrations in PBS with 1% BSA to create a calibration curve. The gut washes were diluted in PBS-T with 1% BSA and added to the plates in series of twofold dilutions. The concentrations of the total and specific IgA were determined from the calibration curve generated for each set of samples using a four parameter logistic curve-fit generated by SigmaPlot software (version 8.0, SPSS Inc).

5.2.2.4.6 Preparation of spleen cell suspensions.

The mice were euthanized by cervical dislocation and their spleens were aseptically removed. Individual spleen cell suspensions were prepared in a Petri dish using curved needles and washed twice with RPMI 1640. The final suspension was adjusted to a final concentration of $1x10^7$ cells /ml in complete RPMI 1640 medium [supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) glutamine, 1% (v/v) Pen-Strep and 2% (v/v) 1M HEPES buffer].

5.2.2.4.7 Cell population of the spleen

The cells (0.025×10^7 cells) were washed twice with cold PBS-supplemented with 2% FBS and then incubated in the dark for 30 minutes at 4ºC with saturated concentrations of FITC-conjugated rat anti-mouse CD4 or FITC-conjugated rat anti-mouse CD8 or FITC-conjugated goat anti-mouse IGM (anti-μ). After incubation, cells were washed three times with PBS-2% FBS and then re-suspended in 500 μl PBS-2% FBS. To exclude dead cells, 2.5 μl propidium iodide (50 μg/ml) were added just before data acquisition. At least 10,000 events were analysed by flow cytometric acquisition, performed in a fluorescent activated cell sorter (FACS Calibur) (BD, Biosciences, Madrid, Spain). Data were analysed by CellQuest software (BD, Biosciences, Madrid, Spain).

5.2.2.4.8 Splenocyte cell culture in the presence of the mitogens

Using sterile 96-well flat-bottomed tissue culture plates, 25 μl of splenocyte suspension ($1x10^7$ cells/ ml) from each mice were plated in triplicate along with 25 μl of a complete RPMI solution of the mitogen [con A (50 μg/ml), CpG ODN (50 μg/ml) plus HBsAg (25 μg/ml), HBsAg (25 μg/ml) alone or without mitogen (control)]. Finally the volume of the well was completed to 200 μl with complete RPMI and incubated at 37ºC with 95% relative humidity and in the presence of 5% CO_2.

5.2.2.4.9 Cytokine production by splenocytes

Spleen cell suspensions were plated with the mitogens (see 2.2.4.8) and incubated in a humidified 5% CO_2 incubator for 24 h (IL-2) and 86 h (INF-γ) at 37ºC. The plates were centrifuged and the supernatants stored at –80ºC until analysis.

IL-2 and IFN-γ cytokines produced by splenocytes were determined by ELISA, following a technique described elsewhere [37].

5.2.2.4.10 Evaluation of the CD69 expression on lymphocytes

After 20 h of incubation in the presence of different mitogens (see 2.2.4.8), cells were washed and re-suspended in cold phosphate buffered saline (PBS) supplemented with 2% fbs (PBS-2% FBS), resulting in a concentration of 0.5 x 10^6 cells/ well. The cells were then incubated in the dark for 30 minutes at 4ºC with saturated concentrations of PE-conjugated hamster anti-mouse CD69 and FITC-conjugated rat anti-mouse CD4 or FITC-conjugated rat anti-mouse CD8 or FITC-conjugated goat anti-mouse IGM (anti-µ). After incubation, cells were washed three times with PBS-2% FBS and then re-suspended in 500 µl of PBS-2% FBS. To exclude dead cells, 2.5 µl propidium iodide (50 µg/ml) was added just before data acquisition. The flow cytometry determinations were done according to the description given above (see 2.2.4.7)

5.2.2.4.11 Lymphoproliferation assay

Splenocytes were obtained and cultured together with the mitogens in a flat-bottomed 96-well plate as described before (see 2.2.4.8). The cells were cultured for 96 hours at 37ºC and on the last 8 h of incubation; each well was pulsed with 1µCi of [methyl-^{3}H] thymidine. 96 well plates with the cells were stored at -20ºC until further analysis. The cells were later thawed and harvested onto a fiberglass filter (filter mats, molecular devices, Skatron, Lier, Norway) using a semiautomatic cell harvester (Scatron Instruments, USA) and DNA thymidine incorporation was counted by standard liquid scintillation techniques with a Beckman LS 6500 scintillation counter (Beckman Coulter Inc, Fullerton, USA). Thymidine incorporation was expressed as counts per minute (cpm).

5.2.3 Statistical analysis

Data were presented as the mean ± S.E.M. for at least three experiments and statistical significance was assessed using one-way ANOVA followed by Dunnett's post test using the GraphPad Prism 4 software. Differences were considered significant when $p<0.05$.

5.3 RESULTS

5.3.1 Characterization of the alginate coated nanoparticles

In a recent publication of our group [28], the development of alginate coated chitosan nanoparticles and the characterization of this new delivery system were reported. Briefly, before coating with sodium alginate, the chitosan nanoparticles have a mean diameter of 643 nm (dynamic light scattering technique) and are positively charged (+37 mV). Scanning electron microscopy (SEM) images of uncoated particles also revealed the presence of small nanoparticles (around 100 nm) and demonstrated that the majority of the coated particles were in a range between 300 and 600 nm. The presence of the alginate coating layer was confirmed both by FTIR and DSC studies and by the observation of the inversion of the zeta potential to -34 mV.

In another study we recently have shown [29] results of the particle uptake by Peyer's patches and the ovalbumin release from alginate coated chitosan nanoparticles. In these studies, ovalbumin was chosen as a model vaccine. Ovalbumin release studies from coated and uncoated chitosan nanoparticles, performed with several pH buffers, allowed us to conclude that the coating with sodium alginate of the ovoalbumin loaded chitosan nanoparticles avoided an ovalbumin burst release observed in the first 30 minutes compared to uncoated chitosan nanoparticles at pH 5.5; 6.8 and 7.4 (phosphate buffer). However at pH 1.2, a burst release was observed for both coated and uncoated nanoparticles. For this reason in the present study a 7% sodium bicarbonate solution was administered immediately before feeding the mice with the nanoparticles with the purpose to increase the pH of the stomach fluids.

5.3.2 CpG ODN and hepatitis B antigen entrapment in coated nanoparticles

Different ratios of Hepatitis B vaccine to chitosan nanoparticles and CpG ODN to chitosan nanoparticles were investigated (data not shown) and the systems with the highest loading efficacy were used for the further studies. It could be shown that hepatitis B antigen and CpG ODN were efficiently associated with alginate coated chitosan nanoparticles. The loading efficacy of Hepatitis B vaccine in the coated nanoparticles

was 85.9 ± 4.7 % (mean ± STDEV) and the mean of the loading capacity was 25.7 μg of HBsAg/ mg of dry chitosan nanoparticles ± 1.42 (STDEV). The loading efficacy of CpG was even better, 98.8 ± 1.29% and the loading capacity 29.85 ± 0.0364 (μg of CpG ODN/ mg of dry chitosan nanoparticles) since the existence of a strong interaction between DNA or oligodeoxynucleotides and the oppositely charged cationic chitosan has already been demonstrated [38, 39].

5.3.3 Confirmation of the structural integrity of hepatitis B antigen

The adsorption of hepatitis B antigen (HBsAg) to chitosan nanoparticles occurred at room temperature, under mild agitation using a phosphate buffer as the external medium of the suspension. The coating with alginate was done under similar conditions, being considered as non aggressive for the entrapment of ODN's, proteins, peptides and vaccines. Nevertheless, a SDS-PAGE analysis followed by a Western blotting was performed in order to confirm the integrity of hepatitis B antigen and the results are shown in figure 1. SDS-PAGE analysis of the bulk vaccine showed the presence of a dominant band with a molecular weight below 30 KD (fig. 1b lane 1) that, according to the literature, was identified to be the 24 kDa polypeptide described by Stephenne [40] and a second protein with a molecular weight between 35 and 55 kD. The same gel revealed identical bands for the entrapped HBsAg (lanes 2, 3 and 4) and there were no additional bands to indicate the presence of fragments. Therefore the integrity of the antigen was not affected by the entrapment procedure. Moreover the Western blot membrane shown in figure 1a demonstrated that the HBs specific antibodies from a serum of a vaccinated mouse recognized the antigen epitopes (fig. 1a; lanes 3, 4 and 5) in a similar way as for the bulk vaccine (fig. 1a; lanes 1 and 2), confirming that the antigenicity of the hepatitis B antigen was not altered after the entrapment into nanoparticles.

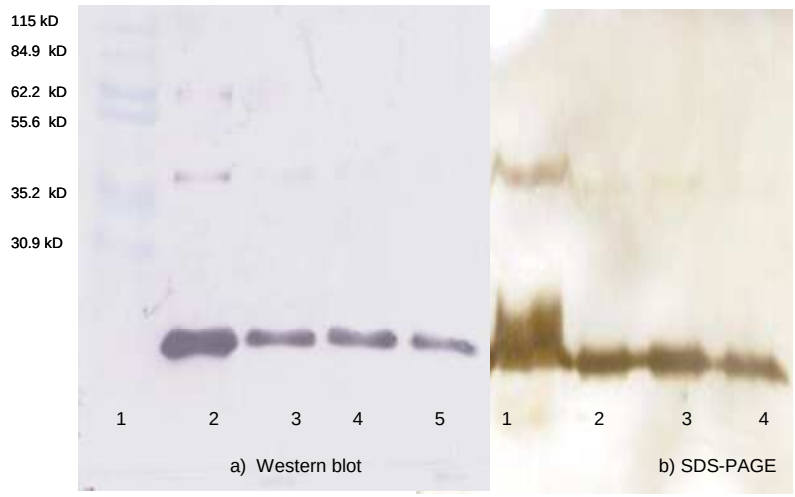


Figure 1 – Western blot and SDS-Page analysis of the released hepatitis B antigen from alginate coated chitosan nanoparticles a). Western blot. Lane1: molecular weight markers, lane2: HBsAg before association with particles in PBS, lane 3, 4 and 5: HBsAg after association with coated nanoparticles and subsequently released overnight in PBS at 37ºC. b) SDS-PAGE silver stained. Lane 1: HBsAg before association with the particles, lane 2, 3 and 4: HBsAg after association with coated nanoparticles and subsequently released overnight in PBS at 37ºC.

5.3.4 Evaluation of cellular immune responses

5.3.4.1 Lymphoproliferative response

The cellular immune response of individual mice was evaluated measuring in vitro proliferation of splenocytes, incubated in the presence of HBsAg or in the presence of HBsAg plus CpG or in the presence of Con A (positive control), respectively. Cells cultured without mitogens were used as a negative control of the experiment and showed as expected only low counts per minute (cpm) values (data not shown). Similar to the negative control were the results obtained with cells incubated only with the antigen (data not shown). The lymphoproliferation in vitro induced by the mixture of the hepatitis B antigen and the immunopotentiator CpG ODN were evaluated in all the groups and are shown in figure 2A. The cellular proliferation observed in all the groups was probably caused by the presence of CpG, since it was previously demonstrated that the HBsAg alone in the concentration used did not induce any proliferative effect. The

same figure (2A) showed that the groups I (HBsAg associated with nanoparticles) and VI (HBsAg and CpG associated with nanoparticles) presented a lower splenocyte proliferative capability when compared with the control group. Nevertheless, due to the large variability of the results, the values are not statistically different from the control group. Even less or no proliferation was observed in groups IV (HBsAg in solution) and V (HBsAg and CpG in solution).

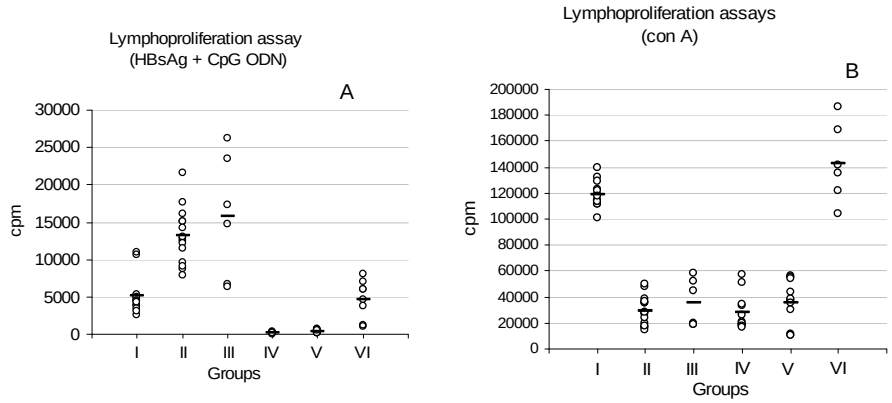


Figure 2 – Lymphoproliferative response after oral administration of the different hepatitis B vaccine formulations. *In vitro* proliferation of individual mouse spleenocytes during a 96 hour period stimulated with: A) HBsAg + CpG ODN, B) Con A. Each circle represents the result of individual samples and the horizontal bar the mean of the group. The results corresponded to Thymidine incorporation and are expressed as counts per minute (cpm).

Finally, figure 2B shows the lymphoproliferative results observed in the presence Con A, a classical mitogen, which served as a positive recall antigen. The groups II, IV and V presented cpm values between 20 000 and 60 000, similar to the control group (group III). These results contrasted with those found for the groups I and VI, where a much stronger proliferative response was observed, showing values between 100 000 and 180 000 cpm.

5.3.4.2 Cell populations

The evaluation of spleen cell populations was done immediately after sacrificing the mice. Each individual value and the means for each group are shown in figure 3. The percentage of CD4+ T-lymphocytes showed to be similar in all groups. On the other hand, slight but statistically differences were found in the CD8+ T-lymphocyte

populations in some treated groups when compared with the control group. In particularly the percentage of CD8+ T-lymphocytes appeared slightly increased in groups IV and V and decreased in groups I and VI.

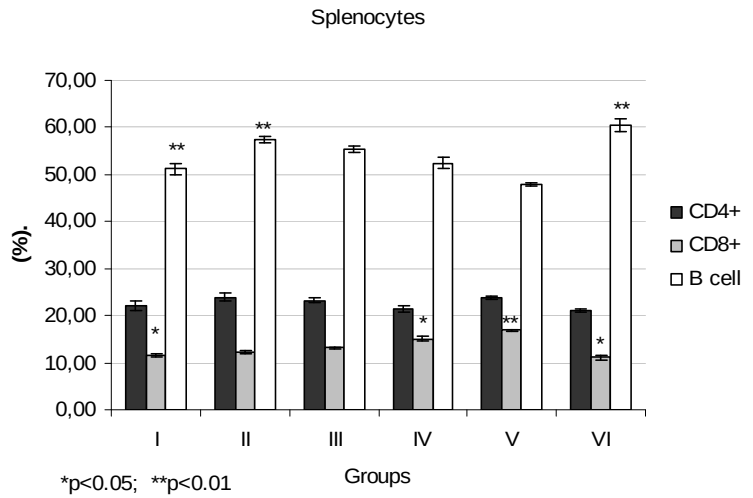


Figure 3 – Percentage of T-lymphocytes CD4+, CD8+ and B+ Lymphocytes in the spleens of mice from the treatment groups (I, II, IV, V and VI) and the control (naïve mice) (group III) determined by FACS analysis. Each bar corresponds to the group geometric mean plus the standard error of the mean (n between 3 and 6). The comparison between the control group (III) and the different treatment groups (I, II, IV, V, VI) was made with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (** p<0.01 and *p<0.05).

The increase of the CD8+ T-lymphocytes with the decrease of the CD4+/CD8+ ratio as well as a significant decrease of the lymphocyte proliferative response to antigen stimulation, appear to be correlated with the immunological tolerance phenomena [41]. The groups IV and V, where antigen or the antigen plus the adjuvant were given in solution, were shown to fulfil these two conditions. On the other hand the association of the antigen with nanoparticles (groups I, II and VI) may have circumvented this undesirable mechanism. Additional studies have to be performed to test this hypothesis.

5.3.4.3 Cytokines production

IL-2 and IFN-γ were assayed in the supernatants from splenocytes cultured in the presence of different mitogens (HBsAg, Con A and the mixture of HBsAg+CpG ODN) and the results are shown in figure 4. The highest IL-2 mean levels were found in Groups

I and VI. Nevertheless these results are not statistically significant due to the high variability within the groups. The production of IFN-γ by the splenocytes was detected in all the groups in the presence of different mitogens. However the highest mean value was detected in the group VI, where CpG and the antigen were administered associated to the coated nanoparticles, with CpG ODN as the main responsible factor for the high IFN-γ production. Therefore, the formulation given to the group VI seemed to be the most effective in the generation of a Th1 profile of immune response.

