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Research Article

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A Novel Inulin Adjuvant Enhanced Local Immune Responses to Influenza Antigen

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ABSTRACT

Sheep efferent lymphatic duct of the prefemoral lymph node was cannulated to collect the draining lymph leaving the lymph node. Efferent draining lymph contains immune cells and immunomodulatory signals that are responsible for early response to antigen and/or adjuvant. The lymphatic duct cannulation model was applied to investigate local immunoreactivity to human influenza virus antigen (Flu ag) mixed with a new derived inulin adjuvant (inulin). The results show that Flu ag mixed with inulin adjuvant provokes lymph node activation including lymphocyte recruitment and elevation of antibodies production and antigen-induced proliferation in cultured efferent lymph cells. Thus inulin derived adjuvants enhance the immunogenicity of weak antigen, making them suitable as good vaccine candidatures.

Keywords - *Inulin; Influenza antigen; Lymphatic cannulation.*

INTRODUCTION

Inulin is a neutral, unbranched polysaccharide containing polyfructofuranosyl-α-D-glucose. It is extracted from dahlia tubers and can be easily dissolved in water. Inulin that is made by precipitation from water is known as alpha inulin, while the other form that is obtained by precipitation from ethanol is known as beta inulin. Both α inulin and β inulin are soluble in water at 37ºC.[1] Gamma inulin is formed from aqueous suspensions of α and β inulin after incubation at 37ºC. γ inulin is insoluble at 37 ºC, thus it is the most stable form of inulin but it can be dissolved at 70-80ºC.[2]

Inulin-derived adjuvants are not toxic and non pyrogenic. It is metabolized in the body into simple sugar and excreted in the urine. In addition, inulin adjuvants are not itself antigen and no side effects have been reportedat any tested species, except minor granuloma in humans when very high doses are injected subcutaneously.[3] Inulin adjuvants have advantages over alum at producing strong Th1 and Th2 immune responses.[4]

Advax™ inulin derived adjuvant that was produced through the National Institutes of Health's Adjuvant Development Program.[5] This adjuvant enhances the immunogenicity against Japanese encephalitis in mice and horses[6], HIV in mice[7], avian (H5N1) influenza in ferrets[8] and African Horse Sickness and Glanders in camels[9], hepatitis B in mice and guinea pigs.[10]

The local lymph node is the first lymphoid organ where antigen is filtered following subcutaneous injection and it's the site where dendritic cells trap, process and present antigens to lymphocytes that immediate the immune responses.[11]

Sheep efferent lymphatic cannulation model has been established to investigate local immune responses to peripheral stimuli.[12] In this model the efferent lymphatic duct of the prefemoral lymph node is cannulated to collect the draining lymph leaving the node. Efferent draining lymph contains immune cells and immunomodulatory signals that are responsible for early response to antigen and/or adjuvant. Thus, utilizing the lymphatic cannulation has demonstrated the ability to understanding immunomodulatory properties of experimental vaccines. The aim of the current study was to investigate the adjuvant activity of a new derived inulin adjuvant (in the present study known as inulin) at the level of the local lymph node.

MATERIALS AND METHODS

Animals

Mature medium merino 3 female sheep, aged form 1 to 2 years were brought from the farm and housed in pens before surgical procedures. After surgery, sheep were kept in metabolism cages. Animals were fed a mixture of lucerne chaff, oats, commercial pellets and water was supplied ad libitum. At the end of each experiment, the sheep were killed by lethal injection of sodium pentobarbitone (Lethabarb, Boehringer Ingelheim). This has been approved by animal ethics of Melbourne University.

Surgery

Sheep were fasted of food and water for 24 hrs before surgery, general anaesthesia was performed by injection the jugular vein with thiopentone sodium (10mg/kg) and anaesthesia was maintained with isoflurane (1.5-2.5%) and oxygen. While still under anaesthesia intramuscular

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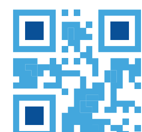


injection of procaine penicillin (1ml dose) and analgesic (Temgesic, 2.2 mg/kg) using a 22 gauge needle. Following recovery sheep were returned to the pens.

