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The immunomodulating properties of human respiratory syncytial virus and immunostimulating complexes containing *Quillaja* saponin components QH-A, QH-C and ISCOPREP™703

Ke-Fei Hu a,d,*, Matthias Regner b, Claire-Anne Siegrist b, Paul Lambert b, Margaret Chen c, Karin Lövgren Bengtsson d, Bror Morein a,d

a Section of Virology, Department of Veterinary Microbiology, College of Veterinary Medicine, Swedish University of Agricultural Sciences, Biomedical Center, Box 585, S-751 23 Uppsala, Sweden

b Center for Vaccinology and Neonatal Immunology CMU, 1 rue Michel-Serret, 1211 Genesa 4, Switzerland

c Department of Vaccine Research, Swedish Institute for Infectious Disease Control, Nobelsväg 18, S-171 77 Stockholm, Sweden

d ISCONOVA AB, Uppsala Science Park, Dag Hammarskjöld’s väg 54A, S-751 83 Uppsala, Sweden

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Keywords: Vaccine; ISCOMs; Cytokine; Th1; Th2

1. Introduction

Human respiratory syncytial virus (HRSV) is a major causative agent of viral lower respiratory tract infection in infants, young children, elderly people as well as immuno-compromised individuals worldwide [1,2]. Similarly bovine respiratory syncytial virus (BRSV) is an important pathogen infecting young calves, resulting in serious economic loses [3]. Attempts to develop a HRSV vaccine have so far been unsuccessful and effective RSV vaccines for bovines are lacking [4]. It has been widely accepted that a strong bias towards a type 2 T helper (Th2) cell response is a critical factor for the disease exacerbation observed after testing of a formalin-inactivated HRSV vaccine candidate [4]. Similar situation was also recorded with a formalin-inactivated BRSV...
commercial vaccine tested in calves [5,6]. Recent studies indicated that IFN-γ (Th1 cytokine) may also contribute to immunopathogenesis [7,8]. Therefore, a successful RSV vaccine is likely one that induces a balanced Th1/Th2 response. In practice, the fine-tuning of vaccine formulation is likely the key to achieve the goal.

Immunostimulating complexes (ISCOMs) induce primarily Th1 or balanced Th1/Th2 responses driven by IL-12 production. The final outcome is also dependent on the antigen used [9,10]. The ISCOM based on enveloped viruses usually contains the envelope proteins, lipids and Quillaja saponins. Through hydrophobic interaction, these three elements form particles of about 40 nm in diameter [11,12]. The comprised Quillaja saponins (Quil A), a purified product from the crude extract of the bark of South American tree Quillaja saponaria Molina, consist of a heterogeneous mixture of saponins, e.g., 23 HPLC fractions have been isolated [13]. All of these components have been found to possess various degrees of adjuvant activities. In particular, three fractions with diverging properties, designated QH-A, QH-B and QH-C, were identified by reverse phase high performance liquid chromatography (RP-HPLC), of which, QH-A and QH-C were regarded of major interest. QH-B was a fraction found to have significant toxicity, eliminating its use as an adjuvant [14]. Further studies [15,16] showed that ISCOM-MATRIX made with QH-A induced higher production of IL-1 and IL-6 than ISCOM-MATRIX made with QH-C in murine peritoneal cells (cytokines known to be important for the early activation of Th-cells [17]).

Later, a more complex picture was revealed by Johansson and Lovgren-Bengtsson [18], using ovalbumin (OVA) ISCOMs formulated with QH-A, QH-C and a formulation designated as 703 ISCOMs [19]: QH-A ISCOMs strongly primed spleen cells to proliferate and to produce the typical Th1 cytokines IL-2 and IFN-γ after in vitro stimulation with OVA. However, this strong induction of Th1 cytokines was not reflected by an enhanced production of OVA-specific IgG2a serum antibodies compared to IgG1 antibodies. QH-C ISCOMs, on the other hand, induced strong antibody responses with a high IgG2a component whereas the corresponding in vitro T cell responses were characterized by a relatively low IFN-γ production. The 703 ISCOM induced the highest titers of IgG2a antibodies with a cytokine profile similar to that of QH-C ISCOMs. Although contrasting with the generally observed correlation of cytokine secretion and IgG isotype profile, these results suggest the possibility of generating different classes of ISCOMs with a range of immunomodulating properties, depending on the Quil A saponin fractions used. In the present study, we examined the potential of ISCOMs containing antigens from human RSV fusion (F) and glyco (G) envelope proteins, and made from each of these three Quillaja saponin fractions to induce differential innate and adaptive immune responses. These observations may further our understanding required for the development of successful RSV vaccines.