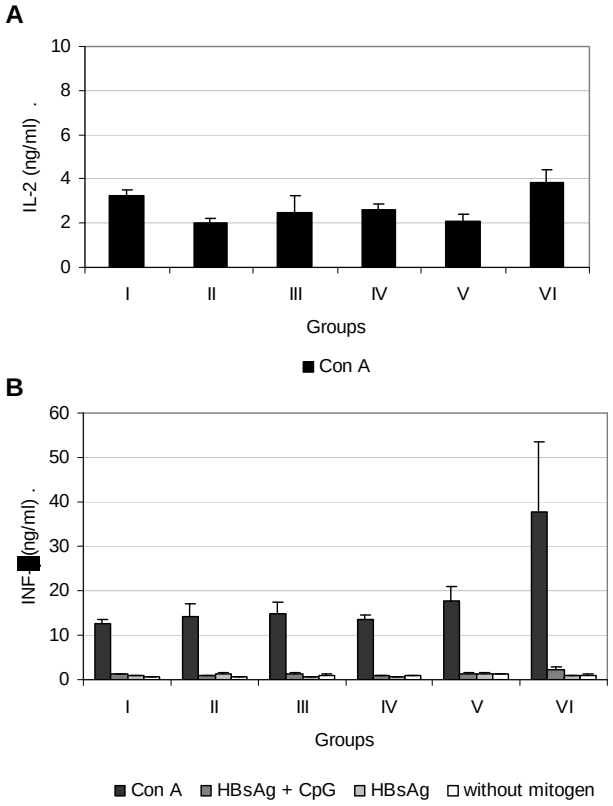
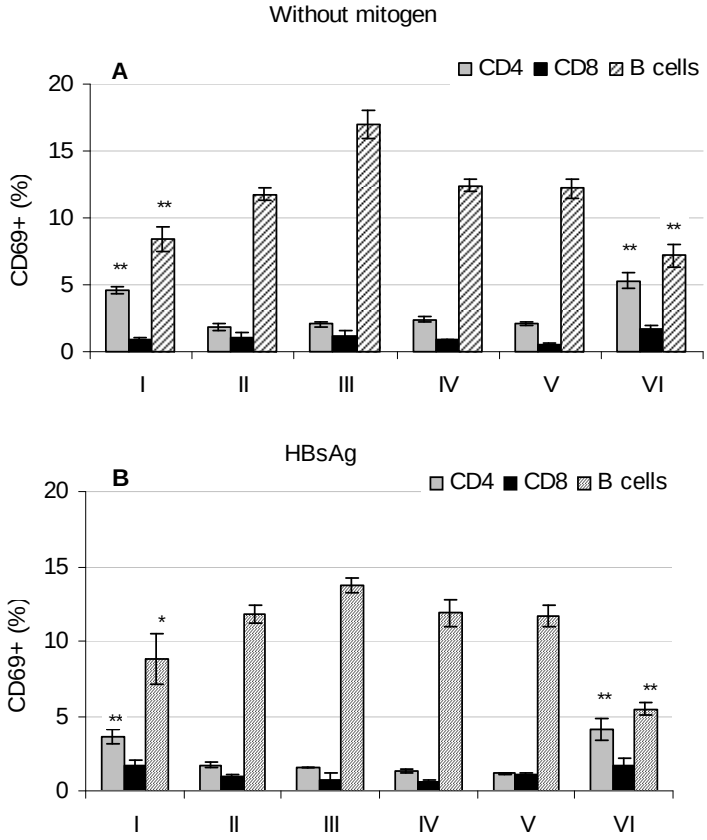


Figure 4 – Cytoquine production by splenocytes of individual mice from each treatment group: A) IL-2 secretion from splenocytes cultured for 24 hours in presence of con A; B) IFN-γ secretion from splenocytes cultured for 86 hours in the absence or in the presence of different mitogens (HBsAg; Con A; HBsAg + CpG ODN). Each bar corresponds to the group geometric mean plus the standard error of the mean (n between 3 and 6). The comparison between control group (III) and the different treatment groups (I, II, IV, V, VI) was made using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (** $p<0.01$ and *$p<0.05$).

5.3.4.4 Expression of the early activation marker CD69

CD69 is a type II membrane protein expressed as a homodimer of heavily glycosylated subunits. Both T and B cells begin to express CD69 within a few hours after stimulation, being recognized as an early activation marker antigen of lymphocytes. In the present study the expression of CD69 in splenocytes of individual mice was evaluated after 20 hours incubation with different mitogens, being the assay with Con A as the positive control of the experiment (figure 5D).



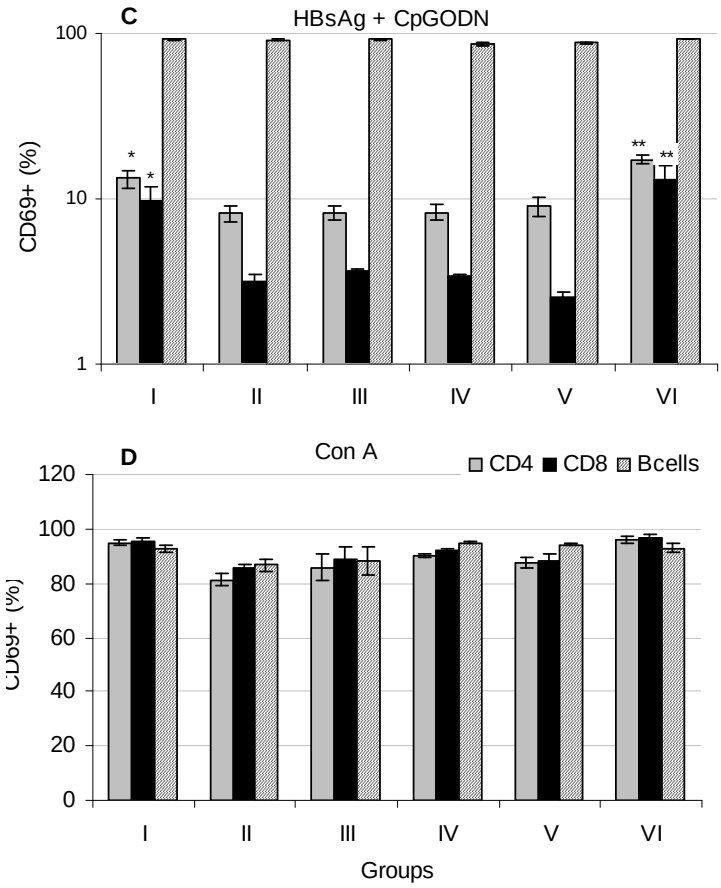


Figure 5 – Expression of the CD69 antigen on B and T splenocytes from different mouse groups in response to different *in vitro* stimulation. The stimulus experienced were the immunostimulators, CpGODN (histogram C) and Con A (positive control; histogram D), the antigen, HBsAg (histograms B and C) or without *in vitro* stimuli (histogram A). The values are means ± SEM of the percentage of the positive CD4 cells that express the CD69 antigen, obtained from individual mice of each group (n between 3 and 6). The comparison between control group (III) and the different treatment groups (I, II, IV, V, VI) was made with the one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (** $p<0.01$ and *$p<0.05$).

Cells from the spleen without in vitro stimulation, normally exhibit low levels of CD69 expression as demonstrated in figure 5A. In spite of that, significant differences in the CD69 expression on CD4+ T-lymphocytes between groups I ($p<0.01$) or VI ($p<0.01$) and the control group were observed. The splenocytes were collected 10 days after the boost and cultured for 20 hours without stimulation, which is considered as a long period

between the antigen exposure and the CD69 expression. These significant higher values were also observed with HBsAg (fig.5B) and HBsAg+CpG (fig. 5C) in vitro stimulated splenocytes. Despite of the lower CD69 expression levels observed on cells, (without in vitro stimulation the values were around 5% and 13-18%) the concordance of the three assays (figure 5A, B and C) gave consistency to the results observed for the groups I and VI. Moreover, in the same groups lower percentages of B lymphocytes CD69+ (8.4 and 7.1% for the groups I and VI respectively) were observed when compared with the control group (17%) (figure-5A and -5B). This result correlates well with the above described lymphoproliferative assay (see 3.4.1.), where the B lymphocytes showed lower capability to proliferate.

Finally, in the same in vitro study it was observed that the CD8+ T-lymphocytes from the groups I and VI, incubated in the presence of the HBsAg and CpG ODN, expressed significant higher percentage of CD69 molecule (9.7% and 12.9 % for the groups I and VI respectively) when compared with the control group (3.6%).

5.3.5 Humoral immune response

5.3.5.1 Systemic antibody response

Serum from the mice was collected before the first immunization and before and after the boost and analysed for the anti-HBsAg IgG. The results are shown in figure 6. With only one boost, two of three groups orally vaccinated with the antigen associated with coated nanoparticles were able to show seroconversion. In fact, the group I vaccinated with the HBsAg associated with coated nanoparticles showed a very low responder number (1/6) whereas the group VI, with the antigen and the adjuvant associated with the nanoparticles showed a better result (2/5). This last result was most probably related with the presence of the also encapsulated adjuvant.

IgG subclass titres were measured in responder mice before and after the boost (data not shown). Oral immunization with HBsAg loaded nanoparticles (group I) induced a Th2 humoral immune response profile (Ig1>Ig2a+Ig2b), however after the boost, the titres of Ig2a+Ig2b (Th1) were stronger, nevertheless lower than Ig1 (Th2). This mixed Th1/Th2 profile of response has been attributed to the HBsAg on its own [42]. On the other side, the oral immunization with the antigen plus the adjuvant loaded nanoparticles

(group VI) induced a Th1 profile humoral immune response, attributed to the presence of the CpG ODN [31]. This kind of immune response, normally associated with a strong immune cell response, has been claimed for the control of intracellular infections. This include viral infections, similar to infections with the hepatitis B virus [43].

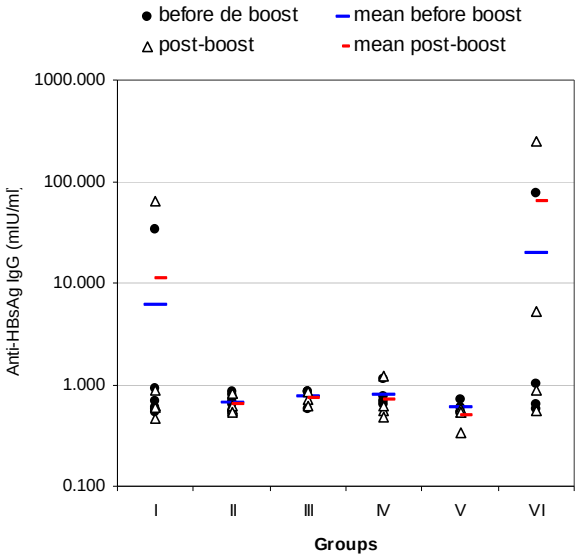


Figure 6 – Serum anti-HBsAg IgG titres of mice immunized with different oral formulations of hepatitis B vaccine. Values are expressed as antibody titres of individual mice taken before the boost and after the boost. Titres were defined as the highest plasma dilution resulting in an absorbance value twice that of nonimmune plasma (1 mIU/ml = mean + 2 SD of the control group).

5.3.5.2 Mucosal anti-HBsAg sIgA

The results of the determination of the IgA in gut washes are shown in figure 7. These results are presented as ratio between the amount of the specific IgA and the total IgA present in the washes of the gut of each mouse. No significant anti-HBsAg IgA was detected in the gut of mice immunized with the solutions of the antigen (groups IV and V). In contrast, detectable anti-HBsAg IgA was quantified in gut washes of some mice from groups I and VI (fig. 7).

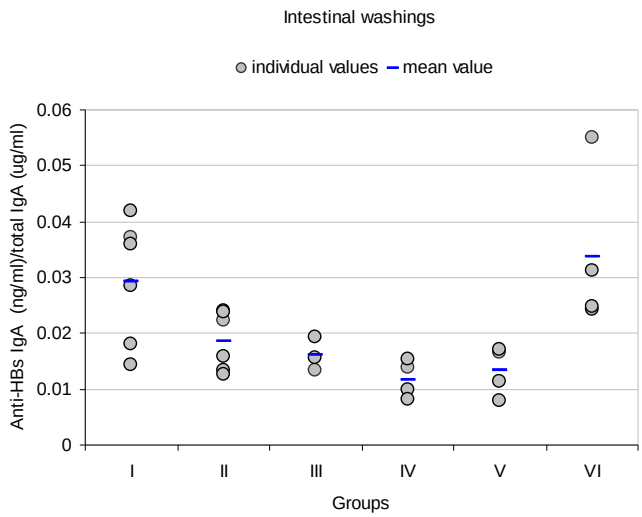


Figure 7 – Secretory anti-HBsAg sIgA profile detected in individual intestine washing samples of mice immunized with different hepatitis B vaccine oral formulations. Each circle represents the result of individual samples and the horizontal bar the mean of the group. Samples with the value equivalent to mean plus twice the SD of the control group (group III), were considered anti-HBsAg sIgA positive. The values were expressed as the ratio between the anti-HBsAg sIgA (ng/ml) and total sIgA (µg/ml)

5.4 DISCUSSION

It has been suggested that the response to orally administered antigens is initiated locally in the gut and then disseminated to the secondary lymph nodes and spleen, while other groups suggest a simultaneous activation of antigen-specific T cells throughout the animal after feeding the antigen [44]. Following these thoughts, it was investigated if a short time oral vaccination scheme with different formulations of hepatitis B antigen was able to induce not only mucosal but also a systemic (cellular and humoral) immune response. One of the parameters studied was the CD69 expression on the B and T-lymphocytes from the spleen. CD69 is a cell membrane receptor and has not been very often used in vaccination experiments. On the other hand, in a short time study, where an extended immune response is not expected, the investigation of an early activation marker may be useful to quickly evaluate different antigen formulations.

In the present study, the administration of the hepatitis B vaccine entrapped in alginate coated chitosan nanoparticles was investigated. In some cases (groups I and VI), those formulations were able to induce a cellular immune response, translated by the highest percentage of CD4+ T-lymphocytes expressing the CD69 and the lowest percentage of CD69+B cells. Moreover, in the same groups, the CD8+ T-lymphocytes have shown to be the most susceptible to CD69 induction when cultured in the presence of the antigen plus the adjuvant. Furthermore, the lymphocytes from those groups (I and VI) also have shown the highest capacity to proliferate and in some cases to produce IL-2 and IFN-γ. This cellular immune response was accompanied by the presence of IgG and IgA HBsAg specific in the serum and on the intestinal mucosa, respectively, however with the presence of non responder mice within the groups (I and VI). In the recent literature, the presence of non responder orally vaccinated mice is frequently reported, especially when the evaluation of the specific antibodies was performed after a single immunization. Moreover, to have a high percentage of seroconverted mice within the groups orally vaccinated, it has been always necessary to administer higher antigen concentrations with successive administration of the vaccine. Commercial injectable hepatitis B vaccines also have this drawback in humans [45]. It was estimated that the priming doses only induce detectable levels of antibodies in 70 to 90% of healthy infants, adolescents and adults. The final boost of the vaccine induces protective levels of anti-HBs antibodies in more than 95% [45] of infants and adolescents. In our study, the vaccination with a solution with 10 μg of the antigen (groups IV and V) did not induce cellular or humoral immune response and the results shown in this work allowed us to hypothesize that immunological tolerance might have been induced. In fact, oral tolerance has been one of the principal obstacles to different strategies designed for the oral administration of the vaccines and its induction seems to be related with the antigen dose [7, 46] and also most probably with the intrinsic properties of the antigen. Identical to our results obtained with a solution of the antigen were found in literature [33]. Even when the dose was increased to 100 μg, the vaccine given orally did not induce at all or in some cases only low anti-HBsAg IgG titers [34]. However, the association of CpG ODN [34] or of PLG microparticles [8] to the same high dose of the vaccine induced a systemic and mucosal immune response.

In order to obtain an immune response, the increase of the antigen dose used may not be sufficient to produce immunological acceptable results. Moreover, those high concentrations may not be economically attractive and hence different solutions have to

be explored. The development of efficient adjuvants for the mucosal route is one of the more promising approaches. One of the examples was recently described in the literature, the CpG ODN. A number of studies has shown that the CpG ODN is an effective immunomodulator molecule for parenteral [31, 47] and mucosal routes [34, 35, 48, 49]. CpG ODN has been shown to induce Th1 profile immune responses with a number of different antigens [31, 50]. The mechanism of adjuvant action of CpG ODN is not completely understood, but it is known that CpG ODN binds to the Toll-like receptor 9 (TLR-9) [51]. Therefore the immunostimulator effect is mediated only after its cell internalization [50]. On the other hand, the entrapment of CpG into nanoparticles with a size larger than a cell, may fail to stimulate immune cells, most probably because the nanoparticles cannot be internalised.

The physical proximity of the antigen and the adjuvant seems to be a requirement for an effective adjuvant effect [52]. This hypothesis could be the key, at least in part, of the lack of an early immune response observed in our study with the group of mice treated with the antigen associated with the nanoparticles and the adjuvant in solution (group II). The hepatitis B antigen antigen associated to the nanoparticles and the adjuvant in solution may have been taken up in different regions of the intestine. According to our own previous studies [29], alginate coated chitosan nanoparticles are able to be taken up by Peyer's patches and the CpG ODN in solution was most probably internalized by the enterocytes. On the contrary in the group VI, antigen and adjuvant, both associated to the nanoparticles, were most probably internalized simultaneously by M-cells of the Peyer's patches and this fact may explain the better results observed in this treatment group.

5.5 CONCLUSION

Alginate coated chitosan nanoparticles are able to entrap efficiently the hepatitis B recombinant vaccine and the CpG ODN used as an adjuvant.

Humoral and cellular immune response were better induced in mice vaccinated with the formulation where the antigen and the adjuvant appeared associated with the nanoparticles. The Th1 profile immune response induced by this formulation is crucial in preventing or overcoming hepatitis B infections, thus making this delivery system a

promising one. However further studies and in time prolonged with additional boosts have to be done in order to evaluate the appearance of desirable new responder mice. Also it has to be demonstrated whether a long lasting cellular and humoral immune response can be induced with this formulation using this challenging oral administration route.

