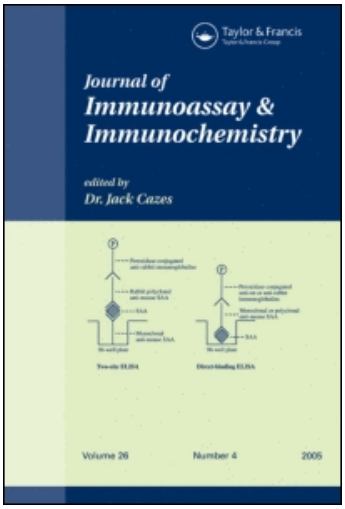

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USING DISTILLED WATER FOR THE EXTRACTION OF MUCOSAL ANTIBODIES AND THE SUBSEQUENT APPLICATION IN RSV NEUTRALIZATION TEST

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ABSTRACT

Virus neutralization (VN) is an important functional test for evaluating RSV vaccines, also encompassing in mucosal secretion of the respiratory tract considering the infection route. In our previous study, an immunogloblin extraction method described by Bergquist et al. was adopted for RSV ELISA, but it was not suitable for virus neutralization test due to the cell toxicity of the 2% saponin solution used for the antibody extraction. In order to overcome this problem, several solvents including distilled water were tested in the present study for the capacity to extract immunogloblins. Antibodies in the extracts were evaluated and compared by ELISA. Distilled water was as efficient as the 2% saponin solution for extraction of total IgA, RSV specific IgA and IgG. More importantly, the organ extracts obtained subsequently could be used for virus neutralization test without causing adverse effect on the cell culture. Therefore, distilled water was finally chosen as the solvent for immunogloblin extraction from mucosal organs when both ELISA and virus neutralization test are required.

(*KEY WORDS*: Respiratory syncytial virus (RSV), Immunostimulating complexes (ISCOMs), Saponin, Antibody extraction, ELISA, Virus neutralization (VN) test)

INTRODUCTION

The induction of virus neutralizing antibodies is an important indicator for efficacy of an respiratory syncytial virus vaccine. Besides reflecting a protective property, lack of protective antibodies may contribute to exacerbation of the disease upon subsequent infection (1,2).

A method for extraction of cytokines (3) and antibodies (4) from lung and intestine was recently developed. Samples based on organ extracts prepared according to this method are larger in volume, easy to obtain and higher in immunogloblin content compared with organ lavage. They are suitable for testing in ELISA and contamination from blood is less than 2% (4), but not for virus neutralization assay where cell toxicity becomes a major obstacle due to 2% saponin included in the solvent used for the antibody extraction. Another less toxic or non-toxic solvent must, therefore, be found, which is suitable for both ELISA and virus neutralization assay.

Thus, our present study was designed to find a solvent to replace the saponin containing solvent. The solvents tested were compared and evaluated in ELISA with regard to capacity to extract total IgA, anti-RSV IgG and IgA. These extracts were analyzed for utility in virus neutralization assay.

MATERIALS AND METHODS

Virus and Cells

The long strain of human RS virus (ATCC VR-26) kindly supplied by Dr Claes Örvell (Huddinge University Hospital, Stockholm) was propagated on MA 104 cells (kidney, Rhesus. ECACC number 85102918). 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 of

glutamine, and 10% fetal calf serum (GibcoBRL, Life Technologies AB, Täby, Sweden).

Mice

Twenty 25-week-old female BALB/c mice used for extraction of non-specific IgA, another twenty female BALB/c mice, 8-12 weeks of age used for RSV ISCOM immunization and extraction of anti-RSV IgG and IgA, were obtained from the Biomedical Centre, Uppsala, Sweden. The mice were screened for viral, bacterial and mycoplasma infections, and kept in accordance with the national guidelines.

Immunizations

Fifteen mice were immunized twice 6 weeks apart intranasally (i.n.), with 5 μg/mouse of RSV ISCOMs (Hu et al., manuscript in preparation). Another 5 mice were kept under the same condition without immunization as control group.