Efferent Cannulation of prefemoral L.N

A 3 to 4 cm incision was made into skin in the flank region overlying the prefemoral L.N and approximately 3 cm below the iliac wing. Blunt incision through subcutaneous tissue was done to exposure the tensor fascialate muscle, which was retracted back to see the efferent prefemoral lymph ducts. A piece of suture was used to ligate the lymphatic duct and the other two were placed loosely around the duct. The adherent fat was removed from the lymph duct and a small transverse incision was made through the membrane of the duct prior to the loose tie with micro scissors. The polyvinyl cannula used was heparinised (CBAS-coated, Carmeda AB, Stockholm, Sweden) and then inserted into lymphatic duct and the two losses ties were tightened to secure the cannula. When more than one efferent duct was found all non cannulated ducts were tied off. The skin was penetrated using post mortem needle and the cannula passed through the skin and secured using a purse string suture. The skin incision was closed using Michel Clips. Lymph was collected into sterile plastic bottles containing 5000 IU heparin (DBL, Australia). It was performed to assess the effect of inulin on local immune responses in prefemoral L.N; total cells outputs and antibodies production.

Injection of sheep

Sheep was injected subcutaneously in the flank with 50 µg Flu ag (CSL Limited, Melbourne, Australia), 100 µg inulin adjuvant, or 50 µg Flu ag admixed with100 µg inulin adjuvant (Vaxine Pty. Ltd., Adelaide, Australia), each in a total volume of 1 ml.

Efferent lymph collection, cell counts, and preparation of lymph samples

Lymph samples were collected prior to the injection and these represent the base line, then the samples were collected every hours after injection in the first day from 9 am until 12 pm, following two days the samples were taken in interval, then one lymph sample was taken every day for 3 weeks to measure antibodies responses.

The lymph volume was immediately recorded after each collection and the white cells and blast cells counted using a coulter counter (industrial D, Coulter Electronics, Uk). RBCs were haemolysed by zapglobulin (Fisher Scientific). Lymph from each collection was centrifuged at 600 x g for 10 min and the lymph plasma stored in aliquots at -20ºC for further analysis; antibodies responses.

Total Ig antibody responses

Antibody responses in efferent lymph plasma were measured using enzyme immune assay (EIA). In briefly; The 96 well plates (Nunc, Roskilde, Denmark)) were coated with 50 µl of 9 µg/ml Flu ag in carbonate buffer (50 mM, pH 9.6) for 1 hr/ RT, and washed twice PBS containing 0.05% Tween 20 (PBST). The plates were block with 200 µl PBS containing 1% Na casein (ICN, USA) and washed one time. Lymph samples were serially diluted (1:100 to 1:218700) in PBS containing 0.05% Tween 20 and 1% Na casein and incubated for 1 hr at room temperature, then wash four times with PBST. A100 µl of rabbit anti ovine immunoglobulin (total Ig) conjugated to horseradish peroxidise (Dilute 1:1000 in diluting buffer) (Silenus,Melbourne, Australia) was added to each well and incubate for 1 hr at RT. A100µl tetramethylbenzidine (TMB) was added to each well and incubated for 30 min at RT.

Finally, the reaction was stopped by adding 50 µl of 0.5 M H_2SO_4 to each well and the absorbance was read at 450 nm using an EmaxMicroplate Reader (Molecular Devices, CA, USA). Individual titres were determined by calculation the midpoint, on a double log scale. The result, expressed as titres by calculating the midpoint, on a double log scale, of the straight line section of the curve using linear regression.

Measurement of the antibody isotypes (IgG1, IgG2, IgM and IgA)

Antibody isotypes responses in efferent lymph plasma were measured using enzyme immune assay (EIA). The procedure was performed in the same manner for total Ig antibody responses except of that lymph samples for IgA detection were serially diluted (1:10 to 1:21870) and 100 µl of (primary antibodies) mouse anti sheep IgG1, IgG2 and IgM immunoglobulin and mouse antibovine IgA (Dilute 1:1000 in diluting buffer) (VMRD Inc., Pullman, WA, USA) were added and incubated for 1 hr at RT. A100 µl of (secondary antibody) rabbit antimouse immunoglobulin conjugated to horse radish peroxidase (HRP) dilute 1:1000 was added to each well and incubated for 1 hr at RT.

In vitro cellular proliferation

The cell culture procedures werecarried out to assess in vitro antigen-specific proliferation as described previously by R.G. Windonet at A 50 µl of Flu ag at concentration of 250ng/ml was added and the results were analysed as a stimulation index (SI) by using the following formula:

$$SI = \frac{\text{mean cpm of stimulated cultures}}{\text{mean cpm of relevant control cultures}}$$

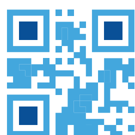
Statistical analysis

Samples of three experiments were analysed using two-way ANOVA and Bonferroni post-hoc test by Graph Pad Prism for Windows version 6 (San Diego, CA, USA). Statistical significant differences were considered as having P values < 0.05.