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CHAPTER

6

IMMUNE RESPONSES BY NASAL DELIVERY OF HEPATITIS B SURFACE ANTIGEN AND CO-DELIVERY OF A CPG ODN IN ALGINATE COATED CHITOSAN NANOPARTICLES[5]

[5] *In preparation for publication*

ABSTRACT

Alginate coated chitosan nanoparticles were previously developed with the aim of protecting the antigen, adsorbed on the surface of those chitosan nanoparticles, from enzymatic degradation at mucosal surfaces. In this work, this new delivery system was loaded with the recombinant hepatitis B surface antigen (HBsAg) and applied to mice by the intranasal route. Adjuvant effect of the delivery system were studied by measuring anti-HBsAg IgG in serum, anti-HBsAg sIgA in feces extracts or nasal and vaginal secretions and interferon-γ production in supernatants of the spleen cells. The mice were primed with 10 μg of the vaccine associated or not with nanoparticles and associated or not with 10 μg CpG oligodeoxynucleotide (ODN) followed by two sequential boosts at three week intervals. The association of HBsAg with the alginate coated chitosan nanoparticles, administered intranasally to the mice, gave rise to the humoral mucosal immune response. Humoral systemic immune response was not induced by the HBsAg loaded nanoparticles alone. The generation of Th1-biased antigen-specific systemic antibodies, however, was observed when HBsAg loaded nanoparticles were applied together with a second adjuvant, the immunopotentiator, CpG ODN. Moreover, all intranasally vaccinated groups showed higher interferon-γ production when compared to naïve mice.

6.1 INTRODUCTION

A safe and effective vaccine to prevent infection with hepatitis B virus (HBV) has been available since 1982, and is recommended by the World Health Organization (WHO) for administration to all infants [1]. Therefore, it is not understandable that the number of chronically infected people continues to be a problem in many parts of the World, including Asia, Africa and the western Pacific region [2]. Some progress in the control of the disease in the western Pacific area were recently reported [1] after the inclusion of hepatitis B vaccine in every national immunization programme. Even though the price of the vaccine had dropped significantly in the last years, there were still some countries that could not afford these immunization programmes. Futhermore, the control of the

spread of the disease through vaccination of infants would undoubtedly prevent a large number of infections among adults, but it will take several decades to be achieved. The implementation of vaccination programmes, at least for the vaccination of high risk adults, like drug users, sex workers and health professionals, would certainly prevent a large number of infections [3].

Recently some questions have been raised concerning the hepatitis B vaccine currently on the market [4]. A significant proportion of healthy individuals (between 2% and 10%) have failed to respond to the currently licensed vaccine [5, 6]. Additionally, rare but serious pathological events have been described during the weeks following a hepatitis B vaccine injection campaign [7], nevertheless no relation between vaccination and the occurrence of the majority of these events has been shown. Moreover, some of these secondary effects have been correlated to the aluminium compounds, used as adjuvant. For example, these adjuvants are know to stimulate IgE antibody formation, which is not desirable in a normal process of immunization (reviewed in [8]). On the other hand, the intramuscular administration of the vaccine currently on the market, is not able to induce mucosal antibodies. The presence of antigen-specific sIgA at mucosal surfaces has been shown to help, when the transmission of the disease is through one of the mucosal routes, which is the case of the sexual transmission of HBV. To note that sexual transmission (either homosexual or heterosexual) in high-risk adults is the main mode of transmission in Europe and North America (regions of low HBV prevalence) [2]. On the other side, the induction of mucosal antibodies seemed to be facilitated if the antigens were administered by a mucosal route together with an appropriate adjuvant. Furthermore, the development of efficient mucosal adjuvants, which allow an easy and inexpensive mass vaccination, with more stable vaccines are urgently required.

Among the mucosal administration routes the nasal mucosa is the most attractive site for the delivery of vaccines. Compared with the more challenging oral vaccination, the nasal vaccination is more efficient at inducing secretory and systemic antibody responses (reviewed in [9]). For this reason, several attempts have been made regarding the intranasal vaccination with hepatitis B, in order to identify an effective mucosal adjuvant that elicits specific mucosal and systemic hepatitis B antibodies. Adjuvants such as recombinant cholera toxin B subunit [10], genetically modified adenovirus [11] expressing the hepatitis B surface antigen, attenuated Salmonella typhimurium [12] expressing the hepatitis B nucleocapsid, or Escherichia coli expressing the hepatitis B nucleoprotein antigen [13] have all been shown to be efficient as intranasal adjuvants.

However, safety concerns have predominantly been the cause for restricting the development and the use of these adjuvants in clinical studies. Synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN) are considered to be potent immunopotentiator adjuvants. It was demonstrated in mice that the intranasal delivery of HBsAg, which alone has no effect, elicits good immune responses when given in combination with CpG ODN [14-16]. Moreover, CpG is superior to cholera toxin (CT) for the induction of humoral and cell-mediated systemic immunity as well as mucosal immune responses (IgA) at local (lung) and distant (intestine) sites [14].

Particulate vaccine delivery systems loaded with HBsAg have also been evaluated by the intranasal route. Some examples already published include polymeric biodegradable poly(lactide-co-glycolide) (PLGA) microparticles [17], cationic particles (SMBV™) [18], cationic lipid emulsion carrying a DNA vaccine [19] and lipid microparticles [20].

Great attention has been paid to the cationic biodegradable polysaccharide chitosan. It is of low toxicity, has mucoadhesive [21] and immunostimulating properties, therefore showing great potential for being used in mucosal vaccines. In a previous study, we have characterized alginate coated chitosan nanoparticles as a novel delivery system [22, 23] designed for the application at mucosal surfaces. The evaluation of the immune response by the oral route has been performed, as well (in process of publication). The present work describes the evaluation of the immune response induced by the intranasal administration of the recombinant hepatitis B vaccine associated with the nanoparticulate delivery system. Additionally, the adjuvant effect of CpG ODN, dissolved in the suspension of the antigen or associated with the coated nanoparticles, was investigated.

6.2 MATERIALS AND METHODS

6.2.1 Materials

6.2.1.1 Polymers

Chitosan was purchased from Primex BioChemicals AS (Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation was 95% (titration method) and the viscosity was 8 cP (measured in 1% solution in 1% acetic acid). A low molecular weight, pharmaceutical grade, sodium alginate (MANUCOL LB®) was kindly donated by ISP Technologies Inc. (Surrey, UK). According to the provider's specifications, the typical values for the percentage of mannuronic and guluronic acid for Manucol LB were 61% and 39%, respectively, with an estimated molecular weight of 18 kDa. Both polymers meet the requirements described in the European Pharmacopoeia for use in pharmaceutical formulations.

6.2.1.2 Antigen, adjuvant and reagents

The hepatitis B surface antigen (HBsAg), (subtype ADW2) was kindly offered by GSK Biologicals (Rixensart, Belgium), Engerix B™ was from GlaxoSmithKline Biologicals (Rixensart, Belgium), Class B CpG ODN (1826) (5'-TCC ATG ACG TTC CTG ACG TT-3') was purchased from Coley Pharmaceutical Group (Ottawa, Canada). Concanavalin A (Con A), phenylmethanesulfonyl fluoride (PMSF) and avidin peroxidase conjugate were from Sigma Chemicals (St. Louis, USA). Certified fetal bovine serum (FBS) and L-glutamine (200 mM) were from Gibco (Invitrogen Co, Paisley, Scotland, UK), 1M HEPES buffer (0.85% NaCl), RPMI 1640 without L-glutamine and Pen-Strep (10,000 U penicillin/ml; 10,000 μg streptomycin/ml) were from Biowhitaker (Cambrex Bio Science, Verviers, Belgium). [Methyl-^{3}H] thymidine (1.0 mCi/ml) was obtained from Amersham Biosciences (UK), R-phycoerythrin (PE)-conjugated hamster anti-mouse CD69, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8 were obtained from BD Biosciences (Madrid, Spain). The FITC-conjugated goat anti-mouse IgM (anti-μ), the anti-mouse IFN-γ and biotin rat anti-mouse

IFN-γ was purchased from PharMingen (San Diego, CA, USA). The mouse IgA ELISA quantification kit was obtained from Bethyl Laboratories, (Montgomery, USA). All reagents used were of analytic grade. All solutions were prepared in ultrapure water.

6.2.2 Methods

6.2.2.1 Preparation of the coated nanoparticles

The preparation of the alginate coated chitosan nanoparticles was performed according to the method previously described [23]. Briefly, chitosan was dissolved at a concentration of 0.25% (w/v) in diluted acetic acid solution. The formation of the particles was achieved after the addition of 3.5 ml of sodium sulfate solution (10% w/v) to 200 ml of the chitosan solution. The resulting suspension was centrifuged for 30 minutes at 3500 rpm (2800 x g) and the supernatant was discarded. The particles were re-suspended in ultrapure water and centrifuged twice. Finally, they were frozen in liquid nitrogen and freeze-dried overnight using a Labconco freeze dry system (Labconco Corporation, Kansas, USA). The dry powder was kept frozen until further use.

The loading of the nanoparticles with HBsAg or with CpG ODN was performed by incubating a solution of HBsAg or the solution of CpG with a suspension of chitosan particles in phosphate buffer at pH 7.4, under mild agitation at room temperature for 120 minutes. The resulting suspensions with the composition of 0.05% (w/v) HBsAg and 0.5% (w/v) nanoparticles and the second with 0.05% (w/v) CpG and 0.5% (w/v) nanoparticles were used in the subsequent coating step. Alginate coated nanoparticles were obtained by mixing equal volumes of the loaded nanoparticles suspension and a buffer phosphate solution of sodium alginate (1% w/v) under magnetic stirring. The agitation was maintained for 20 min. The suspension was then centrifuged for 10 min at 1600 rpm and the supernatant was discarded. The particles were re-suspended in 0.262 mM $CaCl_2$ in 50 mM HEPES buffer solution, kept under agitation for another 10 minutes and immediately administered to the mice.

6.2.2.2 Evaluation of the loading efficacy of HBsAg and CpG ODN in coated nanoparticles.

The loading efficacy of the coated particles was calculated in an indirect way, quantifying the antigen that remained in solution as described before [22]. After the coating with alginate, an aliquot of the particle suspension was centrifuged at 14,000 rpm for 15 minutes and the protein concentration in the supernatant was quantified by micro-BCA-protein assay (PIERCE, Rockford, USA) using a microplate reader with a 570 nm filter.

For the CpGODN the same procedure was followed and the oligodeoxynucleotide concentration quantified by measuring the OD of the supernatants at 260 nm. To eliminate background interference, the supernatant of unloaded particles was processed by the same way. The results refer to the nanoparticle batches used in the vaccination studies.

The loading efficacy (LE) and the loading capacity (LC) were calculated from the following equations:

Eq. 1 - LE (%) = (Total amount of HBsAg or CpG – free HBsAg or CpG)/Total amount of HBsAg or CpG*100

Eq.2 - LC (μg of HBsAg or CpG/mg chitosan nanoparticles dry weight) = (Total amount of HBsAg or CpG – free HBsAg or CpG)/ mg chitosan nanoparticles dry weight.

6.2.2.3 Immunization studies

6.2.2.3.1 Animals:

Seven week-old female BALB/cAnNHsd mice were used (Harlan Iberica, Barcelona, Spain) with four or six mice per group. Animals were housed for acclimatization one week before the experiments at the animal resource facilities of the Faculty of Pharmacy at University of Porto. Animal care, handling and immunization protocols were in accordance with institutional ethical guidelines. They had free access to food and water, with 12h light/dark cycle.

6.2.2.3.2 Treatment groups

Group I - suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg
Group II - suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg plus 10 μg of the adjuvant (CpG ODN) in solution.
Group III - suspension of the mixture of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg and the same particles loaded with 10 μg CpG ODN
Group IV - solution with 10 μg HBsAg
Group V - solution with 10 μg HBsAg and 10 μg CpG ODN
Group VI - untreated or negative control
Group VII – subcutaneous administration of Engerix B (1 μg HBsAg) (positive control)

6.2.2.3.3 Immunization schedule

The primary immunization was followed by two boosts with three week interval, between each immunizations and the mice were sacrificed 4 weeks after the last boost. To evoke an immune response a total volume of 15 μl (7.5 μl in each nostril) of the formulations were administered. The non-anesthetized mice were maintained in supine position and the formulation was deposited in the nasal cavity with the aid of a micropipette with a 10 μl tip. The mice were kept in this position for another 5 min to allow optimal spreading of the formulations on the nasal mucosa.

6.2.2.3.4 Sample collection

Blood samples were taken from the orbital sinus before each boost and by cardiac puncture at the end of the experiment. The sera were prepared by centrifugation and stored at -20ºC until analysis.

Vaginal secretions were collected at the end and 4 days before the end of the experiment, by rinsing with 150 μl of a cold PBS (containing 0.1% bovine serum albumin (BSA)) through the vagina. The 1% BSA-PBS solution was introduced into the vaginal tract of non-anesthetized mice using a Gilson pipette. These 150 μl aliquots were withdrawn and reintroduced six times.

Nasal secretions were collected just at the end of the experiment, by washing the nasal cavity with 200 μl of cold PBS (containing 1% BSA). The washing solution was

forced to pass throughout the trachea of the sacrificed mice and collected from the nostrils side.

The extracts were vortexed and treated with sodium azide and phenylmethyl sulfonyl fluoride (PMSF), allowed to stay at room temperature for at least 15 minutes and then centrifuged (6,000 rpm/15 min/4ºC). The clear supernatants (containing 0.1% sodium azide, 0.1% BSA and 1 mM PMSF) were then stored at –80ºC until tested by ELISA for secretory antibody (sIgA) levels.

Fecal pellets (4-8) were collected four days before the end of the experiment into Eppendorf tubes. The pellets (0.2g/ml) were suspended in PBS (containing 0.1% sodium azide, 0.1% BSA and 1 mM PMSF), vortexed and allowed to rest at room temperature for 1 hour. Solid matter was separated by centrifugation at 14,000 rpm for 15 minutes. The clear supernatants were frozen at -80ºC before being used in ELISA to determine antigen-specific and total IgA.

6.2.2.3.5 Enzyme-linked immunosorbent assays (ELISA) for HBsAg specific immunoglobulins

Ninety-six-well flat-bottomed microtiter plates (Nunc immunoplate maxisorb) were previously coated with the recombinant HBsAg (1 μg/well) in coating buffer (50 mM sodium carbonate, pH 9.6) by overnight incubation at 4℃. The plates were washed 5 times with PBS-T (PBS containing 0.05% Tween-20) and blocked with 3% BSA in PBS-T (200μl/well) for 1 hour at 37ºC. The plates were then washed 5 times with PBS-T and the serial dilutions of each serum (100 μl/well) from the individual mice were tested in triplicate, starting from a 1:100 dilution in PBS-T. The serum was incubated for 2 hours at 37ºC and after washing the plates with PBS-T, they were incubated for an additional 30 min at 37ºC with peroxidase-labeled goat anti-mouse immunoglobulin G and isotypes (anti-IgG1, anti-IgG2a). The bound antibodies were revealed by adding 100 μl/well of 0.5 mg/ml of o-phenylenediamine dihydrochloride (OPD) (Sigma, Spain) in 10 ml of citrate buffer with 10 μl of 30% H_2O_2. The reaction was stopped after 10 minutes with 50 μl of 3M HCl to each well. The absorbance was read out at 492 nm in an automatic ELISA reader (Easy Reader 400, SLT-LABINSTRUMENTS). Elisa titers were expressed as mIU/ml and 1 mIU is the OD mean of the pre-immune serum plus 2 times the standard deviation.

The measurement of IgA was carried out using a mouse IgA ELISA quantification kit (Bethyl Laboratories, Montgomery, Texas, USA) as described by the manufacturer. In order to measure the sIgA levels in the gut, nasal and vaginal secretions, total sIgA and the specific anti-HBs sIgA were determined in the extracts. The results are presented as the anti-HBsAg IgA/total IgA. By this way variations between samples related with the extraction process or stability of the sIgA were minimized.

The standard IgA was diluted to appropriate concentrations in PBS with 1% BSA to create a calibration curve. The extracts were diluted in PBS-T with 1% BSA and added to the plates in series of two-fold dilutions. The concentrations of the total and specific IgA were determined from the calibration curve generated for each set of samples using a four parameter logistic curve-fit generated by SigmaPlot software (version 8.0, SPSS Inc).

6.2.2.3.6 Preparation of spleen cell suspensions

The mice were euthanized by cervical dislocation and their spleens were aseptically removed. Individual spleen cell suspensions were prepared in a Petri dish using curved needles and washed twice with RPMI 1640. The final suspension was adjusted to a final concentration of $1x10^7$ cells /ml in complete RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) glutamine, 1% (v/v) Pen-Strep and 2% (v/v) 1M HEPES buffer.

6.2.2.3.7 Spleen cell populations

The cells (10^6) were washed twice with cold PBS supplemented with 2% FBS and then incubated in the dark for 30 minutes at 4ºC with saturated concentrations of FITC-conjugated rat anti-mouse CD4 or FITC-conjugated rat anti-mouse CD8 or FITC-conjugated goat anti-mouse IgM (anti-µ). After incubation, cells were washed three times with PBS-2% FBS and then re-suspended in 500 µl PBS-2% FBS. To exclude dead cells, 2.5 µl propidium iodide (50 µg/ml) were added just before data acquisition. At least 10,000 events were analysed by flow cytometric acquisition, performed in a fluorescent activated cell sorter (FACS Calibur) (BD, Biosciences, Madrid, Spain). Data were analysed by CellQuest software (BD, Biosciences, Madrid, Spain).