Preparation of Organ Extract

The secretory antibodies were extracted according to Bergquist et al. (4). The mice were injected intraperitoneally with 0.1 ml 1% heparin-PBS under anaesthesia. The mice were exsanguinated, and perfused with 20 ml 0.1% heparin-PBS into the right chamber of the heart through the circulation system. The lungs were removed and trimmed. Each pair of lungs was kept in 3 ml 0.1% heparin-PBS and the weights were recorded. The organs were washed with PBS and stored at -20 °C. Before testing, different concentrations of saponin (Spicoside, Luleå, Sweden) in PBS solution or distilled water was added at a ratio of 1μl/mg of organ weight to corresponding groupwise organs. After overnight extraction at 4

°C, the lung tissue was spun down at 13000 rpm for 10 minutes in a Eppendorf 5415C centrifuge, and the supernatant was collected for antibody analysis.

The upper respiratory tract was excised from the skull by removing the skin and muscles of the lower jaw, the brain and surrounding bones. The sinuses and the nose were treated as described for lungs. For samples to be assayed for virus neutralizing antibody, only distilled water was used for extraction of antibodies to avoid the lytic and cell toxic activity.

Determination of the Total IgA in Lung Extract by A Capture ELISA

Twenty 25-week-old female BALB/c mice were sacrificed and their lung extracts were prepared. The mice were divided into 4 groups (5 mice/group). The left and the right lungs were matched and kept separately. Different solvents used for antibody extraction were as following: Group A, both left and right lungs were treated with 2% saponin; Group B, left lungs were treated with 1% saponin, right lungs were treated with 2% saponin; Group C, left lungs were treated with 0.5% saponin, right lungs were treated with 2% saponin; Group D, left lungs were treated with distilled water, right lungs were treated with 2% saponin.

High binding ELISA plates (Nunc, Denmark) were coated with goat anti-mouse IgA (Nordic Immunology, The Netherlands) 100µl/well (2.5µg/ml) in PBS, at 4 °C overnight. After washing (3 times with PBS-Tween 20), blocking was done by adding 2% BSA in PBS-Tween 20, followed by 1 hour incubation at room temperature under constant shaking. The lung extracts in different solvents were added to the plates with twofold dilutions from 1/20 to 1/5120, 100µl/well. After one hour incubation under the same condition and subsequent washing, biotinylated goat anti-mouse IgA (Southern Biotechnology Associated, Birmingham, USA)

1/10,000 dilution in PBS-Tween 20, 100µl/well was added. After the same incubation, 100µl/well of substrate (K-blue, SVANOVA, Uppsala, Sweden) was added. 10 minutes later, the enzymatic reaction was stopped by adding 50µl/well of 2M H_2SO_4 and the result was read at 450 nm with a Titertek Multiscan Spectrophotometer (Flow Laboratories, Irvine, Scotland). OD values at different dilutions were recorded.

Determination of Anti-RSV IgG and IgA Antibodies by ELISA

ELISA to determine organ extract IgG antibody against RSV was carried out essentially using the method described by Jennings (5). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with 200 ng/well of gradient purified HRSV in 100 µl 50 mM carbonate buffer, pH 9.6, and kept at 4 °C over night. All washings were carried out with phosphate buffered saline containing 0.2% Tween 20 (PBS-Tween). All incubations were done at room temperature for 60 minutes under constant shaking. The organ extracts were titrated in twofold dilutions. The conjugate used was peroxidase-conjugated rabbit anti-mouse immunogloblins (DAKO A/S, Denmark). For IgA levels to RSV in mouse organ extracts, microtiter plates were coated with the purified HRSV, 400 ng/well. Biotinylated goat anti-mouse IgA and HRP conjugated streptavidin were used for the IgA detection. Titers were expressed as reciprocal dilutions of extract giving an OD = mean value of negative control + 2SD.