2. Materials and methods

2.1. Virus and cells

The Long strain of RSV (ATCC VR-26), kindly supplied by Dr. Claes Örvell (Huddinge University Hospital, Stockholm), was propagated on MA 104 cells (ECACC No. 85102918). The cells were grown in full Dulbecco’s modified Eagle medium (FDMEM, National Veterinary Institute, Uppsala, Sweden), supplemented with 100 µg/ml of kanamycin, 2 mM glutamine, and 10% fetal calf serum (Gibco-BRL, Life Technologies AB, Täby, Sweden).

Murine macrophage cell line, RAW 264.7 purchased from ECACC was grown in the same medium as used for MA 104 cells.

2.2. Mice

Female BALB/c mice, 8–12 weeks of age, were obtained from the Biomedical Center, Uppsala, Sweden. The mice were tested free from viral, bacterial and mycoplasma infections and kept in accordance with the national guidelines.

2.3. Quillaja saponin fractions

Three defined Quillaja saponin preparations, QH-A, QH-C, QH-703 (ISCOPREP™703) and the semi-purified Quillaja saponin (Spikoside) containing roughly 25% QH-A, 50% QH-B and 25% QH-C were supplied by ISCONOVA AB (Uppsala, Sweden).

2.4. Preparation of HRSV ISCOMs with Quillaja saponin components QH-A, QH-C ISCOPREP™703 and Quil A

HRSV ISCOMs with different Quillaja saponin compositions (A, C, AC i.e., ISCOPREP™703 and Quil A) were prepared from sucrose gradient purified HRSV, essentially using the method described previously [20,21]. Briefly, 2 ml (1.6 mg/ml) purified HRSV was solubilized with OG (1-O-n-octyl-β-D-glucopyranosid, C14H28O6, Boehringer, Mannheim, GmbH, FRG) at a final concentration of 2% (w/v) for 1 h at 37 °C under constant agitation. The solubilized virus was applied onto a discontinuous sucrose gradient of 2 ml 20% sucrose layer containing 0.5% OG over a cushion of 50% sucrose. After centrifugation at 210,000g at 4 °C in a Kontron TST-41 rotor for 1 h, the sample volume together with the 20% sucrose layer containing viral proteins were collected,
and extra lipids, i.e., cholesterol and phosphatidylcholine, and *Quillaja* saponin, i.e., QH-A or QH-C or ISCOM-REP™703 were added in proportions of protein:cholesterol:phosphatidylcholine:Quillaja saponin = 1:1:1:5 calculated by weight. After extensive dialysis against 0.15 M ammonium acetate at 4°C for 72 h, ISCOMs were formed by hydrophobic interaction among the components. The ISCOM were purified by centrifugation through 10% sucrose at 210,000g in Kontron TST-41 rotor at 10°C for 18 h. The pellet containing the ISCOMs was re-suspended in 200 µl PBS. Protein content in the ISCOMs was determined by amino acid analysis (Aminosyraanalyslaboratoriet, Uppsala, Sweden). Samples of the ISCOMs were submitted for negative staining electron microscopy and their migration patterns were analyzed in 50 to 10% sucrose gradient. Samples of the three ISCOMs were also coated to an ELISA plate and monoclonal antibodies against HRSV F and G envelope proteins of HRSV were used to detect the relative content of HRSV F and G protein in the ISCOMs.

2.5. In vitro stimulation of RAW 264.7 cells with the ISCOMs and detection of released proinflammatory cytokines

The cell concentration was adjusted to 1 × 10^6 cells/ml in medium with 2% FCS, and added to 96 well tissue culture plates (Nunc, Roskilde, Denmark), 100 µl/well. Equal volumes of stimuli with protein concentration of 5 µg/ml and/or saponin concentration of 25 µg/ml were mixed with the seeded cells in duplicated wells. The supernatants were collected after incubation for 24 hours at 37°C in a humid atmosphere with 7% CO₂. The cytokines (mouse GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, TNF-α) were determined with reagents from Bio-Rad 8-plex B assay, and the amount of cytokines were expressed as pg/ml calculated from the standard curves generated in the assay.

2.6. Immunizations

Six groups of mice (1–6), each consisting of five BALB/c mice were immunized subcutaneously (s.c.) twice, 6 weeks apart as follows:

Group 1: 1 µg/mouse of QH-A HRSV ISCOMs.
Group 2: 3 µg/mouse of QH-A HRSV ISCOMs.
Group 3: 1 µg/mouse of QH-C HRSV ISCOMs.
Group 4: 3 µg/mouse of QH-C HRSV ISCOMs.
Group 5: 1 µg/mouse of ISCOM-REP™703 HRSVISCOMs.
Group 6: 3 µg/mouse of ISCOM-REP™703 HRSVISCOMs.