6.2.2.3.8 Splenocyte cell culture in the presence of the mitogens

Using sterile 96-well flat-bottomed tissue culture plates, 25 μl of splenocyte suspension ($1x10^7$ cells/ ml) from each mouse were plated in triplicate along with 25 μl of a complete RPMI solution of the mitogen [Con A (50 μg/ml), CpG ODN (50 μg/ml) plus HBsAg (16 μg/ml), HBsAg alone (16 μg/ml) or without mitogen (control)]. Finally the volume of the well was completed to 200 μl with complete RPMI and incubated at 37ºC with 95% relative humidity and in the presence of 5% CO_2.

6.2.2.3.9 Interferon-γ (IFN-γ) production by splenocytes

Spleen cell suspensions were plated with the mitogens (see 2.2.3.8) and incubated in a humidified 5% CO_2 incubator for 96 h at 37ºC. The plates were centrifuged and the clear supernatants stored at –80ºC until analysis of the IFN-γ by ELISA technique, following a procedure described elsewhere [24].

6.2.2.3.10 Lymphoproliferation assay

Splenocytes were obtained and cultured together with the mitogens in a flat-bottomed 96-well plate as described before (see 2.2.3.8). The cells were cultured for 96 hours at 37ºC and on the last 8 h of incubation each well was pulsed with 1μCi of [methyl-^{3}H] thymidine. These 96 well plates with the cells were stored at -20ºC until further analysis.

The cells were later thawed and harvested onto a fiberglass filter (filter mats, molecular devices, Skatron, Lier, Norway) using a semiautomatic cell harvester (Scatron Instruments, USA) and DNA thymidine incorporation was determined by standard liquid scintillation techniques with a Beckman LS 6500 scintillation counter (Beckman Coulter Inc, Fullerton, USA). Thymidine incorporation was expressed as counts per minute (cpm).

6.2.3 Statistical analysis

Data were presented as the mean ± S.E.M. for at least three experiments and statistical significance was assessed using one-way analysis of variance (ANOVA) followed by

Dunnett's post test using the Prism 4 (GraphPad software, Ca, USA). Differences were considered significant when $p<0.05$.

6.3 RESULTS

6.3.1 Characterization of the vaccine delivery system

In a recent publication of our group, the development of alginate coated chitosan nanoparticles and the characterization of this new delivery system were reported [23]. Briefly, before coating with sodium alginate, the chitosan nanoparticles with a mean diameter of 643 nm (dynamic light scattering technique) were positively charged (+37 mV). Scanning electron microscopy (SEM) images of the uncoated particles also revealed the presence of small nanoparticles (around 100 nm) but demonstrated that the majority of the coated particles were in a range between 300 and 600 nm. More recently, we showed the results of the release studies, choosing ovalbumin as a model vaccine [22]. Ovalbumin release studies from coated and uncoated chitosan nanoparticles, performed in several buffers at different pH values, allowed us to conclude that the coating with sodium alginate of the ovalbumin loaded chitosan nanoparticles avoided an ovalbumin burst release observed with uncoated chitosan nanoparticles at pH 5.5; 6.8 and 7.4 (phosphate buffer) within the first 30 minutes of incubation.

6.3.2 CpG ODN and hepatitis B antigen entrapment in coated nanoparticles

Different ratios of hepatitis B vaccine to chitosan nanoparticles and CpG ODN to chitosan nanoparticles were investigated (data not shown) and the systems with the highest loading efficacy were used for further studies. It was shown that hepatitis B antigen and CpG ODN were efficiently associated with alginate coated chitosan nanoparticles. The loading efficacy of hepatitis B vaccine in the coated nanoparticles was 84.1 ± 3.0 % (mean ± STDEV) and the mean of the loading capacity was 83.9 µg of HBsAg/ mg of dry chitosan nanoparticles ± 2.77 (STDEV). The loading efficacy of CpG

was 97.0 ± 1.3% and the loading capacity was 97.0 ± 0.03 (μg of CpG ODN/ mg of dry chitosan nanoparticles).

6.3.3 Cellular immune responses after intranasal administration of HBsAg loaded nanoparticles.

The percentage of T- and B-lymphocytes was determined in order to examine the possible influence of the HBsAg formulations on the cell populations of the mouse spleen. Figure 1A shows the results of the FACS analysis performed with freshly prepared spleen cell suspensions, using specific mAb against cell surface markers. The percentages of CD8+ and CD4+ T-lymphocytes subpopulations in different groups were shown not to have been significantly different from the control group ($p>0.05$). The group I vaccinated with the HBsAg associated with the coated nanoparticles was an exception and was shown to have a significantly lower percentage ($p<0.05$) of CD8+ T-cells (0.7-fold decrease compared to the control mice), whereas no significant difference in the ratio of CD4+/CD8+ T-cells (Fig. 1B) was observed in the same group when compared with the control group. A slight, but statistically significant difference ($p<0.01$) in the decrease of B cells was observed in the groups vaccinated without nanoparticles (groups IV, V and VII).

We also tested whether the spleen lymphocytes were susceptible to proliferation when cultured in the presence of the antigen or the antigen plus the adjuvant. The comparison between the results obtained with cells cultured without mitogens (negative control of the experiment) (Fig. 2A) and the results of cells incubated only with the HBsAg (Fig. 2B) allowed us to conclude that in general the presence of the antigen in the cell culture proved the ability to induce some division of the cells. However in the concentration used, the stimulation appeared to be non-specific and, in general, the results shown appeared just amplified, when compared with the results without any stimulus. In most cases, the different groups were shown not to be different from the control group. However, two exceptions with a higher mean value ($p<0.01$) were observed: the group vaccinated with the antigen in solution and the group vaccinated with a solution of the antigen in the presence of the adjuvant (groups IV and V). Nonetheless, due to high variability of the individual values within the groups, these

results should not be overestimated. A similar situation was observed for the cells cultured with antigen in the presence of CpG ODN (Fig. 2C).

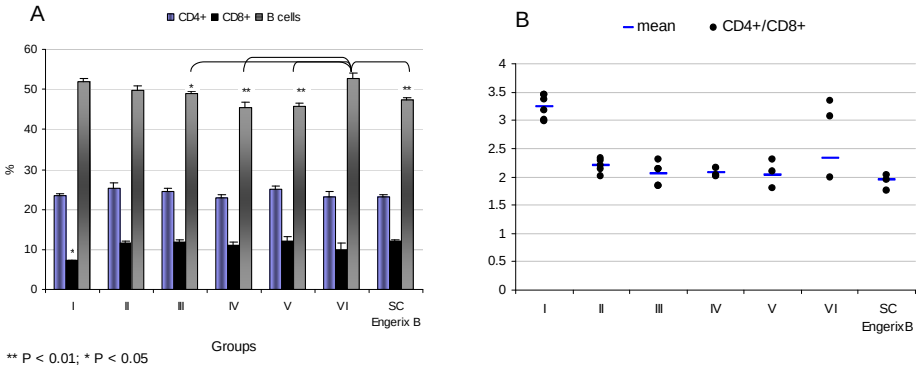

Figure 1 – A) - Percentage of T-lymphocytes CD4+, CD8+ and B+ Lymphocytes in the spleens of mice from groups (I, II, III, IV, V, VI and VII) by FACS analysis. Each bar corresponds to the group geometric mean plus the standard error of the mean. The comparison between the control group (VI) and the different treatment groups (I, II, III, IV, V, VII) was made with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (** p<0.01 and *p<0.05). B) – T-lymphocytes quota between percentage of CD4+ cells and CD8+ cells from individual mouse spleen of each group.

Finally, figure 2D shows the lymphoproliferative results observed in the presence of Con A, a classical mitogen, which served as a positive recall antigen. In this case all the groups vaccinated intranasally showed significantly ($p< 0.01$) lower values. In addition, the group vaccinated with a commercial formulation by subcutaneous injection showed a higher mean value ($p< 0.01$) when compared to the control (group VI).

The quantification of the interferon-γ (IFN-γ) in the supernatants of the different group splenocytes, cultured under the same conditions is shown in figure 3. A direct observation of the results allowed us to conclude that the splenocytes of all the groups vaccinated produced IFN-γ. Moreover, the statistical analysis showed that the amount produced is significantly higher than the amount produced by the cells from the control group in all the groups.

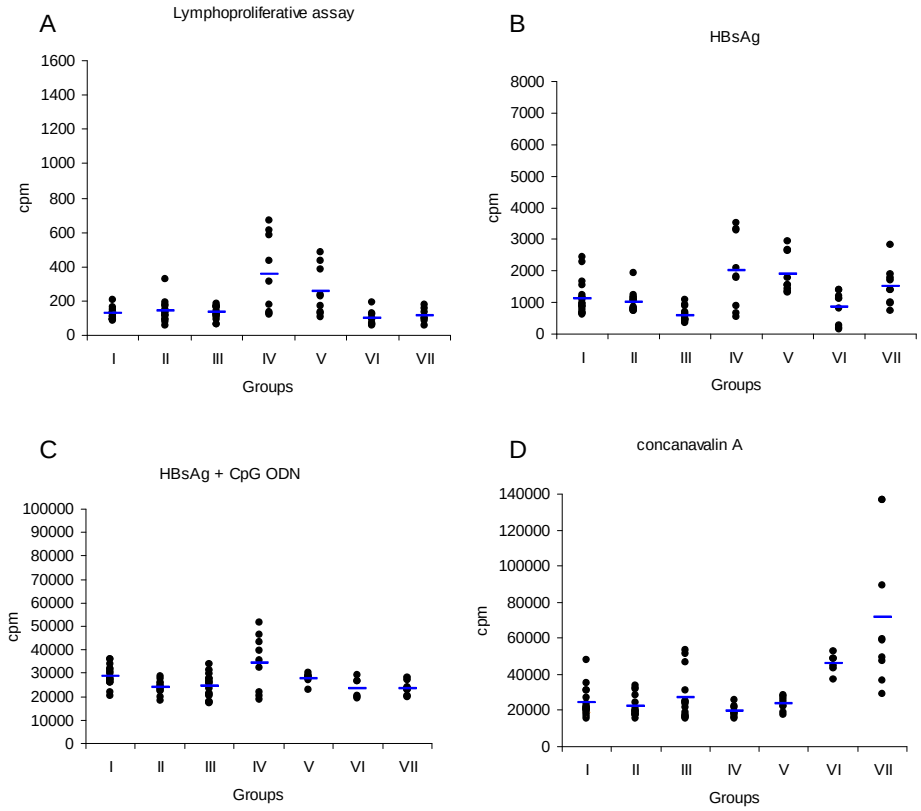

Figure 2 – Lymphoproliferative response after intranasal administration of the different hepatitis B vaccine formulations. *In vitro* proliferation of individual mouse splenocytes during a 96 hour period stimulated with: A) - without stimulus. B) – HBsAg. C) – HBsAg + CpG ODN, D) Con A. Each circle represents the result of individual samples and the horizontal bar the mean of the group. The results correspond to the thymidine incorporation and are expressed as counts per minute (cpm).

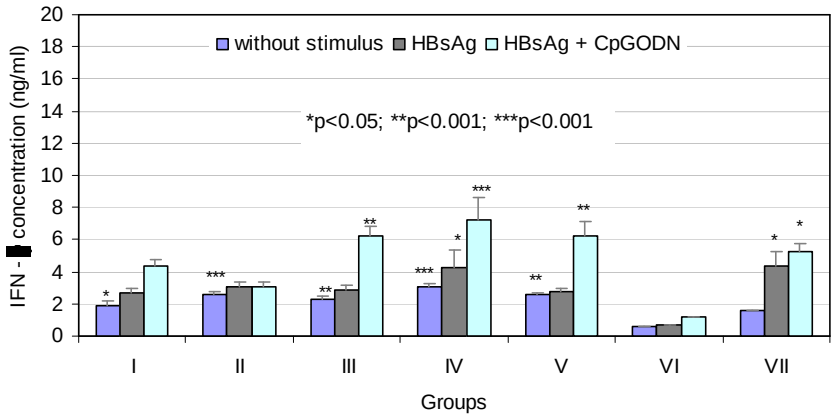

Figure 3- IFN-γ production by splenocytes of individual mice from each treatment group (Groups I-VII). Each bar corresponds to the group geometric mean plus the standard error of the mean (n between 3 and 6). The comparison between the control group (VI) and the different treatment groups (I, II, III, IV, V, VII) was made with a one-way analysis of variance (ANOVA) followed Bonferroni's test.

6.3.4 Humoral immune response to the intranasal administration of HBsAg nanoparticles.

6.3.4.1 Systemic immune response

The positive control group of the experiment received a subcutaneous injection with 1 µg of HBsAg in the presence of aluminium adjuvant (commercial formulation). The highest concentrations of the anti-HBsAg IgG were observed within this group (Fig. 4A). The presence of HBs specific IgG was also observed in some mice within the groups nasally vaccinated with the antigen and in the presence of CpG ODN. The groups I and IV (absence of CpG ODN) did not induce detectable anti-HBs IgG antibodies in the plasma of any mice. Therefore the presence of a strong mucosal adjuvant seemed to be necessary to induce a systemic humoral immune response in mice vaccinated with 10 µg of the HBsAg. On the other hand, the results also showed that within the nasally vaccinated groups the highest IgG titers were found in the groups where the adjuvant was not associated with the nanoparticles (groups II and V).

The anti-HBsAg IgG subclasses present in the responder mice were also evaluated in order to examine the induced Th profile as shown in figure 4B. In the seroconverted nasally vaccinated mice the detected antibodies were predominantly type IgG2 (Th1–like), whereas with the commercial formulation administered subcutaneously, the responses were predominantly Th2 (IgG1>IgG2a).

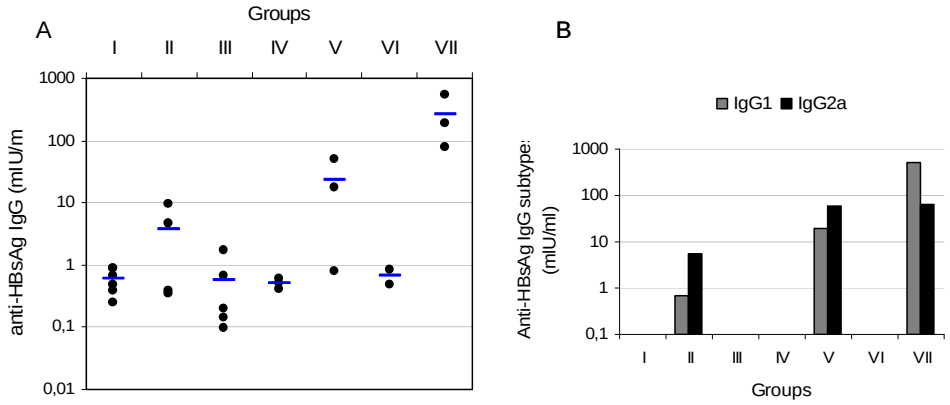

Figure 4 – A) - Serum anti-HBsAg IgG titers of mice nasally immunized with different formulations of hepatitis B vaccine. Values are expressed as antibody titers of individual mice taken at the end of the experiment. The horizontal bar is the mean of the group. B) - Serum anti-HBsAg IgG1 and IgG2a titers of mice nasally immunized with different formulations of hepatitis B vaccine. The bar corresponds to mean titer of responder mice in each group. Titers were defined as the highest plasma dilution resulting in an absorbance value twice that of nonimmune plasma (1 mIU/ml = mean + 2 SD of the control group).

6.3.4.2 Mucosal immune response

Nasal and vaginal washings and the collection of fresh feces were performed at the end of the experiment in order to evaluate the presence of HBsAg specific antibodies (sIgA) on the mucosal surfaces. Detection of sIgA in the nasal washings was possible in almost all the groups nasally vaccinated (Fig. 5A). The exception was group IV vaccinated with a solution of the antigen for which no specific sIgA could be detected. Similarly, it was not possible within this group to detect antibodies in the vaginal washings (Fig. 5B) or in the feces (Fig. 5C), where only one mouse presented a very small amount of the HBsAg-specific sIgA. The entrapment of the hepatitis B antigen into the coated nanoparticles (group I) showed to improved the vaccine inducer ability for the generation of specific

mucosal antibodies. This formulation induced the generation of anti-HBsAg sIgA not only at the nasal mucosa (4/6) (Fig. 5A) but also in vaginal secretions (3/6) (Fig. 5B). The application of the suspension of the nanoparticles associated with both the antigen and the adjuvant (group III) yielded similar results to group I. Group II differs from the group III because in this group the CpG is not associated with the coated nanoparticles. In this case not only the number of responder mice increased (4/5 nasal secretions, 4/5 vaginal secretions) but also the relative amount of the specific antibodies detected was much higher (Fig. 5A and B).