RSV Neutralization Test

The neutralizing activity of pooled organ extracts were assayed by a microneutralization, similar to the method used by Trudel (6) with some

modifications: 100 µl of twofold dilutions of 56 °C inactivated organ extracts were mixed with 100 µl viral suspension containing 100 $TCID_{50}$ of HRSV, long strain. The virus-organ extract mixtures were added to 96-well flat-bottomed microtiter plates (Nunc™, Denmark) , incubated for 4 hours at 37°C. Then 100 µl of 2-day-old MA 104 cells in FDMEM medium containing 2% FCS was added to each well. The plates were finally incubated for 5-7 days at 37 °C in a humidified atmosphere containing 5% CO_2. All tests were performed in quadruplicate. Controls were included where the test organ extract was replaced with negative control organ extract. Titers were expressed as the reciprocal of last dilution exhibiting 50% CPE (ND_{50}/ml).

Statistical Analysis

OD values of non-specific IgA capture ELISA, and ELISA titers of RSV specific secretory IgG, IgA expressed as geometric means of logarithm transformed arithmetical values with 95% Wilcoxon signed rank sum confidence interval were compared with respect to the levels of antibody by Wilcoxon matched pairs signed rank sum test, Kruskal-Wallis test or Mann-Whitney *U* test where appropriate. All calculations were run on a computer using Minitab release 10 *Xtra* software (Minitab Inc., PA, USA).

RESULTS

Comparison of the Capacity of Saponin Containing and Non-saponin Containing Solvents for IgA Extraction from Lungs

The lungs from twenty 25-week-old female BALB/c mice were extracted for non-specific immunogloblins in particular IgA, with different solvents. The total

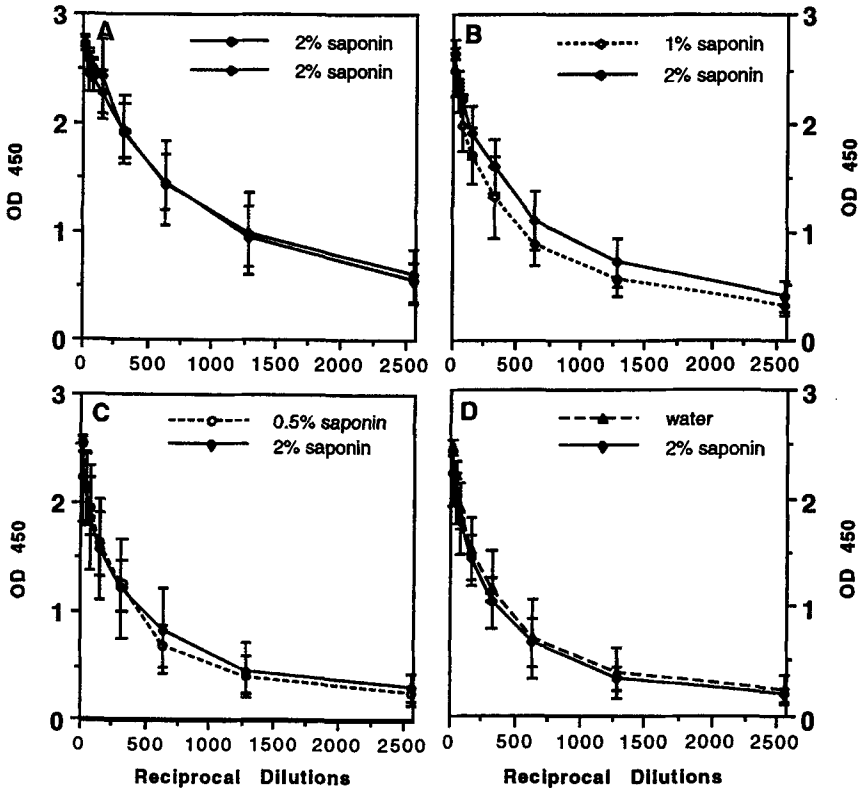

FIGURE 1. Comparison of saponin containing solvents and non-saponin containing solvents for extraction of IgA from lungs as measured by a IgA capture ELISA. Left lungs were treated with 2% saponin as standard control. Right lungs were treated with different solvents: A, 2% saponin; B, 1% saponin; C, 0.5% saponin; D, distilled water. Means of OD ± SD are shown at each dilution (paired left and right lungs. n=5).