Mice in group 7 were kept under the same conditions as un-immunized control.

2.7. Collection of blood and spleen cells

Blood samples for serum were collected twice from retroorbital plexus, 3 weeks after the first immunization and 2 weeks after the second immunization. For T-cell responses, spleen cells were harvested three weeks after the second immunization when the experiment was terminated. The selection of time points for the samplings was based on previous experience [21].

2.8. ELISA for serum anti-HRSV antibodies

Enzyme-linked immunosorbent assays (ELISA) to determine HRSV specific serum antibodies (total immunoglobulins, IgG1 and IgG2a subclasses) were carried out essentially using the method described by Hu et al. [20]. Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with 200 ng/well of purified HRSV in 100 µl PBS containing 0.05% Tween 20 (PBST). All incubations were done at room temperature, under constant shaking, for 60 min. The mouse sera were titrated in threefold dilutions. ELISA employed to determine the total antibody response to HRSV was carried out with a HRP conjugated rabbit anti-mouse immunoglobulins (DAKO A/S, Denmark), whereas IgG1 and IgG2a responses were carried out with biotinylated goat anti-mouse IgG1 and IgG2a antibodies (Southern Biotechnology Associated, Inc., Birmingham, USA), and a HRP streptavidin (Dako-patts, Glostrup, Denmark). The enzyme reactions were visualized by adding 100 µl/well of tetramethylbenzidine (TMB) substrate buffer (TMB, H₂O₂, SVANOVA, Uppsala, Sweden) for 10 min. The reactions were terminated by the addition of 50 µl/well of 2 M H₂SO₄. The optical density was measured at 450 nm with a Titertek Multispec Spectrophotometer (Flow Laboratories, Irvine, Scotland). The titers were expressed as reciprocals of endpoint dilution.

2.9. HRSV specific proliferation in vitro

Spleen cells collected from the mice two weeks after the second immunization were cultured in triplicates in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) at 2.5 × 10⁵ cells/well concentration of 2.5 × 10⁵/well in a total volume of 200 µl per well containing 1 µg/ml or 5 µg/ml UV-inactivated HRSV. The plates were then incubated at 37°C in a humid atmosphere with 7% CO₂. After incubation for 72 h, the cells were pulsed with ³H]thymidine and 16 h later the cells were harvested. The incorporation of thymidine was determined by liquid scintillation. The stimulating index (SI) was calculated by dividing the [³H]thymidine incorporation (expressed as CPM) of cells re-stimulated with
HRSV by CPMs of cells from the same spleen cultured without the antigen.

2.10. Th cytokine production by spleen cells from mice immunized with HRSV ISCOMs and re-stimulation in vitro

Spleen cells from mice collected two weeks after the second immunization were cultured in vitro with or without HRSV as described for the proliferation assay. After incubation for 48 h, the cell culture supernatant was collected and the cytokines IL-4, IL-5, and IFN-γ in the supernatant were measured by a capture ELISA using the reagents from Endogen (Woburn, MA, USA) according to the manufacturer’s instruction. Briefly, microtitre plates (Maxisorp, Nunc, Denmark) coated with monoclonal antibodies against the cytokines and blocked with Assay Buffer were incubated with the supernatant at room temperature overnight and the cytokine standards were also run in parallel with the samples. Bound cytokines were detected by incubation with biotinylated anti-mouse IL-4, IL-5, and IFN-γ antibodies for two hours followed by incubation with HRP-conjugated streptavidin for 1 h. The reaction was visualized by adding substrate buffer (K-blue, SVA-NOVA, Uppsala, Sweden) and 2 M H₂SO₄. The OD values at 450 nm were measured with the spectrophotometer. Concentrations of cytokines in the culture fluid were calculated from interpolation of linear parts of the cytokine standard curves and expressed as pg (picogram)/ml.

2.11. Statistical analysis

ELISA titers are expressed as means with standard errors of means (SEM), and were compared among different groups with respect to the levels of antibody by Mann–Whitney U two-tailed test. All calculations and graphic presentations were run on a Macintosh computer using Prism software version 2.0 (GraphPad Software, Inc, San Diego, CA, USA).