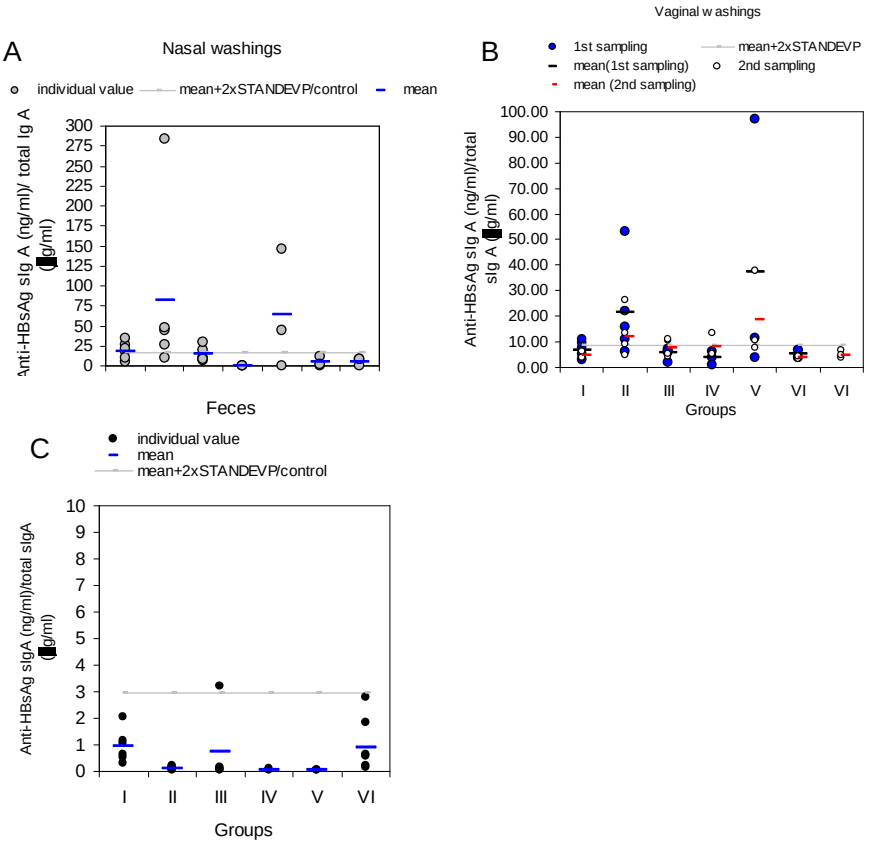

Figure 5 – Secretory anti-HBsAg sIgA profile detected in individual mice samples of immunized mice with different hepatitis B vaccine oral formulations. Each circle represents the result of individual samples and the horizontal bar the mean of the group. A) – nasal washings. B) – vaginal washings. C) – feces. Samples with the value equivalent to mean plus twice the SD of the control group (Group III), were considered anti-HBsAg sIgA positive. The values were expressed as the ratio between the anti-HBsAg sIgA (ng/ml) and total sIgA (mg/ml)

A similar and anticipated result was obtained with the formulation in which the antigen and the adjuvant were not associated with the coated nanoparticles (group VI). In a comparable study performed by McCluskie [25], the intranasal immunization of mice with the same doses of the HBsAg plus the CpG ODN in a solution, also induced the generation of both systemic and mucosal antibodies. Anti-HBsAg sIgA in the lung, gut, saliva, vaginal secretions and in feces could be determined by the same researchers.

Finally, as predictable, the subcutaneous injection of the commercial formulation did not produce any detectable mucosal antibodies in nasal washings extracts.

6.4 DISCUSSION

In several publications it has been convincingly demonstrated that the subcutaneous (sc) or the intramuscular (im) routes of vaccine application are not effective to induce antigen specific mucosal antibodies. In the present work, it was possible to confirm once more that the sc vaccination with a commercial formulation of the hepatitis B (group VII), was unable to induce the generation of detectable sIgA in both, vaginal and nasal secretorial fluids. The induction of mucosal antibodies, with few exceptions described elsewhere for hormonally induced immunomodulation after intramuscular administration of HBsAg [26], seems to be only feasible when a mucosal route of vaccination was chosen and the HBs antigen was associated with an appropriate adjuvant. On the other hand, the importance of inducing mucosal antibodies after a immunization process has been well emphasized in the last few years [27-29]. This aspect is even more relevant for those infections that start their deleterious effects on the mucosal surfaces. In these cases, the infection can be resolved before the causative agent enters the blood. The main protective mechanism is the generation of a local secretory immune response, with secretory IgA (sIgA) antibodies as the primary effectors molecules, but additional humoral and cell-mediated mucosal protective mechanism have also been identified [27, 30].

It is well known that one of the more important modes of hepatitis B virus transmission is the sexual contact with an infected person [31]. Therefore, the induction of the anti-HBsAg sIgA at the urogenital surface would be of great benefit. In the present work, the intranasal administration of a saline solution with 10 μg of HBsAg without any adjuvant (group IV) was not able to induce the generation of mucosal or systemic

antibodies. Similar results were also observed by other groups [10, 14, 18]. Therefore, all the evidences show that an adjuvant is required for the intranasal route, in order to obtain a HBV protective immune response. It has been reported that alum (insoluble aluminium salts), the unique adjuvant approved by the US Food and Drug Administration, is ineffective for the induction of mucosal immunity [32]. Consequently, the investigation of novel, non- toxic adjuvants for the expression of suitable antigens at mucosal surfaces is necessary. In this work, the evaluation of the immune response followed by the intranasal vaccination with the HBs antigen associated to the alginate coated chitosan nanoparticles was evaluated for the first time. This delivery system proved to efficiently encapsulate the HBs antigen and in preliminary studies [22] also showed the capability for internalization by intestinal Peyer's patches through specialized M-cells. We did not study the uptake of the alginate coated chitosan nanoparticles into the epithelium of nasopharyngeal lymphoid tissue (NALT). However, it was demonstrated by Y. Fujimura [33, 34] in an electron microscopic study that the M cells of human NALT are ultrastructurally similar to those in Peyer's paches and colonic lymphoid follicules. On the basis of these facts, we assumed that an identical alginate coated chitosan particle uptake in the NALT would occur. Moreover, to support this hypothesis, the uptake of chitosan nanoparticles into the epithelium of human [35] and trimethyl-chitosan nanoparticles into mice NALT [36] has already been demonstrated. Equally important, in a study performed by Tafaghodi [37], alginate microspheres showed to have a clearance rate in the human nose similar to PLGA microspheres and lower than sephadex microspheres, and were therefore considered as a suitable nasal delivery system. The alginate coating of the chitosan nanoparticles described here, may show a similar behaviour as solid alginate particles on mucosal surfaces, at least during the initial contact with mucosal surfaces.

In the present work, the HBV antigen associated to the chitosan core in alginate coated chitosan nanoparticles was able to induce the generation of mucosal antibodies with strong relevance for nasal secretions. This formulation also induced to some extent a cellular immune response; however the highly required systemic immune response was not induced. The difference between these results and the ones with the solution of the antigen, proved in an indirect way the superior capacity of the chitosan particles in retaining the antigen in the nose cavity. On the other hand, the systemic non-responsiveness in this group could be also explained by a mucosally induced tolerance [9]. This is merely a hypothesis since cellular and molecular contributions of the

immunocompetent cells present in NALT to the generation of tolerance are unknown [9]. However, if this kind of immune response (only mucosal antibodies) would suffice to protect human recipients from a HB infection, especially against transmission by the urogenital tract, it should be further investigated.

Anti-HBsAg IgG antibodies were detected only in the serum of mice immunized with formulations containing a class B CpG ODN (1826). It has been proven that this adjuvant is a potent immunopotentiator and can not be compared with an antigen delivery system the mechanism of which seems to be only related with its capacity of slow release the antigen or to facilitate the antigen internalisation into antigen-presenting cells (APCs) [32]. Conversely, there are strong indications that responses to CpG are dependent on the presence of TLR9 in the cells [38-40]. After interaction with this receptor, the activation of cell signalling pathways occurs, which results in a cascade of immune events [41, 42]. In the present study, the best results were found within the groups vaccinated with the antigen associated with the nanoparticles plus the adjuvant in solution (group II) and in group V, vaccinated with a solution of the antigen plus the adjuvant, being group V with the higher titers. One of the hypothesis to explain this result could be related with the uptake mechanism of the antigen and the adjuvant in the NALT. In the group II, the HBsAg associated with nanoparticles may probably have been retained in the NALT during a period that was probably more prolonged than the time period of retention of the adjuvant or the free antigen. This fact may cause a lower amount of the free antigen in close proximity of the adjuvant when compared with the situation observed in the group V, vaccinated with a solution of the antigen and the adjuvant not associated with the nanoparticles. The proximity between the HBV antigen and the CpG ODN has been demonstrated to be an important condition for the intensity of the antigen specific immune response [43-45]. Some examples were reported in the literature, e.g., the adjuvant activity of a non-coding plasmid was demonstrated only when the plasmid and the HBsAg were co-entrapped in the same liposomes, but not in separate vesicles [45]. Interestingly enough, we have observed the same effect: in fact with group III, where both antigen and adjuvant were associated with separate nanoparticles and the result obtained was similar to the result obtained with group I (i.e. the group without CpG ODN). The results indicated that the association of the CpG ODN to separate chitosan nanoparticles was not a good strategy for the intranasal administration. Further studies are needed to evaluate if the co-encapsulation of the antigen and the adjuvant in the same nanoparticle would produce a better immune

response. On the other hand, the CpG ODN used in this vaccination study is synthesized with a phosphorothioate (PS) backbone and hence is resistant to nuclease degradation. Consequently, the use of this CpG for intranasal vaccination may need no protection. However, the second mechanism by which the nanoparticles may improve the efficacy of the CpG ODN is by a depot effect, which may result in an extended period during which, both, antigen and CpG ODN are available in the application site, therefore increasing their probability for being internalized by the NALT cells [39]. Therefore in this case the encapsulation may be useful, however the poor immunogenic response observed in the group III, led us to hypothesise that the free amount of the CpG ODN was not high enough to stimulate the immune system to respond to the HBV antigen. Therefore, the in vivo CpG ODN amount released from the coated nanoparticles was most probably, inadequate. This phenomenon could be associated with a strong affinity of the CpG ODN's to the cationic chitosan [46, 47]. This hypothesis was addressed in different studies where a poor transfection by chitosan-DNA nanoparticles was observed [48, 49] and dependent on several factors, including the degree of deacetylation and molecular weight of the chitosan, and the chitosan/plasmid charge ratio conferred, essentially by amino amino groups (chitosan) and phosphate groups (DNA) [50, 51].

Nevertheless, these results are still not fully conclusive with regard to the advantages of the association of the CpG ODN to nanoparticles. As referred above, theoretically, suitable nanoparticles may increase the residence time in the NALT tissue and facilitate the internalization of the CpG into the mucosal lymphoid tissues. This would decrease the amount of the immunopotentiator needed for eliciting the desirable adjuvant effect. Once inside the target tissue, the ideal delivery system should release the CpG ODN, while most cell types have the capacity to take up CpG ODN via endocytosis [52] or the CpG ODN internalization in cells would be facilitated by the use of CpG associated to suitable nanoparticles.

Several studies in the literature have shown that chitosan is a biopolymer with characteristics for high loading efficacy of macromolecules like, peptides, plasmid vectors, DNA and vaccines. Promising results of intranasal vaccination using Bordetella bronchiseptica [53], meningococal C conjugate vaccine with mucosal adjuvant LTK63 mutant [54], diphtheria [55, 56] and influenza antigens [57] all associated with chitosan particles or just with chitosan powder have been demonstrated in animal models. More recently, the nasal vaccination in human volunteers by simple syringe insufflations of Menjugate-C associated with chitosan produced geometric mean titers of serum

bactericidal antibody comparable to parenteral immunization. More importantly, the sIgA antibodies were detected in nasal washings [58]. Compared with the above cited vaccines, recombinant hepatitis B surface antigen is considered to be a weaker antigen. Therefore, chitosan based formulations need to be improved, in terms of a delivery system for the simultaneous intranasal administration of the vaccine and an immunopotentiator yielding synergistic effects.

6.5 CONCLUSION

The association of the HBsAg with alginate coated chitosan nanoparticles, administered nasally to mice, gave rise to humoral mucosal immune responses, which was not induced by the HBsAg alone. The generation of systemic, predominantly Th1-type antibodies was observed when the HBV antigen entrapped in the particles was administered simultaneously with the adjuvant, CpG ODN, in solution. Future work will focus in the improvement of the chitosan nanoparticles in order to better control the in vivo CpG ODN release from the particles and the co-entrapment of the antigen and the adjuvant, in order to elicit not only mucosal antibodies but also, if possible, systemic humoral immune response.

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CHAPTER

7

ALGINATE COATED CHITOSAN NANOPARTICLES ARE AN EFFECTIVE SUBCUTANEOUS ADJUVANT FOR HEPATITIS B SURFACE ANTIGEN[6]

[6] *In preparation for publication*

ABSTRACT

We recently described a delivery system that is composed of a chitosan core to which the hepatitis B surface antigen (HBsAg) was adsorbed and then coated with sodium alginate as the second polymer. In this work, the alginate coated chitosan nanoparticles were evaluated as a subcutaneous adjuvant for HBsAg. A high anti-HBsAg IgG titer (2271 ± 120 mIU/ml), with the majority of antibodies being of Th2 type, was observed 4 weeks after the boost. The specific antigen immune response was 5.3-fold higher than the mean value found for the control group immunized with the saline solution of the antigen. However, regarding the cellular immune response, no important differences were observed for the antigen-specific splenocyte proliferation or for the secretion of Th1 (IFN-γ) and Th2 type cytokines (IL-4). Additionally, the co-delivery of antigen-loaded nanoparticles and the adjuvant, CpG ODN 1826, was investigated. The increase in anti-HBsAg IgG titers was not statistically different from the first group; however, an increase of the IgG2a/IgG1 ratio from 0.1 to 1.0 and an increase ($p<0.01$) of the IFN-γ production by the splenocytes stimulated with the HBV antigen was observed. Finally, a third formulation, designed as antigen and adjuvant co-adsorption in the same uncoated chitosan nanoparticles was also evaluated, but no additional benefits were found in comparison to the other formulations.

The enhancement of the immune response, observed with the antigen-loaded nanoparticles demonstrated that chitosan is a promising platform for parenteral HBsAg delivery and when co-administered with the CpG ODN, results in a mixed Th1/Th2 type immune response.

7.1 INTRODUCTION

Chitosan, a biodegradable and biocompatible polysaccharide with immunological activity [1, 2], which acts both as a bioadhesive [3] and an efficient absorption enhancer material [4], has also been regarded as a promising polymer for the formulation of vaccine delivery systems, especially for the application to mucosal surfaces [5]. Recently, we have designed a delivery system composed of a chitosan core to which the hepatitis B

surface antigen (HBsAg) was adsorbed and then coated with sodium alginate. This delivery system was recently evaluated for the mucosal routes of immunization.

On the other hand, the evaluation of chitosan as an adjuvant for parenteral vaccination has been less studied and, in most cases, the results of the vaccination were reported together with the results of intranasal or oral vaccination studies, making the possible value of chitosan as an adjuvant for parenteral routes less perceptible in the scientific literature. Generally speaking, the development of safe novel adjuvants is necessary not only for the more challenging environment of the mucosal surfaces, but also for parenteral vaccination, to maximize the efficacy of new or already available vaccines. In the last few years this idea became even more urgent since the newer generation of antigens are predominantly purified recombinant proteins, which are often poorly immunogenic. Additionally, new generation of adjuvants may also allow vaccination strategies to be applied to novel areas, including “therapeutic” vaccines designed to control allergies, auto-immune diseases, malignancies, drug dependencies, neural diseases, or fertility [6].

Despite the efficacy of hepatitis B virus vaccines, immunization failure may occur and can sometimes be explained by several factors such as improper storage or administration of the vaccines, advanced age, chronic liver disease, and immunosuppression. Another important factor causing a non-responder rate of up to 10%, seems to be a genetically determined resistance [7, 8]. Moreover, all the conventional licensed hepatitis B vaccines in use contain alum as adjuvant. Although being a potent B cell stimulator, alum is less effective in inducing a Th1 response by the intramuscular route [9]. The Th1 type immune responses, characterized by secretion of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), opsonizing antibodies such as the IgG2a isotype, and strong cytotoxic T-lymphocyte (CTL) induction, are necessary for the control of intracellular infections, such as viral infections [10-12]. In contrast, the development of a strong Th2 response, which is characterized by the secretion of IL-4 and IL-5 cytokines and antibodies such as IgG1 and IgE, is more useful in combating extracellular infections [12].

The viral clearance in acute, self-limited hepatitis B virus infection correlates with increasing CTL and T-helper cell activities and the occurrence of anti-HBs antibodies [13]. In contrast, patients suffering from chronic hepatitis B have insufficient or absent immune responses [9, 13]. On the other hand, experimental data support the hypothesis that the enhancement of HBV- specific immune reactions could have some beneficial

effects in the therapy of chronic hepatitis B [13]. Therefore, it has been suggested [9] that the administration of the hepatitis B vaccine alone, or in combination with diverse cytokines, should be evaluated in future studies for treatment of chronic hepatitis B virus infection. The usefulness of this therapeutic vaccination may be further improved if the vaccine was able to induce a stronger Th1 immune response.

The differentiation of an antigen specific CD4 T helper subset (Th1 or Th2) takes place at the time of priming, and the type of stimulated CD4 subset will depend on a number of factors, including the cytokine environment [10]. The adjuvant used can alter the cytokine environment at the site of the primary immune response [10]. Therefore the selection of an appropriate adjuvant is the first step for a successful induction of an appropriate immune response.

In the present study, the above mentioned alginate coated chitosan nanoparticles were investigated for the first time as adjuvants for the subcutaneous vaccination with the recombinant hepatitis B surface antigen. Moreover, this paper investigates the co-administration of CpG ODN 1826, a potent adjuvant in mice that was shown to induce Th1 type immune response in combination with a number of different antigens, such as e.g. influenza virus [14], hepatitis B antigen [15-17], and tetanus toxoid [18], with the nanoparticles.