extracted IgA was measured by a capture ELISA as described in Materials & Methods. The results were compared by Wilcoxon matched pairs signed rank sum test. The graphic results are shown in Figure 1 (A to D). There was no significant (P=0.789) difference between the left and the right lungs with regard to the quantity of IgA extracted by 2% saponin (Figure 1A), which provided a basis

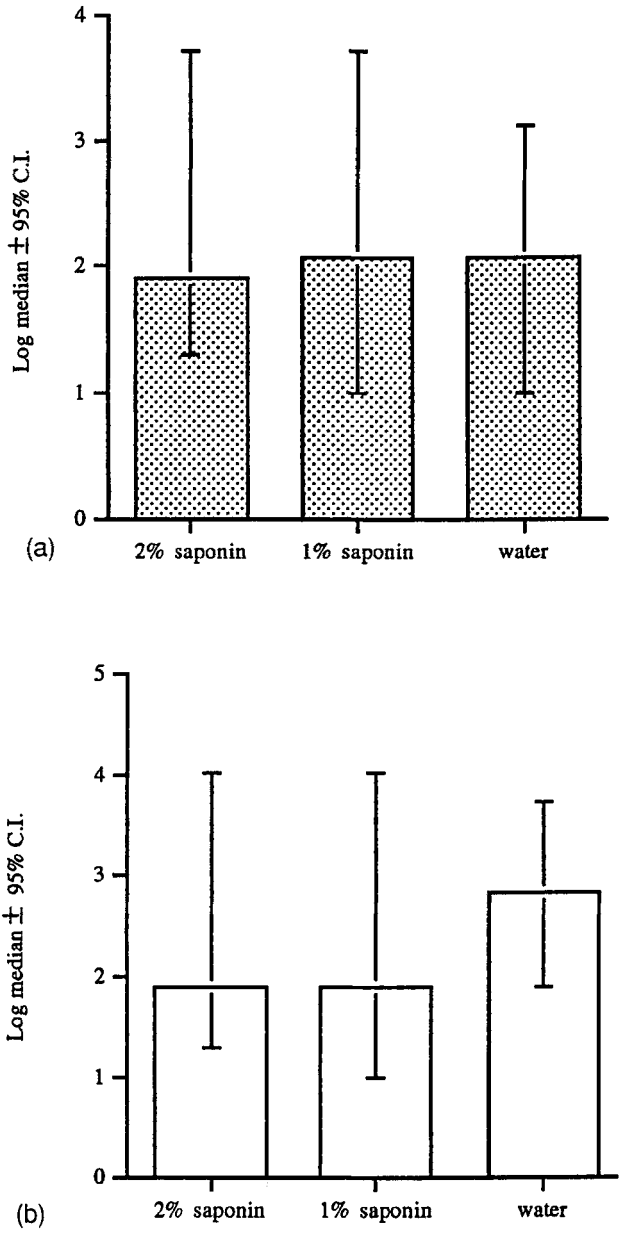

FIGURE 2. Comparison of anti-RSV IgG (2A) and IgA (2B) in secretions extracted with 2% saponin, 1% saponin and distilled water from lungs of mice immunized with RSV ISCOMs as measured by RSV specific ELISA. (n=5).

for further comparisons using different solvents to treat the left and the right lungs. From Figure 1 B to D, 1%, 0.5% saponin and the non-saponin containing distilled water were used for the extraction. The amount of IgA extracted with various solvents were compared using the paired right lungs as standard treated with 2% saponin. There was no significant difference between the different solvents used with regard to the capacity to extract IgA. Hovwever, higher OD values were obtained when distilled water was used for extraction compared to extracts carried out with 2% saponin. Extracts performed with the other two saponin-containing solvents gave lower OD values than the extracts with the 2% saponin. Thus, destilled water was selected as the candidate solvent for the further comparative studies with 2%, 1% saponin in RSV specific IgG, IgA ELISAs.

