Synthetic oligodeoxynucleotide containing CpG motif induces an anti-polysaccharide type 1-like immune response after immunization of mice with *Haemophilus influenzae* type b conjugate vaccine

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Abstract

Synthetic oligodeoxynucleotides containing CpG motifs [immunostimulatory sequences (ISS)] have been described as potent adjuvants of type 1 immune responses when co-administered with protein or peptide vaccines. To investigate their role in the immune response to polysaccharides (CHO), different preparations of anti-*Haemophilus influenzae* type b (Hib) conjugate vaccine were administered to mice. The unconjugated CHO did not induce the synthesis of specific antibodies even in the presence of ISS. On the other hand, anti-CHO-specific antibodies significantly increased in the presence of ISS, when tetanus (TT) or diphtheria [cross-reacting material (CRM)] toxoid-conjugated CHO were used to immunize mice. The adjuvant effect was also observed for the immune response against the carrier protein (TT and CRM). ISS insured an early and long-lasting specific IgG production. The effects of ISS on the anti-CHO immune response could be attributed to the amplification of the T help provided by the carrier. The analysis of anti-CHO IgG subclasses showed a significant increase of IgG2a and IgG3 in the presence of ISS. ISS caused a rapid release of IL-12 and IFN-γ in sera from treated mice. This data provide a first evidence for the ability of ISS to induce an anti-CHO type 1-like immune response and demonstrate that ISS have the potential to increase host antibody response against both the CHO and the protein component of a conjugated vaccine.

Introduction

Polysaccharides (CHO) are considered T-independent antigens because of their inability to specifically activate T cells (1). Moreover, the ability to respond to purified CHO is age dependent and children <18 months usually are unable to mount an anti-CHO antibody response (2). The conjugation of CHO to a carrier protein improves the immunogenicity because of the T-dependent help conferred by the protein (3,4). Thus, also in the case of responses to conjugated CHO, it can be speculated that the type of Th cells (Th1 or Th2) can modulate the amount and the isotype switch of anti-CHO IgG. Antigen-specific CD4+ cell responses can be divided into type 1 and type 2 on the basis of cytokine secretion and effector function (5). Type 1 responses involve Th1 cells that differentiate in an IL-12 (produced by macrophages) and IFN-γ (produced by NK and T cells) milieu. On the contrary, type 2 responses involve IL-4-dependent differentiation of Th2 cells, and are associated with IL-5 secretion and decreased macrophage activation (6).

Recently, it has been shown that along with whole bacterial DNA, synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides in particular base contexts [immunostimulatory sequences (ISS)] (7) cause activation of...
ISS as adjuvants of polysaccharide conjugate vaccines

B and NK cells (8, 9) as well as antigen-presenting cells (APC) (10). When co-administered in experimental animals with proteins or peptides, ISS act as potent adjuvants and induce type 1 immune responses (11) even with poor immunogenic antigens (12). Nonetheless, when used to increase an anti-CHO immune response, ISS not only failed in their adjuvant effect, but significantly reduced the synthesis of anti-CHO antibodies (13). This effect was interpreted by the authors as a probable consequence of a B cells polyclonal activation (13). However, at odds with T-independent antigens, the immune response to conjugated CHO relies upon different basis and the use of ISS as adjuvant could influence the amount and the subclasses of anti-CHO antibody produced following the vaccination.

Haemophilus influenzae type b (Hib) possesses a CHO capsule of polyribosyl ribitol phosphate. Antibodies against the specific capsular CHO have been shown to be protective against Hib. Several conjugate vaccines, used in infants during the first year of life, contributed to the decline of incidence of Hib-related diseases (14). The commercially available vaccines use different carrier proteins, such as tetanus toxoid (TT) or diphtheria toxoid (DT).

In this work, the effect of adding ISS to unconjugated Hib CHO as well as to TT- or cross-reacting material (CRM)-conjugated Hib vaccines was investigated in mice by studying whether ISS may influence the immune response to conjugated CHO. The antibody responses to both the CHO and the carrier as well as anti-CHO-specific IgG subclasses were evaluated. Furthermore, the cytokines production in mice vaccinated in the presence or absence of ISS and their kinetics of release in the serum was investigated, to obtain evidence of the type 1/type 2 specificity of the immune response to conjugated CHO. The aims of this study were to contribute to the understanding of the mechanisms involved in the T cell dependency of anti-CHO immune response, and to identify possible advantages of using ISS in the anti-Hib vaccination in terms of antibody response against both the CHO and the protein component of a conjugated vaccine.