7.2 MATERIALS AND METHODS

7.2.1 Materials

7.2.1.1 Polymers

Chitosan was purchased from Primex BioChemicals AS (Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation was 95% (titration method) and the viscosity 8 cP (measured in 1% solutions in 1% acetic acid). A low molecular weight pharmaceutical grade sodium alginate (MANUCOL LB®) was kindly donated by ISP Technologies Inc. (Surrey, UK). According to the provider's specifications, the typical values for the percentage of mannuronic and guluronic acid for Manucol LB were 61%

and 39%, respectively, with an estimated molecular weight of 18 kDa. Both polymers meet the requirements described in the European Pharmacopoeia for use in pharmaceutical formulations.

7.2.1.2 Antigen, adjuvant and reagents

The hepatitis B surface antigen (HBsAg), (subtype ADW2) was kindly offered by GSK Biologicals (Rixensart, Belgium), Engerix B™ was from GlaxoSmithKline Biologicals (Rixensart, Belgium), Class B, CpG ODN (1826) (5'-TCC ATG ACG TTC CTG ACG TT-3') was purchased from Coley Pharmaceutical Group (Ottawa, Canada).

Concanavalin A (Con A), phenylmethanesulfonyl fluoride (PMSF), avidin peroxidase conjugate and the BCIP/NBT- purple liquid substrate system for membrane were from Sigma Chemicals (St. Louis, USA). Certified fetal bovine serum (FBS) and L-glutamine (200 mM) were from Gibco (Invitrogen Co, Paisley, Scotland, UK), 1M HEPES buffer (0.85% NaCl), RPMI 1640 without L-glutamine and Pen-Strep (10 000 U penicillin/ml; 10 000 μg streptomycin/ml) were from Biowhitaker (Cambrex Bio Science, Verviers, Belgium). [methyl-^{3}H] thymidine (1.0 mCi/ml) was obtained from Amersham Biosciences (UK), R-Phycoerythrin (PE)-conjugated hamster anti-mouse CD69, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8 were obtained from BD Biosciences (Madrid, Spain). The FITC-conjugated goat anti-mouse IgM (anti-μ), the anti-mouse IFN-γ and biotin rat anti-mouse IFN-γ was purchased from PharMingen (San Diego, CA, USA). The mouse IgA ELISA quantification kit was obtained from Bethyl Laboratories, (Montgomery, USA). All others reagents used were of analytic grade. All solutions were prepared in ultrapure water.

7.2.2 Methods

7.2.2.1 Preparation of the coated nanoparticles

The preparation of the alginate coated chitosan nanoparticles was performed according to the method previously described [19]. In brief, chitosan was dissolved at a concentration of 0.25% (w/v) in diluted acetic acid solution. The formation of the particles was achieved by the addition of 3.5 ml of sodium sulfate solution (10% w/v) to 200 ml of

the chitosan solution. The resulting suspension was centrifuged for 30 minutes at 3500 rpm (2800 x *g*) and the supernatant was discarded. The particles were re-suspended in Millipore water and centrifuged twice. Finally they were frozen in liquid nitrogen and freeze-dried overnight using a Labconco freeze dry system (Labconco Corporation, Kansas, USA). The dry powder was kept frozen until further use.

The loading of the chitosan nanoparticles with HBsAg was performed by incubating a solution of HBsAg with a suspension of chitosan particles in phosphate buffer of pH 7.4 under mild agitation at room temperature during 120 minutes. The resulting suspension with the composition of 0.015% (w/v) HBsAg and 0.5% (w/v) nanoparticles was used in the subsequent coating step. Alginate coated nanoparticles were obtained by mixing equal volumes of the HBsAg loaded nanoparticle suspension and a solution of sodium alginate in phosphate buffer (1% w/v) under magnetic stirring. The agitation was maintained for 20 min. The suspension was then centrifuged for 10 min at 1600 rpm and the supernatant was discarded. The particles were re-suspended in 0.262 mM $CaCl_2$ in 50 mM HEPES buffer solution, kept under agitation for another 10 minutes and immediately administered to mouse group I. Group II received the same 1% particle suspension to which previously CpG ODN was added. Finally, a 1% chitosan nanoparticle suspension, which contained CpG and the antigen but was not coated with alginate, was administered to mouse group III.

7.2.2.2 Evaluation of the loading efficacy of HBsAg in coated and uncoated nanoparticles.

The loading efficacy of the coated and uncoated nanoparticles was calculated by an indirect way, quantifying the antigen that remained in solution as described before [20]. After the coating with alginate, an aliquot of the particle suspension was centrifuged at 14,000 rpm for 15 minutes and the protein concentration in the supernatant was quantified by micro-BCA-protein assay (PIERCE, Rockford, USA) using a microplate reader with a 570 nm filter.

The loading efficacy (LE) and the loading capacity (LC) were calculated from the following equations:

Eq. 1 - LE (%) = (Total amount of HBsAg – free HBsAg)/Total amount of HBsAg *100

Eq.2 – LC (μg of HBsAg /mg nanoparticles dry weight) = (Total amount of HBsAg – free HBsAg)/ mg chitosan nanoparticles dry weight.

7.2.2.3 Immunization studies

7.2.2.3.1 Animals:

Seven week-old female BALB/cAnNHsd mice were used (Harlan Iberica, Barcelona, Spain) with four mice per group. Animals were housed for acclimatization one week before the experiments at the animal resource facilities of the Faculty. Animal care, handling, and immunization protocols were in accordance with institutional ethical guidelines. The mice had free access to food and water, with a 12h light/dark cycle.

7.2.2.3.2 Treatment of groups (see also table 1)

Group I - suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg
Group II - suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg plus 20 μg of CpG ODN in solution.
Group III - suspension of the chitosan nanoparticles loaded with 10 μg HBsAg and 20 μg CpG ODN
Group IV – Phosphate buffer saline (PBS; pH 7.4) solution with 10 μg HBsAg (reference group)
Group V - untreated or control

Table1 – Resume of the formulations

	Group I	Group II	Group III	Group IV
Chitosan NP	X	X	X	-
Alginate coating	X	X	-	-
HBsAg loaded on NP	X	X	X	-
HBsAg solution	-	-	-	X
CpG ODN loaded on NP	-	-	X	-
CpG ODN in sol.	-	X	-	-

7.2.2.3.3 Immunization schedule

The primary immunization was followed by one boost with three week intervals between the immunizations. The mice were sacrificed 4 weeks after the boost. To evoke an immune response, a total volume of 100 µl of the formulations was subcutaneously administered to non-anesthetized mice.

7.2.2.3.4 Collection of samples

Blood samples were taken from the orbital sinus before the boost and by cardiac puncture at the end of the experiment. The sera were prepared by centrifugation and stored at -20ºC until analysis.
Fecal pellets (4-8) were collected in Eppendorf tubes four days before the end of the experiment. The pellets (0.2g/ml) were suspended in PBS (containing 0.1% sodium azide, 0.1% BSA, and 1 mM PMSF), vortexed, and allowed to rest at room temperature for 1 hour. Solid matter was separated by centrifugation at 14,000 rpm for 15 min. The clear supernatants were frozen at -80ºC until determination of antigen-specific and total secretory IgA by ELISA.

7.2.2.3.5 Enzyme-linked immunosorbent assays (ELISA) for HBsAg specific immunoglobulins

Ninety-six-well flat-bottomed microtiter plates (Nunc immunoplate maxisorb) were coated before use with the recombinant HBsAg (1 µg/well) in coating buffer (50 mM sodium carbonate, pH 9.6) by overnight incubation at 4ºC. The plates were washed 5 times with PBS-T (PBS containing 0.05% Tween-20) and blocked with 3% BSA in PBS-T (200µl/well) for 1 hour at 37ºC. The plates were then washed 5 times with PBS-T and the serial dilutions of each serum (100 µl/well) from the individual mice were tested in triplicate, starting from a 1:100 dilution in PBS-T. The serum was incubated for 2 hours at 37ºC and after washing the plates with PBS-T, the plates were incubated for additional 30 min at 37ºC with peroxidase-labeled goat anti-mouse immunoglobulin G and isotypes (anti-IgG1, anti-IgG2a, anti-IgG2b, and anti-IgG3). The bound antibodies were revealed by adding 100 µl/well of 0.5 mg/ml of o-phenylenediamine dihydrochloride (OPD) (Sigma, Spain) in 10 ml of citrate buffer with 10 µl of H_2O_2. The reaction was stopped

after 10 minutes with 50 µl of 3M HCl to each well. The absorbance was read at 492 nm in an automatic ELISA reader (Easy Reader 400, SLT-LABINSTRUMENTS). ELISA titers were expressed as mIU/ml, and 1 mIU is the mean OD of the pre-immune serum plus two times the standard deviation.

The measurement of IgA was carried out using a mouse IgA ELISA quantification kit (Bethyl Laboratories, Montgomery, Texas, USA) as described by the manufacturer. In order to measure the sIgA levels in the feces, total sIgA and the specific anti-HBs sIgA were determined in the extracts. The results are presented as the anti-HBsAg IgA/total IgA. By this way, variations between samples related with the extraction process or stability of the sIgA were minimized.

The IgA standard was diluted to appropriate concentrations in PBS with 1% BSA to create a calibration curve. The gut washes were diluted in PBS-T with 1% BSA and added to the plates in series of two-fold dilutions. The concentrations of the total and specific IgA were determined from the calibration curve generated for each set of samples using a four parameter logistic curve-fit generated by SigmaPlot software (version 8.0, SPSS Inc).

7.2.2.3.6 Preparation of spleen cell suspensions

The mice were euthanized by cervical dislocation and their spleens were aseptically removed. Individual spleen cell suspensions were prepared in a Petri dish using curved needles and washed twice with RPMI 1640. The final suspension was adjusted to a final concentration of $1x10^7$ cells/ml in complete RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) glutamine, 1% (v/v) Pen-Strep, and 2% (v/v) 1M HEPES buffer.

7.2.2.3.7 Spleen cell populations

The cells (10^6) were washed twice with cold PBS supplemented with 2% FBS and then incubated in the dark for 30 minutes at 4ºC with saturated concentrations of FITC-conjugated rat anti-mouse CD4 or FITC-conjugated rat anti-mouse CD8 or FITC-conjugated goat anti-mouse IGM (anti-µ). After incubation, cells were washed three times with PBS-2% FBS and then re-suspended in 500 µl PBS-2% FBS. To exclude dead cells, 2.5 µl propidium iodide (50 µg/ml) were added just before data acquisition. At least 10,000 events were analysed by flow cytometric acquisition, performed in a

fluorescent activated cell sorter (FACS Calibur) (BD, Biosciences, Madrid, Spain). Data were analysed with CellQuest software (BD, Biosciences, Madrid, Spain).

7.2.2.3.8 Splenocyte cell culture in the presence of the mitogens

Using sterile 96-well flat-bottomed tissue culture plates, 25 μl of splenocyte suspension ($1x10^7$ cells/ ml) from each mouse were plated in triplicate with 25 μl of complete RPMI solution of the mitogen [Con A (50 μg/ml), CpG ODN (50 μg/ml) plus HBsAg (16 μg/ml), HBsAg (16 μg/ml) alone, or without mitogen (control)]. Finally the volume of the well was filled up to 200 μl with complete RPMI and incubated according to the following conditions (see 2.2.3.9 and 2.2.3.10.).

7.2.2.3.9 Cytokine production by splenocytes

Spleen cell suspensions were plated with the mitogens (see 2.2.3.8) and incubated in a humidified 5% CO_2 incubator for 48h (IL-4, IL-10) and 96h (IFN-γ) at 37ºC. The plates were centrifuged and the clear supernatants stored at –80ºC until analysis of the cytokines by ELISA technique described elsewhere [21].

7.2.2.3.10 Lymphoproliferation assay

Splenocytes were obtained and cultured together with the mitogens in flat-bottomed 96-well plates as described before (see 2.2.3.8). The cells were cultured for 96 hours at 37ºC and during the last 8 h of incubation, each well was pulsed with 1μCi of [methyl-^{3}H] thymidine. The 96 well plates with the cells were stored at -20ºC until further analysis. The cells were later thawed and harvested onto a fiberglass filter (filter mats, molecular devices, Skatron, Lier, Norway) using a semiautomatic cell harvester (Scatron Instruments, USA) and DNA thymidine incorporation was counted by standard liquid scintillation techniques with a Beckman LS 6500 scintillation counter (Beckman Coulter Inc., Fullerton, USA). Thymidine incorporation was expressed as counts per minute (cpm).

7.2.3 Statistical analysis

If not referred to another method, the data were presented as the mean ± S.E.M. for at least three experiments and statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's post test for comparing the vaccinated groups with the control group. The Kruskal-Wallis test followed by Dunn's multiple comparison test, was used for comparing the cytokine values between the groups using the Prism 4 (GraphPad software, CA USA). Differences were considered significant when $p<0.05$.

7.3 RESULTS

7.3.1 Characterization of the vaccine delivery systems

Our group recently reported on the development of alginate coated chitosan nanoparticles and the characterization of this new delivery system [19]. Briefly, before coating with sodium alginate, the chitosan nanoparticles have a mean diameter of 643 nm (dynamic light scattering technique) and are positively charged (+37 mV). Scanning electron microscopy (SEM) images of uncoated particles also revealed the presence of small nanoparticles (around 100 nm) and demonstrated that the majority of the coated particles were in a range between 300 and 600 nm. More recently [20], we published release studies with ovalbumin as a model vaccine. Ovalbumin release studies from coated and uncoated chitosan nanoparticles, performed in several buffers at different pH values, allowed us to conclude that the coating with sodium alginate of the ovalbumin loaded chitosan nanoparticles prevented an ovalbumin burst release observed in uncoated chitosan nanoparticles at pH 5.5; 6.8, and 7.4 (phosphate buffer) within the first 30 minutes of incubation.

7.3.2 Hepatitis B antigen entrapment and CpG adsorption to nanoparticles

Hepatitis B antigen was efficiently associated with alginate coated chitosan nanoparticles. The loading efficacy of hepatitis B vaccine in the coated nanoparticles was 77.1 ± 3.0 % (mean ± STDEV) and the mean of the loading capacity was 23.1 μg of HBsAg/mg of dry chitosan nanoparticles ± 2.1 (STDEV). The adsorption efficacy of CpG to the chitosan nanoparticles was 97.0 ± 1.3% and the loading capacity was 29.0 ± 0.03 (μg of CpG ODN/mg of dry chitosan nanoparticles).

7.3.3 Cellular immune response to SC administration of HBsAg associated with the chitosan nanoparticles

The study of the spleen cell populations of the mice subcutaneously vaccinated with different chitosan based formulations was done immediately after sacrificing the mice. The freshly prepared spleen cell suspensions of each mouse were fluorescently labelled and analysed by FACS in order to evaluate the hypothetical influence of the HBV vaccine associated with chitosan formulations on the cell composition of the spleen. A significantly higher percentage of B lymphocytes was observed (Fig. 1) in group I (p<0.01), vaccinated with HBV antigen associated with coated nanoparticles than in group II (p<0.05), vaccinated with the antigen associated with the coated nanoparticles plus the CpG ODN in solution. The percentage of CD4+ and CD8+ T-lymphocytes in the spleen was shown not to be significantly different from the percentages found for the naïve mice group, 21% (CD4+) and 13% (CD8+), respectively.

Furthermore, possible changes in the splenocyte function were also studied. One of the parameters observed was their ability to proliferate (figure 2) and to produce cytokines (figure 3) after being stimulated with HBsAg, HBsAg+CpGODN, and Con A (positive control). As expected, after 96 hours of culture without any external stimulation (negative control), the splenocytes did not exhibit significant proliferative activity. Group IV was an exception, with a mean value statistically higher (p<0.05) than the control group; however the variability was high (Fig. 2A). The same behaviour was observed when the splenocytes from the same group IV (vaccinated with a solution of the HBsAg) were stimulated *in vitro* with the antigen (Fig. 2B) or with the antigen + CpG ODN (Fig. 2C), but not with Con A (Fig. 2D).

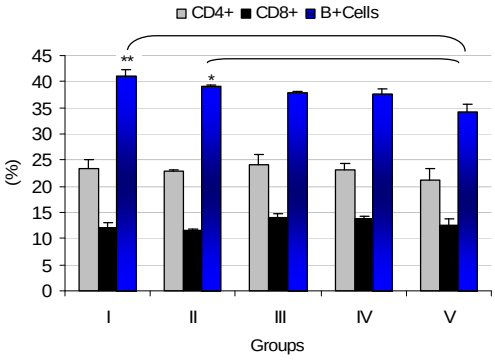

Figure 1 – A) - Percentage of T-lymphocytes CD4+, CD8+ and B+ Lymphocytes in the spleens of mice from groups (I, II, III, IV and V) by FACS analysis. Each bar corresponds to the group geometric mean plus the standard error of the mean. (** p<0.01 and *p<0.05).