Distilled Water Efficiently Extracts Anti-RSV IgG and IgA from Lungs and Upper Respiratory Tract

The upper respiratory tract is a different immunological compartment than the lungs and should therefore be analyzed separately. Samples of lung extracts from 15 RSV ISCOM immunized mice were divided into 3 groups with 5 mice per group. The groups of samples were treated with 2% saponin, 1% saponin and distilled water respectively. Anti-RSV IgG (Figure 2A) and IgA (Figure 2B) ELISA were carried out as described in Materials & Methods. High IgG and IgA titres were detected in the extracts from all groups. The results were analyzed statistically with the Kruskal-Wallis Test. No significant difference was recorded in ELISA titers between the 3 different solvents in regard to IgG (P=0.673) and IgA (P=0.339). The ELISA titers indicated that the 3 solvents were inter-exchangeable in extracting anti-RSV IgG and IgA. Extracts from lungs of non-immunized animals had all reciprocal titers below 1:30 (not shown).

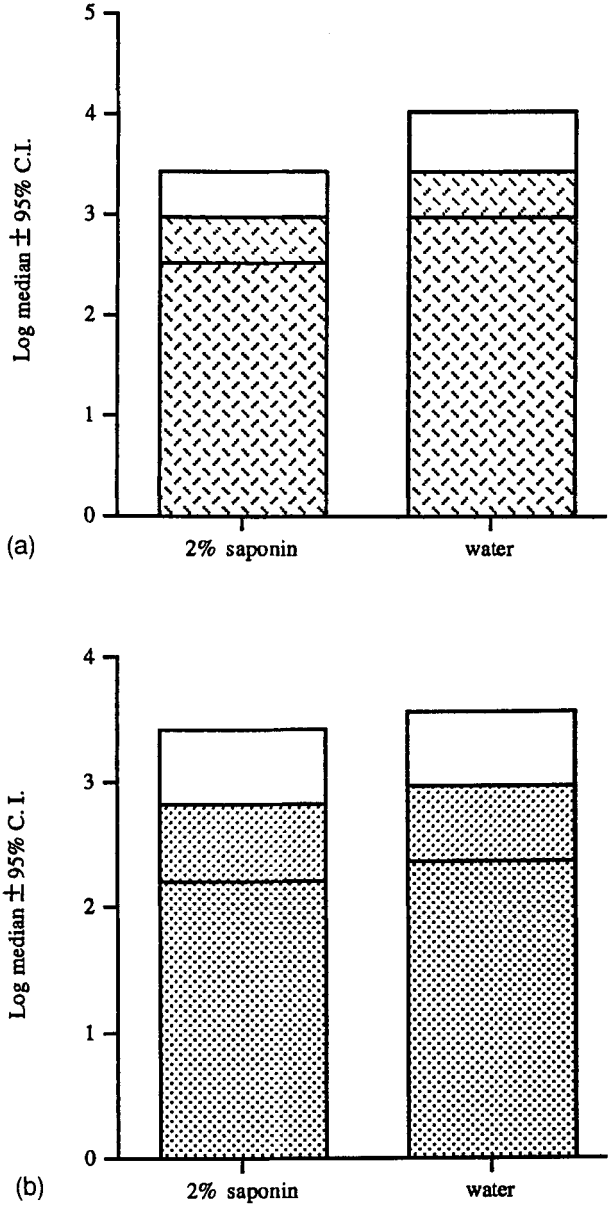

FIGURE 3. Comparison of anti-RSV IgG (3A) and IgA (3B) in secretions extracted with 2% saponin and distilled water from the upper respiratory tracts of mice immunised with RSV ISCOMs as measured by ELISA (each organ was divided into 2 equal paired parts and treated with 2% saponin or distilled water respectively. n=15).