Methods

Immunization protocol

Hib CHO and Neisseria meningitidis group A CHO (MenA) were a kind gift from Chiron (Siena, Italy). The DT (CRM197)-conjugated CHO and the TT-conjugated CHO used were part of the commercially available anti-Hib vaccines (Vaxem Hib; Chiron and Act-Hib; Pasteur Mérieux MSD, Lyon, France respectively) as well as the anti-tetanus vaccine (Imovax Tetano, Pasteur Mérieux MSD).

Phosphorothioated CpG ODN and non-ISS-containing ODN (M-ODN) were synthesized (M-Medica, Firenze, Italy) according to published sequences (7) (TGACTGTGAACGTCGATGA and TGACTGTAAGCTTCCGAGATGA respectively).

Groups (n = 5–10) of female BALB/c or CD1 mice (6–8 weeks old; Charles River, Calco, Lecco, Italy) were immunized by intradermal (i.d.) injection of 2.5 µg/mouse of CHO or conjugated-CHO (CHO–TT or CHO–CRM) in combination with M-ODN or ISS at 50 µg in a total volume of 50 µl. In some experiments, mice were immunized by s.c. injection.

Unconjugated CHO and CHO–CRM conjugate vaccine were administered in a three-dose schedule (0, 10 and 20 days). CHO–TT conjugate vaccine was administered in a two-dose schedule (0 and 14 days). M-ODN or ISS were administered in the first inoculum only. Where indicated, CHO–CRM or ISS were used at decreasing concentrations (2.5–0.67 and 40–1.25 µg respectively). Unconjugated CHO was also administered i.d. mixed with 4 IU of anti-tetanus vaccine per mouse (mix CHO/TT) or s.c. in incomplete Freund’s adjuvant (IFA), alone or in combination with ISS.

Collection of samples

Plasma was collected by retro-orbital puncture 40 days after the first inoculum of CHO or CHO–CRM and 21 days after the first inoculum of CHO–TT, unless specified in selected experiments. Samples were frozen at −80°C until use.

Evaluation of the immune response

Anti-Hib CHO serum antibody levels were determined by ELISA. A human albumin-conjugated Hib CHO (HSA–CHO; gift from Chiron) was used at 10 µg/ml in PBS (100 µl/well) to coat flat-bottomed 96-well microtiter plates (Dynatech, Chantilly, VA) overnight at 4°C. The HSA–CHO conjugate was used to detect anti-CHO antibodies because it binds to the plates better than unconjugated CHO and does not react with anti-TT or -CRM antibodies elicited by the conjugate vaccines used. Plates were then incubated for 1 h at 4°C with 2% BSA in PBS (post-coat). Sera (non-immune controls and test sera) diluted 1:100 and 2-fold dilutions (1:50–1:3400) of the reference serum in PBS containing 0.2% BSA and 0.1% Tween 20 were added to appropriate wells. After overnight incubation at 4°C and washings, plates were incubated with anti-IgG (IgG), anti-IgM (IgM) or anti-IgA (γ chain specific) goat anti-mouse antibodies conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After a 1 h incubation and washings, p-nitrophenylphosphate (Sigma, St Louis, MO) was added as a substrate. The reaction was blocked with 2 M NaOH and the absorbance evaluated at 405 nm using the Microtiter reader Victor-1420 multilabel counter (EG & G Wallac, Turku, Finland). All tests were designed to compare in the same ELISA plate sera from mice belonging to different experimental groups. To minimize plate-to-plate variations, data were normalized using, as reference in each plate, a hyperimmune mouse serum containing anti-Hib CHO antibody (kind gift from Pasteur Mérieux MSD) and expressed as ELISA units (EU). Mice were considered responders when the serum anti-CHO EU values were >2 times the mean EU value of non-immune mice.