3. Results

3.1. The three ISCOMs are morphologically identical and all incorporate more F protein than G protein

No morphological differences were observed among the three ISCOMs. All showed typical ISCOM structures, i.e., cage-like spherical particles with a diameter of around 40 nm (Fig. 1(a)). The RSV antigens and ISCOM structures were found in the same fraction of a sucrose gradient centrifugation. In agreement with our previous observation [20], both F and G glycoproteins were incorporated into each of the ISCOMs. The three ISCOM formulations contained more F than G proteins. The ratio F/G declined slightly in the order QH-A > QH-C > 703-ISCOMs (Fig. 1(b)).

3.2. ISCOMs stimulate RAW 264.7 cells to produce a number of pro-inflammatory cytokines

Fig. 1. (a) The ISCOM particles. All three ISCOM formulations showed identical cage-like spherical particles with a diameter of around 40 nm under electronic microscope. This is a representative photo of the three ISCOMs. (b) The relative F and G envelope protein content in three HRSV-ISCOM formulations as measured by anti-F and -G monoclonal antibodies. Different HRSV-ISCOMs were made from *Quillaja* saponins, QH-A, QH-C resp.703 and coated to ELISA plates. The incorporation of F and G proteins was detected by anti-F and anti-G monoclonal antibodies. The relative contents of F and G in 1 μg of total protein was measured as OD value at 450 nm.

The murine macrophage cell line, RAW 264.7 was used to determine the capacity of the different ISCOM formulations to induce secretion of eight selected cytokines. LPS was used as a positive control showing that the cells were able to produce all cytokines investigated but IL-2. The predominant proinflammatory cytokine induced by C and 703 ISCOM formulations was TNF-α, which was produced at an almost 100-fold higher level than after LPS stimulation. Purified inactivated HRSV was a weaker inducer of cytokines than ISCOMs made from Quil A (semi-refined saponin), which strongly induced secretion of a number of cytokines, most notably IL-1β, TNF-α, GM-CSF and IL-5. Both 703 and QH-C
ISCOMs induced a cytokine secretion pattern that closely resembled that induced by Quil A ISCOM, whereas QH-A ISCOM triggered a cytokine profile strikingly different from the other ISCOM formulations, i.e., TNF-α was absent, which was the predominant cytokine after stimulation with QH-C and 703 ISCOMs. IL-5 was substantially reduced. In general, the QH-A ISCOM-induced a cytokine pattern that more resembled the profile induced by purified HRSV than the profiles induced by the other ISCOM formulations. IL-10 production triggered by the various ISCOM formulations as well as by LPS was insignificant compared to the background from the control cells (Fig. 2).

3.3. The RSV 703 ISCOMs are the strongest inducer of total anti-RSV antibodies and IgG2a

The total serum antibody responses induced by the three tested ISCOM formulations were measured at week 3 after the first immunization and at week 2 after the second immunization by the ELISA. In the majority of cases, significant ($P < 0.05$) differences were found between the different ISCOM formulations when the groups of animals immunized with the same dose (1 or 3 µg) were compared, i.e., 703 ISCOMs > QH-C ISCOMs > QH-A ISCOMs. For details, see Fig. 3.

This picture was also corroborated by the IgG2a levels, i.e., 703 ISCOMs induced the strongest IgG2a response followed by QH-C and QH-A ISCOMs (Fig. 4).

3.4. The three ISCOM formulations differ in their capacity to prime T cell proliferation

We next tested the ability of the three different ISCOM preparations to prime T cells for proliferative responses to in vitro stimulation with inactivated HRSV as antigen (Fig. 5). With the high antigen restimulation dose of 5 µg/ml, splenocytes from mice primed in vivo with QH-A ISCOMs (group 1 and 2) proliferated most
strongly. Splenocytes from mice immunized with 703 ISCOMs were the only ISCOM formulation, which responded with proliferation to 1 μg/ml of the antigen (group 6). Splenocytes from QH-C ISCOMs immunized mice exhibited low proliferative responses to in vitro restimulation compared to the other two formulations.

In general, an increasing sensitivity to the low dose of antigen used for the restimulation was observed in the order QH-A, QH-C and 703 (Fig. 5).

The cytokine pattern showed that all three formulations gave rise to strong IFN-γ production (more than 10,000 pg/ml), but comparatively weaker IL-4 (<200 pg/ml) and IL-5 (<750 pg/ml) responses. The IFN-γ/IL-4 and IFN-γ/IL-5 ratios indicated that 703 ISCOMs were the strongest inducer of Th1 cytokines, followed by QH-C and QH-A ISCOMs. The various degrees of Th1 dominated cytokine responses are in agreement with the IgG2a levels in the serum (Fig. 6).