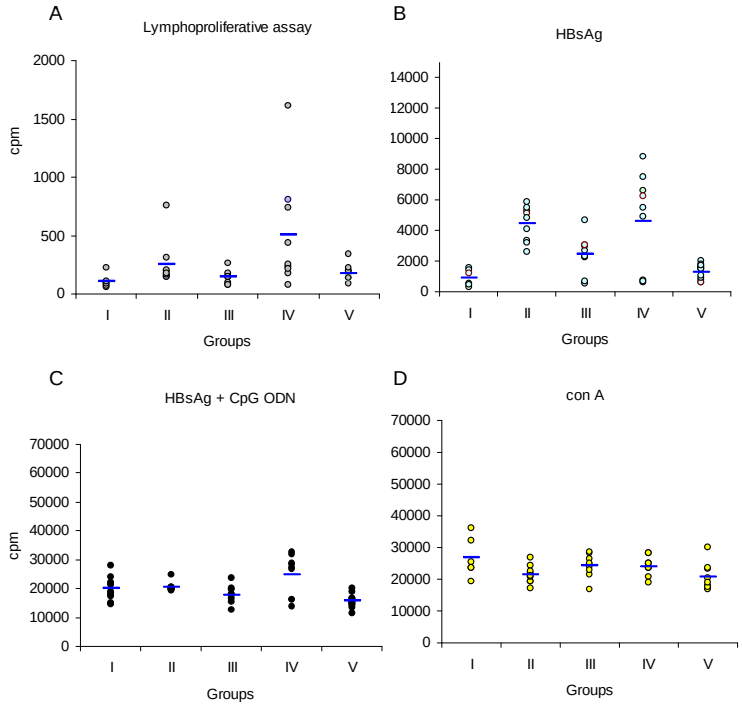

Figure 2 – Lymphoproliferative response after sc administration of the different hepatitis B vaccine formulations. *In vitro* proliferation of individual mouse splenocytes for a 96 hour period stimulated with: A) - without stimulus. B) – HBsAg. C) – HBsAg + CpG ODN, D) - Con A. Each circle represents the result of individual samples and the horizontal bar the mean of the group. The results corresponded to the thymidine incorporation and are expressed as counts per minute (cpm).

Another group that showed a statistically higher (p<0.05) proliferative response was the group II, vaccinated with the HBsAg associated with the coated nanoparticles + the adjuvant in solution. This last result had a different significance. The higher proliferative response was selective to the presence of the antigen (p<0.01), HBsAg (Fig. 2B) or the antigen plus the CpG ODN (p<0.05) (Fig. 2D). Moreover, after the incubation with the different compounds, the production of cytokines was analysed in the culture supernatants. It was not possible to observe detectable amounts of IL-10 in the supernatants of splenocytes cultured without mitogenic compounds or cultured in the presence of the HBsAg. Additionally, the concentration of IL-10 was only weakly increased (Fig. 3A) in the group IV cultures stimulated with Con A.

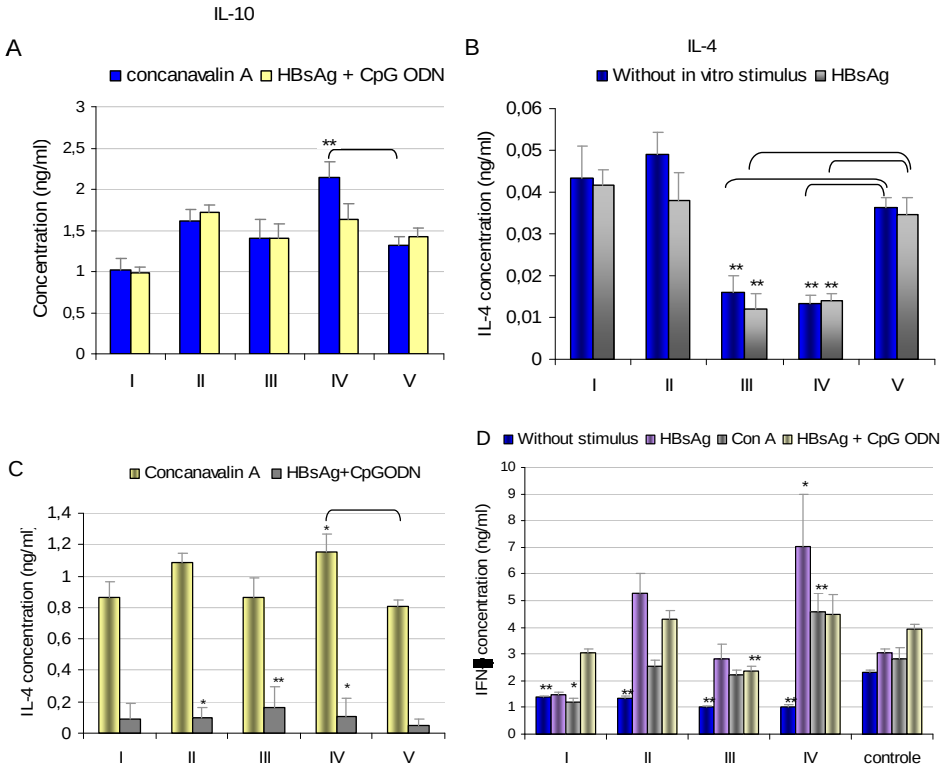

Figure 3- Cytokine production by splenocytes. The spleens were harvested at 4 week post boost and suspension of individual spleen cells were cultured with either medium alone or in the presence of different stimulus (HBsAg, Con A and HBsAg+CpGODN). Each bar corresponds to the group geometric mean plus the standard error of the mean (n=9). A) – Data are IL-10; B) and C) – Data are IL-4; D) – Data are IFN-γ

Next we wanted to examine the profile of the cell mediated immune response, specifically IFN-γ (Th1) and IL-4 (Th2) cytokines. The production of IL-4 was inhibited in the mouse groups vaccinated with the solution of HBsAg and in the group vaccinated with uncoated chitosan nanoparticles associated with the antigen and to the adjuvant, groups III and IV, respectively. This characteristic was observed in the cultures without mitogen, as well as in the cultures stimulated with the HBsAg. Groups I and II, vaccinated with the HBsAg associated with coated nanoparticles and in the second case with coated nanoparticles + adjuvant, did not show any change in IL-4 production, which may indicate that a Th2 immune response was not predominant. Nonetheless, following *in vitro* stimulation of the splenocytes with a solution containing HBsAg + CpG ODN, a small, but significant IL-4 enhancement was observed in almost all the vaccinated groups (Fig. 3C). On the other hand, this effect was not confirmed with the splenocytes cultured in the presence of Con A (positive control), except for the group IV, where a statistically higher mean value ($p<0.05$) was observed again. At the same time, low amounts of interferon-γ in the supernatants of the splenocytes, cultured for 96 hours without any mitogen, when compared with the non vaccinated mice (control group), were observed in all vaccinated groups while a simultaneous enhancement was observed in group IV, when the splenocytes were stimulated with the antigen HBsAg and with Con A. Furthermore, comparison within HBsAg vaccinated groups showed that an increase in the amount of IFN-γ, produced by splenocytes cultured in the presence of the HBsAg ($p<0.01$) or in the presence of con A ($p<0.05$), in group II (with CpG) occurred when this group was compared with group I (without CpG). This result was expected since the ability of CpG ODN adjuvant to induce Th1 type cytokines has been previously demonstrated [10]. Also of note, is the comparison between the two groups vaccinated with formulations containing the CpG ODN. In group II, the adjuvant was in solution and in group III the adjuvant was adsorbed together with the antigen to the surface of the chitosan nanoparticles. A statistical significant ($p<0.01$) decrease for the amount of the γ-IFN produced in the second group referred (Group III) was observed for the cells *in vitro* stimulated with the antigen plus the adjuvant.

According to these cytokine data, a clear Th1 cellular immune response, induced by each formulation, was not observed. On the contrary, a Th1/Th2 mixed response was observed in the group vaccinated with a saline solution of the antigen. This mixed Th1/Th2 response has been attributed to the HBsAg alone [22].

7.3.4 Humoral immune response following subcutaneous administration of HBsAg associated with nanoparticles.

7.3.4.1 Systemic immune response

The association of the HBV antigen (group I) to the alginate coated chitosan nanoparticles induced a strong immune response that was 5.3-fold higher than the mean titer found for group IV, vaccinated with the antigen without any adjuvant (Fig. 4A).

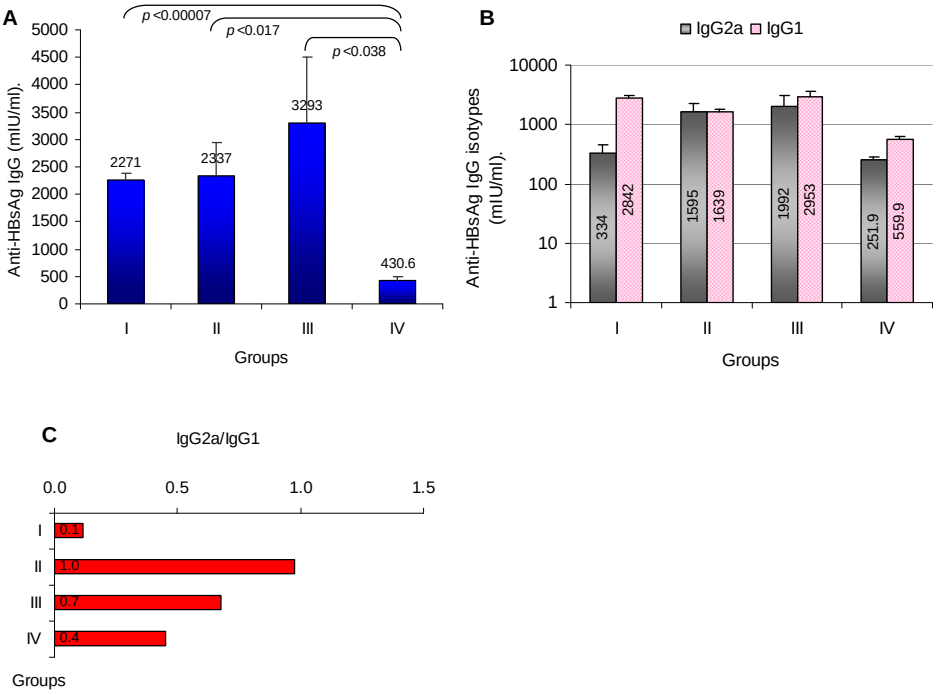

Figure 4 – A) - Serum anti-HBsAg IgG titers of mice immunized with different formulations of hepatitis B vaccine. Values are expressed as antibody titers of individual mice taken in the end of the experiment. The horizontal bar is the mean of the group. T-test was used for analysis of significance between each group and the group IV. B) - Serum anti-HBsAg IgG1 and IgG2a titers of mice immunized with different formulations of hepatitis B vaccine. The bar corresponds to mean titer in each group. Titers were defined as the highest plasma dilution resulting in an absorbance value twice that of nonimmune plasma (1 mIU/ml = mean + 2 SD of the control group).

In group I, both anti-HBsAg IgG1 and IgG2a were detected in the serum, however there was a clear predominance of IgG1 (Fig. 4B). The addition of the CpG ODN to the formulation of the group I, given to group II, resulted in an increase in anti-HBsAg IgG2a antibody titers and in a decrease of anti-HBsAg IgG1 antibody titers. Thus, the main impact was the breaking of the clear predominance of the Th2 immune response for the induction of a mixed Th2/Th1 response (Fig. 4C). Moreover, the mean IgG titers are not different between the groups I and II. Consequently it can be concluded that the immune response differences between the two groups are only qualitative. Finally, the group III was vaccinated with HBsAg + CpG ODN adsorbed to the uncoated chitosan nanoparticles. A strong HBsAg-specific IgG immune response was observed and was 7.7-fold higher than the mean value found for the control group, immunized with the saline solution of the antigen. Moreover, comparing group I with group III, the values of the anti-HBsAg IgG are not significantly different. However, similar to group I, the group III showed a predominance of the IgG1 antibody subtype (Th2 profile immune response). The amount of CpG ODN administered to both groups II and III, was equal. In the group III, the CpG ODN, associated with the chitosan nanoparticles and therefore less available to interact with the immune cells, seemed to exert a smaller effect, resulting in the decreased ratio of IgG2a/IgG1 of 1.0 in the group II to 0.7 in the group III (Fig. 4C).

7.3.4.2 Mucosal immune response

In this study the evaluation of anti-HBsAg sIgA antibodies in the faeces extracts was done with fresh faeces, collected two days before the end of the experiment. As expected, since the induction of mucosal antibodies seemed to be complicated, after the subcutaneous route of administration of the vaccines, the ratio anti-HBsAg IgA/total IgA of the vaccinated groups was not different from the control group (Naïve mice) (Fig. 5).

7.4 DISCUSSION

We recently described a new delivery system that is composed of a chitosan core to which the antigen was adsorbed and thereafter coated with sodium alginate. One advantage of this delivery system is that the antigen is encapsulated under non-stressful

conditions, with a high probability that the biological properties of the antigen will remain intact, which has been confirmed in our own previous studies (results not shown). Coated chitosan nanoparticles were originally designed for targeting and transporting recombinant protein antigens to mucosal surfaces, in particular the oral mucosa. In this study, we evaluated for the first time the potential of the chitosan nanoparticles as an adjuvant for the hepatitis B surface antigen administered by a subcutaneous route. The adjuvant effect of the alginate coated chitosan nanoparticles for HBsAg (group I) could be clearly demonstrated, by the production of high anti-HBsAg IgG titers, with a clear dominance of Th2 type antibodies (IgG1>IgG2a). A similar situation (IgG1>IgG2a) was recently reported with a different type of nanoparticle and antigen. For example, nanoparticles of poly(D, L-lactic-co-glycolic acid) encapsulated tetanus toxoid, were used as a model protein vaccine [18] and also with liposomes encapsulated influenza subunit [23], both of which were administered intramuscularly.

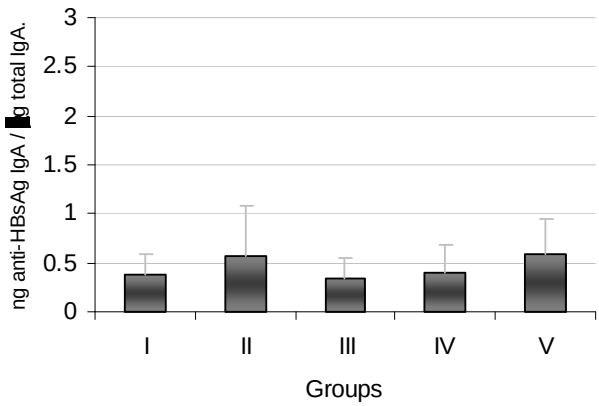

Figure 5 – Secretory anti-HBsAg sIgA profile detected in individual mice samples of mice faeces colleted in the end of the experiment. Samples with the value equivalent to mean plus twice the SD of the control group (Group V), were considered anti-HBsAg sIgA positive. The values were expressed as the ratio between the anti-HBsAg sIgA (ng/ml) and total sIgA (mg/ml).

Comparing the results of group I (HBsAg loaded nanoparticles) with the results observed with the group vaccinated with the solution of the antigen (Group IV) it can be concluded that even though nanoparticles had stimulated both Th1 and Th2 type antibodies, IgG2a antibodies was induced in a lower proportion, resulting in a decrease of the IgG2a/IgG1 ratio from 0.4 to 0.1. This means that the induced mixed Th1/Th2 profile was shifted towards the Th2 profile, when the alginate coated chitosan

nanoparticles were used as an adjuvant. The enhancement of the Th2 response by a formulation with chitosan nanoparticles after intranasal immunization with genetically detoxified diphtheria toxin was also recently reported [24].

In a previous study (intranasal vaccination), we investigated the induction of an immune response to alginate coated chitosan nanoparticles loaded with HBsAg. In the same study, we also included a subcutaneous study with a commercially available hepatitis vaccine. For this reason, we did not include such a test with a commercially available vaccine in the present study. Although the set-up of the nasal study with the commercially available vaccine was slightly different compared with the study presented here, nevertheless a similar Th2 profile can be observed with both alum (commercial formulation) and coated chitosan nanoparticles.

In addition, after the statistical analysis of the cellular immune responses, it was possible to draw attention to the higher percentage of B cells in the spleen and a higher proliferation capability of the spleen cells from group I stimulated with Con A, when compared with the control group (group V). In contrast, an up-regulation of Th2 type cytokines (i.e., IL-4) *ex vivo* produced by the splenocytes was not observed. On the other hand, downregulation of IFN-γ may indicate that natural killer T (NKT) and T cells (Th1 type cells), which are both IFN-γ producers [13], were less activated in comparison to the same cells from the unvaccinated mouse group.

It has been demonstrated that a cell-mediated immune response, and in particular a Th1 response, is important not only for preventing, but also for overcoming HBV infections [13, 25] and eliminating the virus from the infected cells [12]. At the same time, it has been suggested that the conventional vaccine is able to elicit a high humoral immune response, but failed to elicit a cell-mediated immune response, which makes it ineffective for treatment of chronic hepatitis B infection [26]. Therefore the study of new potential adjuvants with emphasis on their capacity to induce a cell-mediated immune response has been reported in the literature [26-28]. For this reason, we decided in this study to co-administer HBsAg loaded nanoparticles and CpG ODN 1826 (group II), which is recognized for its ability to re-direct the Th bias toward a Th2 type immune responses in mice [10, 16, 17, 29]. In fact, in group II an increase of the anti-HBsAg specific IgG2a antibodies and a slight decrease of the IgG1 was observed, but the total HBsAg specific IgG antibodies were not significantly different from the previous group (Group I). Therefore, in this study, the ability of the CpG ODN to induce a Th1 profile immune response was again confirmed; however, an additive effect with the

nanoparticles was not observed, most likely because chitosan nanoparticles appeared to be an already strong adjuvant. A similar situation was recently described with tetanus toxoid loaded PLGA microspheres and CpG ODN in solution [18].