To assess the specificity of anti-CHO ELISA, 50 µl of 10-fold dilutions (2000–0.0002 pg/ml) of Hib CHO or MenA were added to HSA–CHO-coated plates immediately before 50 µl of the reference serum diluted 1:50. The plates were then treated as reported above.

The antibody response to the carrier proteins was evaluated as previously described (15). Briefly, TT or CRM were used to coat microtiter plates at 10 µg/ml in carbonate-bicarbonate buffer overnight. After washings and a 1 h post-coat, sera diluted 1:100 in PBS-T were added and incubated for 3 h at room temperature. Bound antibodies were detected using
alkaline phosphatase-conjugated goat anti-mouse IgG as described above.

Subclasses of anti-Hib CHO IgG \(^+\) sera were determined using rat anti-mouse IgG subclass mAb labeled with biotin (PharMingen, San Diego, CA) and horseradish peroxidase-conjugated streptavidin (PharMingen). After the addition of \(\alpha\)-phenylenediamine (Sigma fast; Sigma) the reaction was stopped with 2 N \(\text{H}_2\text{SO}_4\) and the absorbance was evaluated with the plate reader at 490 nm. A pool of sera from mice vaccinated s.c. with CHO-conjugate vaccine was used as reference serum. In this serum, IgG1 and IgG3 were respectively the most and the least represented anti-CHO IgG subclasses. Thus, 1000 U for IgG1, IgG2a and IgG2b, and 100 U for IgG3 were arbitrarily chosen as anti-CHO subclass contents of the reference serum (16). In each assay 2-fold dilution of the reference serum were tested. The results were expressed relative to the arbitrary units (AU) of the reference serum.

**Cytokine determination**

Groups of CD1 mice were vaccinated i.d. with CHO–TT in the presence or absence of ISS in the first inoculum or treated with 50 µg/mouse of ISS and saline 14 days apart. Three mice per group were sacrificed at 6, 24, 48 and 72 h after the first and the second inoculation. Sera within each group were pooled and the kinetics of cytokines secretion evaluated using commercially available ELISA kits (R & D, Minneapolis, MN) according to the manufacturer’s procedures.

**Statistical analysis**

Data were expressed as arithmetic mean ± SD and analyzed by the Statview 4.1 program (Abacus Concepts, Berkeley, CA). Data were analyzed for normal distribution and the statistical significance of the difference between groups was determined by the two-tailed unpaired Student’s \(t\)-test. Differences were considered significant with \(P < 0.05\).

**Results**

**Anti-CHO antibodies following vaccination with CHO, CHO–TT or CHO–CRM in the presence or absence of ISS**

Mice were immunized with CHO, CHO–CRM or CHO–TT in the presence or absence of ISS and anti-CHO antibodies were detected by ELISA. The use of a protein-conjugated CHO (CHO–HSA) to coat ELISA plates allowed a good and reproducible CHO binding to the plates, and the detection of anti-CHO antibodies without the interference of anti-TT or anti-CRM antibodies elicited by the conjugated vaccines used. In fact, sera from TT and CRM vaccinated mice did not react in anti-CHO ELISA (data not shown). Anti-Hib CHO ELISA was specifically inhibited by soluble Hib CHO, but not by another polysaccharide such as MenA (Fig. 1A). The reactivity of the hyperimmune serum used as reference serum in all the anti-CHO ELISA is shown in Fig. 1(B). The titer of this serum corresponded to 1:3200.

In preliminary experiments we determined the optimal dose and schedule for each conjugate vaccine without ISS. CHO–CRM vaccine gave the best results when administered 3 times (day 0, 10 and 20) at 2.5 µg/mouse, while CHO–TT did not capable to induce a significant specific response even when co-administered with ISS in BALB/c mice. Data were expressed relative to the arbitrary units (AU) of the amounts of soluble CHO from Hib, at odds with the literature indicated the i.d. as the route of injection of ISS controls: \(P < 0.05\) constantly with a low anti-CHO IgG production, the adjuvant effect of ISS was magnified (CHO–CRM + ISS versus CHO–CRM, \(P < 0.001\)). In the absence of ISS, the majority of BALB/c mice vaccinated with CHO–TT showed high levels of anti-CHO IgG, thus rendering less evident the adjuvanticity of ISS. The unconjugated CHO was not capable to induce a significant specific response even when co-administered with ISS i.d. in saline and s.c. in IFA (data not shown). In addition, no anti-CHO response was observed following immunization with the Hib CHO non-chemically bound to TT, irrespective on the co-administration of ISS the CHO/TT mixture resulted in elevated anti-TT (see below), but not anti-CHO antibodies.