4. Discussion

Successful RSV vaccines need to be able to induce a Th1 skewed or Th1/Th-2 balanced immune response. It is generally conceived that the enhanced immunopa-thology observed in clinical trials of a formalin-inacti-vated RSV vaccine candidate was, at least in part, due to a strong bias of Th2 cytokine type of response. Therefore, extensive research has been focused on Th1-driving adjuvants and antigen delivery systems, tailoring a desired balance of cytokine responses. In the present study, a group of HRSV ISCOMs containing the different Quillaja saponins namely QH-A, QH-C and a combination of QH-A and -C (703) were generated with a record of Th1 profiles but still exhibiting diverging immuno-modulating properties [15,18].

The acquired immune response cannot start without a preceding innate immune response. The innate response is triggered by antigen presenting cells (APCs) exposed to, e.g., adjuvants and immunogens, resulting in antigen presentation and cytokine production, which in turn initiates the acquired immune response. Therefore, the innate responses has the potential to be used to make preliminary predictions of the capacity of vaccine formulations to induce acquired immune responses, i.e., the goal of vaccination [22]. Thus, cytokines produced by the innate cells in response to stimulation by vaccine formulations will quantitatively and qualitatively determine the nature of subsequent acquired immune responses. We have previously shown that HRSV ISCOMs stimulate murine peritoneal cells to produce proinflammatory cytokines, such as IL-1α, IL-6, and TNF-α [23]. Studies carried out by Villacres-Eriksson et al. [24] using influenza virus envelope antigens and by Smith et al. [25] using OVA-ISCOMs for parenteral
and mucosal modes of immunization showed a strong Th1 profile with IL-12 as a key factor. This was further corroborated in the present study in a murine macrophage cell line. In addition, here we observed a marked diversity in the capacity of ISCOMs comprising different fractions of Quil A saponin to induce a range of cytokines. Both QH-C and 703 were potent inducers of TNF-α and IL-5 and retained a secretion pattern similar to that of Quil A, whereas QH-A ISCOM only induced low levels of cytokine secretion. This suggests that the adjuvanticity of the comparatively crude Quil A may reside in the different sub-components. None of these formulations induced IFN-γ production, which was observed with a strong Th1 control antigen Mycoplasma mycoides subsp. mycoides (MmmSC) causing contagious bovine pleuropneumonia (CBPP) (un-published observation). This indicates that these ISCOMs are not as potent as CBBP as a Th1 inducer, at least in the macrophage cell line tested. IL-12 was absent even with CBBP, which may reflect the cell line's limitation. Whether such an innate cytokine profile is advantageous for optimal priming of an anti-RSV memory response needs to be judged by further studies including immunopathology. The OVA is a weak antigen and might, therefore, better reflect the inherent modulatory effect of an adjuvant.

The antibody responses and cytokine profile indicate that various degrees of Th1 biased immune responses have been evoked in mice by the three formulations. The strongest Th1 inducer are 703 ISCOMs followed by QH-C and QH-A ISCOMs. This contrasts with a report by Johansson and Lovgren-Bengtsson [18], in which OVA ISCOMs instead of RSV ISCOMs were used. They documented that the strongest proliferative as well as IFN-γ responses were induced by QH-A ISCOMs. The biggest difference between our and their studies is the antigens used. OVA is known as a weak antigen with little immunomodulating capacity. RSV, on the other hand, particularly when a large amount of F protein is incorporated, will assert strong Th1 modulating effect. The combined effect between the adjuvant and antigen has to be taken into consideration. Possibly requiring higher doses of QH-A to overcome or complement the immunomodulatory effect of RSV antigens, which is possible in view of that QH-A is very well tolerated even by new-born mice.

BRSA ISCOMs formulated with the crude Quillaja saponin abstract namely Quil A has been tested in two separate animals trials conducted in calves (Hu et al. submitted; Willman et al. Vaccine, in press). Full protection was achieved in these trials. In this case, serum virus neutralizing antibodies and IgA at high levels were detected in the vaccinated animals. However, in another trial conducted in Balb/c mice with HRSA ISCOMs prepared with the crude saponin seemed to give some side effects while virus replication was inhibited [26]. Therefore, caution should be observed when predicting vaccine performance from result obtained from one species to another or even in the mouse model, which strain or inbred strain is used.

It is also possible to generate other HRSA ISCOM formulations with various saponin fractions with immuno-modulating properties diverging from the three tested ones. Animal experiments aiming at examining their protective potential and immunopathological profiles of the thee ISCOM formulations are currently underway, which will guide us to find a formulation inducing the desired immunity in the target species, i.e., man and cattle. The prospect to formulate an RSV ISCOM formulation with an optimal Th1/Th2 balance is in reach particularly in view of the versatile properties of the ISCOM concept.

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