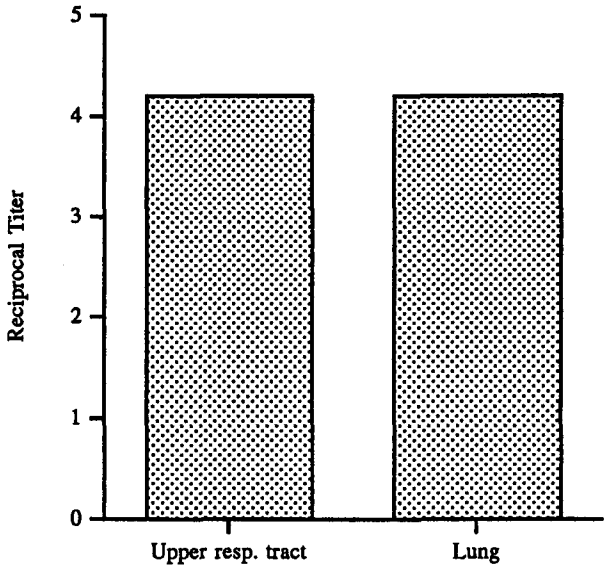

FIGURE 4. RSV neutralization titers in secretions of the upper respiratory tract and the lungs of mice immunised with RSV ISCOMs extracted with distilled water. The values represent ND_{50}/ml (pooled organ extracts).

The upper respiratory tract from each of the 15 mice, immunized intranasally with RSV ISCOMs, was divided randomly into 2 equal parts, and extracted with 2% saponin or distilled water. Anti-RSV IgG (Figure 3A) and IgA (Figure 3B) ELISA was carried out as described in Materials and Methods. The ELISA results were analyzed by Paired Wilcoxon Signed Rank Test. Extraction with distilled water yielded significantly ($P=0.001$) higher anti-RSV IgG titers than samples extracted with 2% saponin. There was no significant difference in anti-RSV IgA titers between samples extracted with distilled water and samples extracted with 2% saponin. Extracts from lungs of non-immunized animals had all reciprocal titers below 1:30 (not shown).

Anti-RSV Virus Neutralizing Antibodies Can Be Measured in Samples from Lungs and Upper Respiratory Tract Extracted with Distilled Water

Secretions used for virus neutralization assay were extracted with distilled water instead of 2% saponin. The neutralization assay was carried out on pooled samples. In secretions from both the upper respiratory tract and the lungs, virus neutralizing antibody titers of about 40 ND_{50}/ml were detected after i.n. immunization with RSV ISCOMs (Figure 4). Samples extracted with 2% saponin, on the other hand, still exhibited visible cell toxicity in our system at saponin concentrations ranging from 20-40 ng/μl, which covered the virus neutralization titers making the results unreadable. Samples from non-immunized animals did not contain virus neutralizing activity (not shown).

DISCUSSION

The extraction procedure for immunogloblins with distilled water described here did not only solve the cell toxicity problem in virus neutralization assay caused by saponin used in the extraction, but also yielded comparatively high amounts of immunogloblins from small laboratory animals. Virus neutralization assay usually requires comparatively large volumes (25μl x 4 in a micro-neutralization test) with adequate virus neutralizing capacity. Using the method described here, the volume for upper respiratory tract was 1300±140μl, for lungs was 250±70μl (both were mean ± SD, n=20) from the 8-12 week old RSV ISCOM immunized mice. Both of the volumes were large enough for running the micro-neutralization test several times. Extraction of immunogloblin with distilled water from other mucosal organs, such as genital tract and intestinal tract is also feasible (Hu et al. manuscript in preparation), which makes the extraction method suitable for taking out a number

of organs and even parts of organs to compare immune responses, e. g. various regions of the gut for evaluation of anti-HIV antibodies.

It is anticipated that, during the immunogloblin extraction, distilled water may exert lysis by osmotic pressure on the cells and the cells may break, releasing cell content and intracellular immunogloblins into the water. The osmotic and pH balances will, therefore, be largely restored by the released biological fluid, and the biological functions of the immunogloblins are preserved as indicated in our ELISA and virus neutralization test.

Our results have convincingly shown that distilled water can be used to replace saponin in the solvent used for extraction of total IgA, RSV specific IgG and IgA. More importantly, such extracts could be successfully used in virus neutralization tests by evading the lytic activity of saponin. Although the procedure was used on BALB/c mice and RSV, it could be expanded to the other animals and viruses where both ELISA and virus neutralization assay are of interest.

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