The adjuvant effect of ISS was also tested in the outbred CD1 mouse strain. Inasmuch as in preliminary experiments CD1 mice showed good immune responses to the CHO-conjugate vaccines when immunized s.c. and data from the literature indicated the i.d. as the route of injection of ISS (26), we chose CD1 mice to test the adjuvant effect of ISS in dependence on the injection route. No significant differences in the mean anti-CHO IgG levels were observed following immunization with CHO-conjugate vaccine inoculated i.d. or s.c. (i.d. versus s.c., \(P > 0.05\)), but the number of responding mice was increased when the vaccine was given i.d. (Fig. 2A). When the effect of ISS was tested in dependence on the administration route, it was observed that the adjuvant activity was statistically significant only when ISS were administered without ISS. CHO

![Fig. 1. Specificity and sensitivity of anti-Hib CHO ELISA. (A) Increasing amounts of soluble CHO from Hib, at odds with N. meningitidis CHO (MenA), specifically inhibit the binding of reference sera. (B) Titration of the hyperimmune pooled serum used in all the assays as reference serum.](image-url)
In the vaccination adjuvants for CHO-conjugate vaccines. When ISS were co-
fist dose of vaccines, not only was the antibody response more vigorous, but also its persistence was enhanced. The adjuvant effect was evident in inbred as well as in outbred mouse strains, when the i.d. administration route was used. However, the adjuvant activity of ISS was clearly observed only when the CHO was chemically linked to a carrier protein: no anti-CHO antibodies were detected immunizing mice in the presence of ISS with CHO or CHO/ protein vaccine mixtures.

Dose-dependent adjuvant activity of ISS
BALB/c mice were immunized i.d. with CHO–CRM vaccine (2.5 µg/mouse) and different doses of ISS in the first inoculum to assess the minimal amount of ISS required for a measurable adjuvant activity. A significant increase of anti-CHO specific IgG levels was observed when ISS was used at 40 and 20 µg (CHO–CRM + ISS 40 µg and CHO–CRM + ISS 20 µg versus CHO–CRM, P < 0.001). Irrespective on the use of ISS, unconjugated CHO, even if mixed with anti-TT vaccine (mix CHO/TT) did not elicit a significant anti-CHO IgG response. Data are the mean values ± SD of at least two independent experiments. Mice were considered responders when the serum anti-CHO EU values were >2 times the mean EU value of non-immune mice. In each experiment 10 mice per group were used.

Table 1. Anti-CHO specific IgG after anti-Hib vaccination in the presence or absence of ISS

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Anti-CHO IgG (mean EU ± SD)</th>
<th>No. of responder mice (%)</th>
<th>Anti-CHO IgG in responding mice (mean EU ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.119 ± 0.004</td>
<td>0/20 (0)</td>
<td>–</td>
</tr>
<tr>
<td>CHO–CRM</td>
<td>0.382 ± 0.138</td>
<td>18/20 (90)</td>
<td>0.413 ± 0.112</td>
</tr>
<tr>
<td>CHO–CRM + ISS</td>
<td>1.018 ± 0.452</td>
<td>20/20 (100)</td>
<td>1.018 ± 0.452</td>
</tr>
<tr>
<td>CHO–TT</td>
<td>1.157 ± 0.96</td>
<td>25/30 (83.3)</td>
<td>1.225 ± 0.732</td>
</tr>
<tr>
<td>CHO–TT + ISS</td>
<td>1.560 ± 1.06</td>
<td>26/30 (86.6)</td>
<td>1.713 ± 0.961</td>
</tr>
<tr>
<td>CHO</td>
<td>0.149 ± 0.06</td>
<td>4/20 (20)</td>
<td>0.244 ± 0.064</td>
</tr>
<tr>
<td>Mix CHO/TT</td>
<td>0.098 ± 0.04</td>
<td>0/20 (0)</td>
<td>–</td>
</tr>
<tr>
<td>Mix CHO/TT + ISS</td>
<td>0.122 ± 0.08</td>
<td>1/20 (5)</td>
<td>0.329</td>
</tr>
</tbody>
</table>