A more simple formulation was studied using mouse group III. In this case, we tested the effect of the co-adsorption of the antigen and the CpG ODN together in chitosan nanoparticles. This nanoparticle formulation was easily prepared by simple mixing of the chitosan nanoparticles with the adjuvant and the antigen, and was then immediately administered to the mice, avoiding the additional coating procedure with sodium alginate. Furthermore, as a second hypothetical advantage of this delivery system, it could present multiple copies of the antigen on its surface, an effect which has been shown to be optimal for B cell activation [30]. Moreover, the co-adsorption of the antigen and the CpG ODN to the same nanoparticles, has been indicated as the ideal formulation [31-34]. However, comparing the results with those of mouse group II, where the adjuvant was in particle formulation as a free suspension, this group (Group III) showed a lower IgG2a/IgG1 ratio. In addition and in comparison with a recent study [23] which showed comparable IgG2a/IgG1 ratios in mice containing the CpG ODN as adjuvant in their formulations, a mixed Th1/Th2 immune response can be consider for group III. Additionally, although the mean value of the total specific IgG has been higher in group III, the value was not statistically different when compared with group II, due to the high polydispersion of the values obtained. A decrease in IFN-γ production was also observed. Therefore, all the observations seemed to support the idea that the soluble CpG ODN was more accessible and could interact with the cells better, perhaps due to its increased availability in the formulation of the group II.

In the present study, the evaluation of the cellular and humoral immune responses was done 4 weeks after the last boost. To better evaluate the effect of the CpG ODN released from the chitosan particles longer sampling might be useful.

Other studies on particulate delivery of CpG, either surface adsorbed [35] or in liposomes [32] or in PLGA nanoparticles [18], found a stronger effect when CpG ODN was encapsulated in the delivery system in comparison with CpG ODN in solution. This feature was considered to be important since it would allow for a decrease in dosage of CpG ODN administered while obtaining the same effect. Two different mechanisms can be discussed to explain our results. The first is related with the frequently reported strong affinity between the CpG ODN and the cationic chitosan polymer [36, 37], that may cause a slow release of the CpG *in vivo*. Therefore, the amount of free CpG, ready to be

internalized by the target cells is lower when compared with the situation where the CpG ODN was given in solution. The second one is that the internalization of the CpG ODN into the cells is a prerequisite before it can bind to Toll-like receptor 9 (TLR9) present within a number of mouse immune cells and subsequently trigger the immune response [34, 38]. Supported by theory and also by some experimental evidence [39], the internalization of the CpG ODN would be facilitated, if it administered in associated with chitosan nanoparticles. Additionally a protective effect against the CpG ODN enzymatic degradation by the chitosan biopolymer, especially in the enzyme rich oral mucosa should also be considered as previously reported [39-41]. Moreover in this study, the size of the administered particles seemed to be adequate for their internalization, although hypothetically an *in vivo* aggregation of the particles may also take place, with a concomitant increase in size. This cannot be totally excluded and will be evaluated in future studies. Finally, even under the assumption that chitosan nanoparticles have been internalized to some extent, the release of the CpG ODN from the particles after intracellular uptake should also be considered as an important issue which can influence the efficacy of a vaccine formulation.

Due to their unique and interesting properties, recently reviewed in several papers [3, 42-45], chitosan and chitosan nanoparticles have been used in mucosal delivery systems for vaccination using several antigens and mucosal routes [24, 46-49]. However their potential as adjuvants for parenteral vaccination has been less studied. In a very recent study, [50] a chitosan solution was explored as an adjuvant for subcutaneous vaccination of mice with a model antigen. It was found that chitosan enhanced the antigen-specific antibody titers over five-fold and antigen-specific CD4+ lymphocytes proliferation over six-fold. Mechanistic studies performed by the same authors revealed that the antigen depot and a transient cellular expansion in draining lymph nodes induced by chitosan may explain its adjuvant properties [50].

7.5 CONCLUSION

For the first time, in this present study, the adjuvant effect of alginate coated chitosan nanoparticles for the hepatitis B surface antigen was evaluated after subcutaneous application in mice. A potent enhancement of the humoral immune response was

observed with predominance of Th2 type antibodies. The Th profile immune response was re-directed to Th1 type when the antigen loaded chitosan nanoparticles were co-delivered with the CpG ODN 1826 in solution. A third formulation, in which the antigen and the adjuvant were both adsorbed to chitosan nanoparticles was also evaluated. No additional benefits were observed with this formulation 4 weeks after the last boost. However, the manufacturing simplicity of this last formulation makes it a potential basis for future formulation improvements. All tested formulations have shown a potential for the improvement of the currently licensed HBV vaccines, in particular in rendering them useful for the treatment of chronic hepatitis B, where a strong Th-1 cellular immune response induction is required. However, the controlled delivery of the antigen and the CpG ODN from the chitosan nanoparticles should be further investigated with respect to enhanced longevity of the immune response, as well as their ability to produce and maintain the Th1-type cellular and humoral immune response.

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CHAPTER

8

FINAL DISCUSSION & FUTURE PERSPECTIVES

The development of novel vaccine adjuvants together with an optimized delivery platform is becoming as important as the development of novel vaccines. This idea is especially true for the new generation of antigens, including those identified through genomic screening of microorganisms, which are predominantly recombinant purified proteins but often poorly immunogenic. Additionally, increasingly scientific evidences of the advantages, associated with the production of mucosal antibodies following oral, nasal and even pulmonary immunization, especially for the microorganisms that invade the body through mucosal surfaces, strongly supports the necessity to do more studies on efficient adjuvants.

An even important argument is related with the dependence that exists between successful mass vaccination programmes, especially in underdeveloped countries, but also in situations of a bioterrorism, and the availability of novel adjuvants that allows the rapid production of more stable, cheaper and “needle free” vaccines. Having this in mind, our first ambition was the design of chitosan nanoparticles based delivery system for oral vaccination.

Due to its unique properties, the use of chitosan in life science became and is being a research subject of considerable interest [1-8]). Several *in vitro* studies, showing the immune stimulating activity of the biopolymers with macrophages and dendritic cells, have been published in the scientific literature. A similar approach was also performed by us *in vitro,* using primary cultures of splenocytes incubated in the presence of the biopolymer. Stronger induction of the CD69 expression, not only on B cells, but also in T-Lymphocytes was observed by us. However extrapolations to the *in vivo* situation should be made carefully. Therefore, it is of great interest and it is in our plans to further investigate the *in vivo* stimulation of the immune cells, not only in spleen but especially in the Peyer’s patches, following subcutaneous and oral administration of chitosan to mice.

The use of chitosan based formulations for mucosal vaccination has been already widely used in several studies with different antigens. For example, in an oral vaccination study with diphtheria toxoid (DT) [9], adsorbed to chitosan nanoparticles, a strong enhancement, when compared with the delivery of the antigen alone, of both, systemic and local mucosal immune responses against DT were observed, after six applications in mice. In another study, the conjugated group C meningococcal vaccine, adsorbed to similar chitosan particles was also given orally (data not published), however an immune response could not be elicited. As a final example, in which Toxoplasma gondii GRA1 protein and the DNA vaccine loaded chitosan particles were used, the type of immune response seemed to be largely depend on the prime/boost regimen [10]. In all these studies, the chitosan particles had very similar properties; therefore the variations in the immune response obtained in these studies are certainly related with the characteristics of the antigen used and also with prime/boost regime.

The interpretation of the different results found in the scientific literature stimulated us to think about the possibility to improve the properties of these chitosan nanoparticles. The antigen adsorption to the surface of the chitosan particles has been regarded as a convenient and safe loading method,

however for low porous nanoparticles, the antigen would be more exposed to the adverse environment of the gastrointestinal fluids. Hence it looked reasonable to us that a subsequent coating of the antigen, adsorbed onto chitosan nanoparticles might provide a better protection during their passage through the gastrointestinal tract. As a result of these considerations sodium alginate has been chosen as the second polymer layer because of its insolubility in the acid environment of the stomach and, like chitosan, of its mucoadhesive properties.

In the second chapter of this thesis the development of chitosan nanoparticles, loaded with a model antigen and subsequently, coated with calcium chloride crosslinked alginate is described. According to our hypothesis, and verified by our experimental results, the use of the calcium, as alginate crosslinker, strengthened the coating, and so contributed for the delayed antigen release from the particles. Moreover, the neutralization of part of the negative charges of the alginate molecule, resulting in a less negative zeta potential of the coated nanoparticles and this aspect, together with the size of the coated nanoparticles, is believed to have been important for the success of the subsequent studies of the particle's internalization by M-cells in gut.

In contrast to what was found in literature, the methodology developed in this work allowed the obtainment of coated particles still in the nanosize range, which is one of the requisites for the uptake by M-cells (reviewed in several references [11, 12]). In fact, our uptake studies in rat Peyer's patches of the coated chitosan nanoparticles (3rd chapter) clearly showed the internalisation of the particles. This promising result was important to support the decision to continue with vaccination studies in mice. However, and despite of being frequently found in the scientific literature, these uptake studies are in the most of the cases only qualitative. They do neither allow the access to information about the percentage of the nanoparticles internalized nor give details about the mechanism of internalization or about the residence time in the Peyer's patches. Moreover, the type of immune cells that preferentially are attracted to the site of the vaccine entrance and subsequently facilitate the recognition of the vaccine by professional antigen-presenting cells is not known. The same hold for the total or partial uptake of the vaccine into these cells, which will dictate the type of immune response [13]. As future prospects based on this discussion, a better understanding of the interaction of the particles with the cells of mucosal surfaces, required for the optimization or development of more efficient vaccine delivery systems, is dependent on more detailed and quantitative uptake studies.

The role of the alginate coated chitosan nanoparticles as adjuvant was evaluated for the hepatitis B vaccine. Following subcutaneous administration of the antigen associated with the nanoparticles, a 100% seroconversion and 5.2 fold enhancement of the anti-HBsAg IgG was observed when compared with the solution of the antigen. The mechanism responsible for that result was not further investigated in detail and should be clarified in the future. However, we are aware about the difficulties that such mechanistic adjuvant studies can involve. For instance, the adjuvant effect of the widely used aluminium compounds is still not fully understood at the present time, and

continuous is an important research subject. In a recent review, Lindblad [14] has summarized as the most probable mechanisms the gradual release and delay clearing of the adsorbed antigen from the inoculated aluminium compound depot. However and also in accordance with the same author, over the last two decades the role of T cells and cytokine profiles following the application of aluminium adjuvants have been described as well. In the studies of this thesis, a similar gradual release of the adsorbed Hepatitis B antigen from the inoculated coated nanoparticles may have occurred. A little knot, observed on the site of the injection may indicate that a depot was formed, a fact which supports this hypothesis. But there is also the possibility that some of the coated nanoparticles have been internalized by local cells and therefore were originating different mechanisms.

Finally, oral and nasal vaccination simply with the HBsAg entrapped into coated nanoparticles induced a low or no seroconversion rate. In the recent literature, the presence of non responder mice in the orally vaccinated groups is frequent reported, especially when the evaluation of the specific antibodies was performed after a single immunization. Moreover, to get a high percentage of seroconverted mice within the orally vaccinated groups it was always necessary to administer higher antigen concentrations (10-fold more from what was used in this work) together with a successive administration of the vaccine, especially when these results are compared with the intramuscular or subcutaneous route of administration [15]. Commercial injectable hepatitis B vaccines also have this drawback in humans. Therefore the recombinant hepatitis B vaccine will become a very challenge for vaccinations through mucosal routes.

Regarding mucosal immune response we have found that for the subcutaneously vaccinated mice groups the concentration of anti-HBsAg sIgA in fecal extracts was below the detection limit of the ELISA method. In contrast, the detection of mucosal antibodies in gut washes after oral vaccination studies, was possible within mice groups vaccinated with the antigen entrapped into coated nanoparticles. The same holds for nasal and vaginal secretions within the nasal vaccination groups.

Recently, Holmgren & Czerkinsky [16] reviewed some mucosal immunity concepts like the one of the “common mucosal immune system” whereby immunocytes activated at one site disseminate immunity to remote mucosal tissues rather than to systemic sites. However, the appearance of a higher or lower antigen specific sIgA concentration, in a specific mucosal tissue is dependent of the vaccination route. For instance, the oral immunization may induce substantial antibody responses in the small intestine, mammary and salivary glands and, according with the same authors, it is relatively inefficient in evoking an IgA antibody response in the distal segments of the large intestines, tonsils or female genital tract mucosa in humans [16]. On the other hand, the intranasal vaccination seems to be more efficient to evoke mucosal antibodies in the upper airway mucosa, regional secretions (saliva and nasal secretions) and cervicovaginal mucosae in humans, without evoking an immune response in the gut. Our observations in mice are in accordance with these findings (chapter 6).

The choice of the administration route is an important aspect and should be carefully taken into consideration to where we want the induction of a mucosal immune response.
It was found that the sc vaccination with the hepatitis B vaccine entrapped into alginate coated chitosan nanoparticles elicited the enhancement of the humoral immune response with a predominance of the Th2 type antibodies. Similar observations were made after oral vaccination with the same nanoparticles associated with HBsAg. For all administration routes investigated (in, oral and sc) the immune response profile was re-directed to mixed Th2/Th1 profiles or towards Th-1 profile, when the antigen entrapped into coated nanoparticles was co-delivery with the CpG ODN 1826. These formulations have shown a great promising potential for the improvement of the currently licensed HBV vaccines, in particular to make them useful also for the treatment of chronic hepatitis B, where a strong Th-1 cellular immune response induction is required. Additionally, it would be worth examining if the controlled delivery of the antigen and the CpG ODN from the chitosan nanoparticles is also able to enhance the longevity of the immune response.
Finally, regarding CpG ODN 1826, the importance of its entrapment into the alginate coated chitosan nanoparticles is related with the route of administration. For oral vaccination, the necessity of protecting the CpG oligodeoxynucleotides from the enzymatic and acidic adverse environment of the gastrointestinal tract is higher than for the intranasal or subcutaneous routes. Therefore in the oral vaccination study, the best result was obtained within the mice group vaccinated with HBsAg and CpG ODN encapsulated in alginate coated chitosan nanoparticles. On the contrary, intranasal and subcutaneous vaccination with both the HBsAg and the CpG ODN encapsulated into the same delivery system have induced a weaker immune response when compared with the group where the CpG ODN was administered free in phosphate buffer saline solution together with the HBsAg in the nanoparticles. A possible explanation for this unexpected observation is most probably related with the CpG ODN *in vivo* release profile from the chitosan nanoparticles which results in a lower available concentration of the CpG ODN for interaction with the Toll-like receptor 9 (TLR-9. A second concurrent hypothesis is related with the possibility of the nanoparticles for being internalized by the cells that express the TLR-9.

In summary a new nano-sized chitosan based delivery system for mucosal vaccination of the hepatits antigen is described in this work. The delivery system is composed of a chitosan core, to which the antigen was adsorbed, and was subsequently coated with sodium alginate. The appropriateness of the methodology for a high efficiency encapsulation of the hepatitis B antigen and of the CpGODN was demonstrated. Alginate coated chitosan nanoparticles have shown to be non-cytotoxic and their ability to be taken up by the M-cells of the Peyer's patches was demonstrated in rats.
The study of the adjuvant effect of alginate coated chitosan nanoparticles was performed by subcutaneous, oral and intranasal vaccination routes (see table 1). The vaccination studies have shown that alginate coated chitosan nanoparticles have an adjuvant effect for the hepatitis B

antigen when administered subcutaneously, however for the mucosal routes the association of an immunopotentiator is required.

As future prospects of my research the advantages of these nanoparticles over the currently used adjuvant (alum) in the commercial formulation of the hepatitis B vaccine will be assessed for the subcutaneous route. In these new studies the long-term protection should also be evaluated. Moreover, the association of the CpG ODN to the pre-formed suspension of the HBsAg loaded particles would allow a new application for the vaccine in treatment of chronic hepatitis B. To confirm this hypothesis additional studies have to be performed with a suitable animal model.

Furthermore with adequate improvement of the delivery system and of the prime/boost regimens, the suspension of the nanoparticles containing the antigen and the CpG ODN will be investigated in order to have a higher seroconversion rate. Finally, the delivery system has shown promising capabilities for being evaluated in the future also with other vaccines.

Table1 – Resume of the antibodies

Groups		Anti-HBsAg IgG	Anti-HBsAg IgG2a/IgG1	Anti-HBsAg sIgA Faeces	Anti-HBsAg sIgA Gut washes	Anti-HBsAg sIgA Vaginal secretions	Anti-HBsAg sIgA Nasal secretions
NP +HBsAg	sc	+	<1	-	na	na	na
	nasal	-	na	-	na	+	+
	oral	+	<1	-	+	-	na
NP +HBsAg CpG-sol	sc	+	1	-	na	na	na
	nasal	+	>1	-	na	+	+
	oral	-	na	-	-	-	na
NP +HBsAg NP + CpG	sc	Na	na	-	na	na	na
	nasal	+	na	-	na	+	+
	oral	+	>1	-	+	-	na
uNP+CpG+HBsAg	sc	+	<1	-	na	na	na
	nasal	-	na	-	na	na	na
	oral	-	na	-	na	na	na
HBsAg + CpG	sc	na	na	-	na	na	na
	nasal	+	>1	-	na	+	+
	oral	-	na	-	-	-	na
HBsAg	sc	+	<1	-	na	na	na
	nasal	-	na	-	na	+	-
	oral	-	na	-	-	-	na
Engerix B	sc	+	<1	-	na	-	-

NP – alginate coated chitosan nanoparticles; uNP – uncoated chitosan nanoparticles; sc – subcutaneous administration; nasal – nasal administration; oral – oral administration

Na = non applicable; += positive mean; - =negative mean

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