BALB/c mice were immunized i.d. with 2.5 µg/mice of conjugated Hib CHO (CHO–CRM and CHO–TT), unconjugated Hib CHO (CHO) or Hib CHO mixed with 4 IU anti-tetanus vaccine (mix CHO/TT) in the presence or absence of 50 µg ISS in the first inoculum. Specific anti-CHO IgG were detected by ELISA at the end of the vaccination schedules and expressed as EU after normalization using an anti-CHO hyperimmune serum as reference serum. The co-administration of ISS with CHO–CRM vaccine increased the anti-CHO IgG response. (CHO–CRM versus control, P < 0.05; CHO–CRM + ISS versus control, P < 0.001; CHO–CRM + ISS versus CHO–CRM, P < 0.001). The immune response to the CHO–TT vaccine was greater than the response to the CHO–CRM vaccine in BALB/c mice. The use of ISS further increased the anti-CHO IgG levels (CHO–TT versus CHO–CRM + ISS, P < 0.05). Irrespective on the use of ISS, unconjugated CHO, even if mixed with anti-TT vaccine (mix CHO/TT) did not elicit a significant anti-CHO IgG response. Data are the mean values ± SD of at least two independent experiments. Mice were considered responders when the serum anti-CHO EU values were >2 times the mean EU value of non-immune mice. In each experiment 10 mice per group were used.

To determine the influence of ISS on the persistence of specific antibodies, CD1 mice were tested for the anti-CHO IgG levels 120 days after the vaccination (Fig. 2B). Four months after the vaccination, mice inoculated s.c. showed mean IgG levels higher than controls, but the effect of ISS on the persistence of specific antibodies was not statistically significant (s.c. and s.c. + ISS versus controls, P < 0.05; s.c. versus s.c. + ISS, P = 0.67). On the other hand, when the vaccine was administered i.d., the adjuvant effect of ISS in the persistence of specific antibodies was clearly evident (Fig. 2B). The mean of anti-CHO IgG levels in ISS-treated mice was higher than controls and the use of ISS increased significantly the maintenance of high IgG levels in a greater number of mice (i.d. versus controls, P < 0.05; i.d. + ISS versus controls, P < 0.001; i.d. versus i.d. + ISS, P < 0.05). Thus, it can be conceived that the i.d. administration is crucial for the adjuvant effect of ISS and that ISS administration in outbred populations increase the frequency of responders to the vaccination.

All together, these data indicate that ISS are effective adjuvants for CHO-conjugate vaccines. When ISS were co-administered in the first dose of vaccines, not only was the antibody response more vigorous, but also its persistence was enhanced. The adjuvant effect was evident in inbred as well as in outbred mouse strains, when the i.d. administration route was used. However, the adjuvant activity of ISS was clearly observed only when the CHO was chemically linked to a carrier protein: no anti-CHO antibodies were detected immunizing mice in the presence of ISS with CHO or CHO/ protein vaccine mixtures.

Influence of ISS on the dose of CHO-conjugate vaccines
Preliminary experiment showed that the use of 2.5 µg of CHO-conjugate vaccine resulted in the maximal antibody response. BALB/c mice were vaccinated with decreasing doses of CHO–CRM, in the presence of 50 µg/mouse of ISS in the first inoculum, to verify the adjuvant effect of ISS with lower amounts of the conjugate vaccine. The mean anti-CHO IgG level in mice vaccinated with doses as low as 0.67 µg of CHO–CRM in the presence of ISS was higher than that obtained with the standard 2.5 µg dose without ISS (data not shown). These data indicate that by the use of ISS the dose of CHO–CRM vaccine needed to obtain the same levels of serum anti-CHO specific IgG can be reduced to one-fourth.

Influence of ISS on the anti-CHO IgG subclasses detected following s.c. or i.d. immunization with conjugated vaccine
The specific IgG subclasses pattern induced by a vaccination is indirect evidence for the preferential type 1 or type 2 immune response evoked by the antigen and/or for the influence of the adjuvant. ISS are described as inducer of type 1 immune response. In this contest, it was interesting to analyze the IgG subclass production against a classical T-independent antigen such as a CHO upon the adjuvant effect of ISS in the CHO-conjugate vaccine model. When CD1 mice were immunized s.c. in the absence of ISS, IgG1 was the main anti-CHO IgG subclass detected (Fig. 4A). A single 50 µg dose of ISS in the first inoculum determined the increase of IgG2a and IgG2b with a dramatic increase of IgG3 (IgG2a s.c. versus IgG2a s.c. + ISS, P < 0.005; IgG2b s.c. versus
Fig. 2. Role of ISS in the production of anti-CHO specific IgG after the vaccination of CD1 mice with CHO–TT, in dependence on the administration route. (A) CD1 mice were vaccinated s.c. or i.d. with CHO–TT, in the presence or absence of ISS. Specific anti-CHO IgG were detected by ELISA at the end of the vaccination schedule (day 21) and expressed as EU after normalization using an anti-CHO hyperimmune serum as reference serum. Each bar represents the IgG production of a single mouse. Mice were considered responders when the serum anti-CHO EU values were >2 times the mean EU value of non-immune mice (dotted line: cut-off). The difference in the mean IgG levels obtained with CHO–TT i.d. or s.c. was not statistically significant (\( P > 0.05 \)) as well as the difference in the mean IgG levels of mice immunized s.c. with or without ISS, in spite of the fact that the number of mice responding to the vaccination s.c. in the presence of ISS was increased (cut-off = 0.279 EU). On the other hand, the presence of ISS in the i.d. administration significantly increased the mean level of anti-CHO IgG (i.d. versus i.d. + ISS, \( P < 0.05 \)). (B) The adjuvant role of ISS was even more evident when sera from mice immunized i.d. were tested 4 months after the vaccination. The i.d. immunization in the presence of ISS resulted in a marked increase in the number of mice with high levels of anti-CHO IgG (i.d. versus controls, \( P < 0.05 \); i.d. + ISS versus controls, \( P < 0.001 \); i.d. versus i.d. + ISS, \( P < 0.05 \)). On the contrary, in spite of the fact that mice immunized s.c. showed an anti-CHO IgG level significantly higher than untreated controls (cut-off = 0.229 EU), no statistically significant difference was observed in dependence on the use of ISS (s.c. and s.c. + ISS versus controls, \( P < 0.05 \); s.c. versus s.c. + ISS, \( P = 0.67 \)). Data are representative of two independent experiments. In each experiment eight mice per group were used.

IgG2b s.c. + ISS, \( P < 0.01 \); IgG3 s.c. versus IgG3 s.c. + ISS, \( P < 0.001 \). When the vaccine was administered i.d. (Fig. 4B) an IgG3 level higher than that obtained with the administration s.c. was observed. A single 50 µg dose of ISS in the first inoculum determined an anti-CHO IgG subclasses profile that characterizes type 1 responses. In particular, a relative increase of IgG2a and IgG3 was observed (IgG2a i.d. versus IgG2a i.d. + ISS, \( P < 0.001 \); IgG2b i.d. versus IgG2b i.d. + ISS, \( P = 0.4 \); IgG3 i.d. versus IgG3 i.d. + ISS, \( P < 0.05 \)). Interestingly, ISS did not affect the mean anti-CHO IgG1 production, that remained at a level similar to that obtained in their absence, irrespective of the route of inoculum.
Fig. 3. Adjuvant activity of ISS depending on the dose. ISS were used at decreasing amount (40, 20, 10, 5, 2.5 and 1.25 µg/mouse) in the first i.d. inoculum in six groups of BALB/c mice (five mice per group), with the standard 2.5 µg dose of CHO–CRM vaccine. The results are expressed as the percentage increase of anti-CHO IgG levels in mice immunized with different doses of ISS, with respect to the mean IgG level of mice vaccinated without ISS (*P < 0.05; **P < 0.005).

Fig. 4. Role of ISS in the production of anti-CHO IgG subclasses, in dependence on the administration route. CD1 mice were immunized with CHO–TT and anti-CHO-specific IgG subclasses detected by ELISA. The anti-CHO subclass levels of a reference serum were arbitrarily defined as 1000 U for IgG1, IgG2a and IgG2b, and 100 U for IgG3. The results are expressed relative to the AU of the reference serum. (A) Mice immunized s.c.: the presence of ISS in the vaccine significantly increased the specific anti-CHO IgG2a, IgG2b and IgG3. The anti-CHO IgG1 level was not affected. (n.s. = non significant; **P < 0.005; ***P < 0.001). B) Mice immunized i.d.: the presence of ISS in the vaccine significantly increased the specific anti-CHO IgG2a and IgG3. The IgG1 and IgG2b levels were not affected (n.s.; ***P < 0.001; ****P < 0.05). Data are mean values ± SD of two independent experiments. In each experiment eight mice per group were used.

Influence of ISS on the antibody response against the carrier protein

We examined whether the increased anti-CHO response obtained in the presence of ISS paralleled the anti-carrier IgG synthesis. As shown in Fig. 5, the use of ISS determined a significant increase of anti-CRM IgG both in BALB/c and CD1 mice (BALB/c versus BALB/c + ISS, P < 0.005; CD1 versus CD1 + ISS, P < 0.001). These data matched a significant increase of anti-TT IgG both in BALB/c and CD1 mice (CHO–TT versus CHO–TT + ISS, P < 0.05) immunized with CHO–TT vaccine in the presence of ISS. In spite of the fact that no anti-CHO IgG was measurable upon vaccination with mixed CHO/TT, the 4 IU anti-tetanus vaccine used in the mixed CHO/TT induced anti-TT antibody levels similar to those obtained with CHO–TT conjugate vaccine. Overall these data indicate that the use of ISS increases the antibody responses to both the conjugated CHO and the carrier.

Influence of ISS on serum cytokine levels in mice vaccinated with conjugated Hib CHO

It has been shown that one of the reasons for the adjuvant effect of ISS is their ability to induce the synthesis of cytokines by APC, B lymphocytes and NK cells. We measured the in vivo cytokine production after the administration of ISS alone or the CHO–TT vaccine in the presence or absence of ISS. Table 2 shows the serum cytokine levels in CD1 mice treated with CHO–TT vaccine (two doses: day 0 and 14), with ISS (ISS day 0 and saline at day 14) or with CHO–TT vaccine and ISS in the first inoculum. Collection of samples started 6 h after the vaccination. Mice immunized with CHO–TT vaccine in the absence of ISS showed a peak of cytokines release at 24 h, with IL-6 and IFN-γ already detectable at 6 h. At 48 h the serum cytokine level was lower than at 24 h. There was a low IFN-γ serum release and a detectable (~10 pg/ml) production of IL-5 at 24 h after the injection, that was mainly characterized by a continuous release of IL-5 from 6 to 72 h after the first inoculum; no measurable IL-5 was detected (data not shown). The co-administration of vaccine and ISS caused the release of high cytokine levels already detectable at 6 h. The highest levels of IL-12 and IFN-γ were respectively observed at 6 and 48 h after the injection. No measurable IL-5 was detected (data not shown). These data indicate that the i.d. inoculum of ISS induces the systemic release of type 1-defining cytokines and that the serum release is enhanced when the conjugated vaccine is co-administered.
This finding can be considered as a consequence of a type 1-like immune response (19) induced by ISS. It is remarkable that these IgG subclasses fix the complement (20) and their production is particularly desirable in anti-capsulated bacteria immune responses.

We also studied the cytokine secretion after administration of ISS alone or ISS and CHO–TT vaccine, in comparison to the CHO–TT vaccine in the absence of ISS. We confirmed that ISS induce the production of IL-12 and IFN-γ, that define type 1 immune responses, but with a kinetics of secretion slightly different to that previously described (21–23), probably because of the different route of inoculum (i.d. in our experiments, i.p. in others). In addition, we could demonstrate that the production of IL-12 and IFN-γ was greatly increased when ISS was administered together with the CHO–TT vaccine, indicating that ISS and the vaccine have a summative stimulating activity on the innate immunity (24).

The effects of ISS on the anti-CHO immune response can be attributed to the amplification of the T help provided by the carrier. We observed in fact that the increase of anti-CHO antibodies paralleled the increase of anti-carrier antibodies in the presence of ISS. Thus, our data confirm the hypothesis that after immunization with CHO-carrier conjugate vaccines, anti-CHO-specific B cells precursors receive help by CD4+ T cells (T<sub>h</sub>) that are simultaneously activated by the carrier processed and presented by professional APC. In addition, anti-CHO-specific B cells, capturing the conjugate vaccine through the CHO, could process the carrier. CHO-specific B cells could then present epitopes derived from the carrier to carrier-specific T<sub>h</sub> cells, thus receiving help in a cognate interaction (25). In any case, spatial and temporal vicinity between CHO-specific B and carrier-specific T cells are required: the activation of the innate immunity and the cytokine milieu induced by ISS were not sufficient to trigger B cells to initiate an anti-CHO antibody production when using uncon-
jugated CHO, even if administered s.c. in IFA (26), or non-chemically bound to anti-tetanus vaccine. Hib capsular CHO behaves like an apten and our results confirm that cell-to-cell contacts of B cells, T cells or APC are essential to determine B cell maturation and Ig secretion even in the presence of soluble help provided by cytokines (27).

Finally, we showed that the inoculation route is relevant for both the anti-CHO response and the adjuvant effect of ISS. In general, the administration i.d. was shown to be more effective than the s.c. in inducing anti-CHO antibody and in maintaining high amount of detectable anti-CHO antibodies at the end of a 4 month follow-up. The adjuvant effect of ISS, measured as an increment of anti-CHO antibody levels, was significant when vaccines were administered i.d., but the anti-CHO subclass pattern was influenced by ISS also when administered s.c.. Differences in antigen-specific antibody levels have already been shown in dependence on the route of both soluble and DNA-based vaccine delivery (16,28). A possible interpretation of these differences may rely on the assumption that antigens encounter different APC when delivered in different body compartments. Vaccines administered i.d. have probably more chance to be captured by dermal dendritic cells (DC) and be delivered to regional lymph nodes (18). Antigen presentation by highly efficient APC such as DC (29) and the maintenance of antigen memory in terms of MHC-peptide complexes (30) on the membrane of mature DC for long periods may be responsible for the differential outcome of the i.d. vaccination.

In conclusion, this study demonstrates that the response to a CHO can be modulated by ISS, if the CHO is conjugated to a carrier protein. This observation gives indirect further evidence on the need of cell-to-cell contact between CHO-specific B cells, APC and carrier-specific T\(_h\) cells for the differentiation of plasma cells secreting anti-CHO antibodies. Finally, this work provides first evidence for the ability of ISS to induce an anti-CHO type 1-like immune response and shows that the adjuvant activity of ISS in the immune response to proteins can be exploited to foster anti-CHO responses when using CHO-carrier conjugate vaccines. Shams and Heron (31) showed that Hib-conjugate vaccines induced neutralizing antibody against the carrier protein (TT). However, serum levels of anti-TT antibody were shown (32–34) to be so low to require further anti-TT vaccinations to reach protective levels. It is tempting to speculate that the use of ISS could be useful in vaccines composed by a CHO conjugated to a carrier protein (such as TT or CRM) to obtain a simultaneous serum levels of anti-TT antibody were shown (32) to be so low to require further anti-TT vaccinations to reach protective levels. It is tempting to speculate that the use of ISS could be useful in vaccines composed by a CHO conjugated to a carrier protein (such as TT or CRM) to obtain a simultaneous

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## Abbreviations

- **APC**: antigen-presenting cells
- **CHO**: polysaccharide
- **CRM**: cross-reacting material
- **DC**: dendritic cells
- **DT**: diphtheria toxoid
- **EU**: ELISA units
- **Hib**: *Haemophilus influenzae* type b
- **i.d.**: intradermal injection
- **IFA**: incomplete Freund’s adjuvant
- **ISS**: immunostimulatory sequences
- **ODN**: oligodeoxynucleotides
- **TT**: tetanus toxoid

## References

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