RESULTS

Inulin induces cell recruitmentin the local lymph node

Flu ag alone and Fluag+inulin were injected subcutaneously into the draining area of contralateral prefemoral lymph nodes. Figure 1 shows the total cells outputs of thenodes through the efferent ducts after injection in sheep 1. The total cell output increased from the 24 x10^6 $h^{-1}$of the pre-injection level to reach the peak of four-fold of the post-injection of Flu ag+inulin (80 x10^6 h^{-1}) at day 9 before returning to baseline by day 22. Whereas, following injection of Flu ag alone, the cell output slightly increased between 13 x10^6 $h^{-1}$of the pre-injection level and 45 x10^6 $h^{-1}$by 4 day post-injection, the total cell output then dropped to 24 x10^6 h^{-1}. Similar responses were observed in two additional animals injected with Flu ag alone and Flu ag+inulin (data not shown). The number of total cells outputs in efferent lymph in the three animals following



Flu ag+inulin treatment was significantly higher than Flu ag alone ($P < 0.05$) by 9, 10 and 11day.

Inulin stimulates antibodies production in the local lymph node

The quantitative and qualitative levels of antigen specificantibody production in efferent lymph were induced by the injection of Flu ag+inulin. As shown for Sheep (Figure 2), total Ig antibody responses increased at 5 days after injection and reached the peak at 10 days (50 x10^3 titre). However, antibody production level in efferent lymph from Flu ag alone were remained at pre-injection levels. For all sheep, the total peak of Ig titres after injection of Flu ag+inulin were significantly higher ($P < 0.005$) than those after Flu ag alone. The peak titres for antigen-specific IgG1, IgG2, IgA and IgM responses after injection of Flu ag+inulin were increased than with Flu ag (data not shown).

In vitro cellular proliferation

In vitro antigen-specific proliferation responses were measured in efferent lymph cells following injection of Flu ag+inulin and Flu ag. As shown for sheep 1 in Figure 3, before injection, antigen-specific proliferation was at low levels (SI < 2). Proliferation after injection with Flu ag+inulin was fivefold greater than that for Flu ag alone at 8 day. Similar cellular proliferation responses were observedin the three sheep. In these animals the proliferation after injection with Flu ag+inulin was significantly higher ($P < 0.005$) than with Flu ag alone at 8 day. Cell proliferation responses stimulated with the positive control mitogens concanavalin A was approximately reached up to 100 SI (Data not shown).

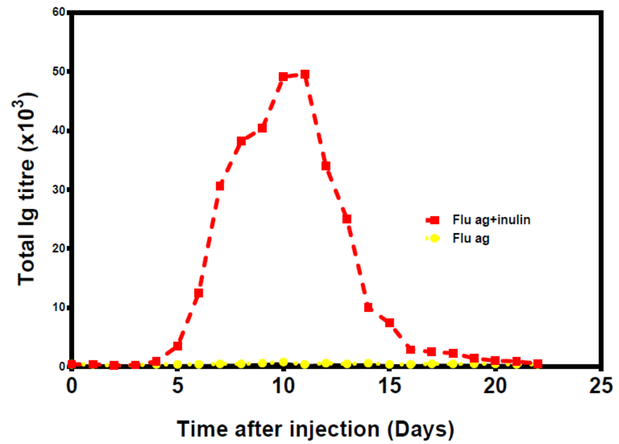


Figure 2: Total Ig responses (titres) in efferent lymph following injection of Flu ag Flu ag+inulin.

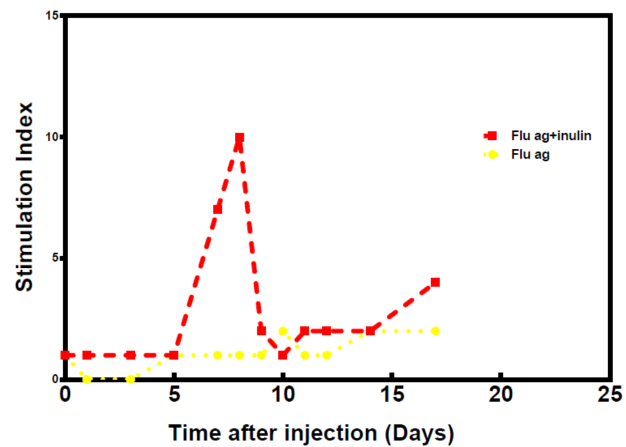


Figure 3: In vitro antigen-specific proliferation following injection of Flu ag+inulin and Flu ag alone.

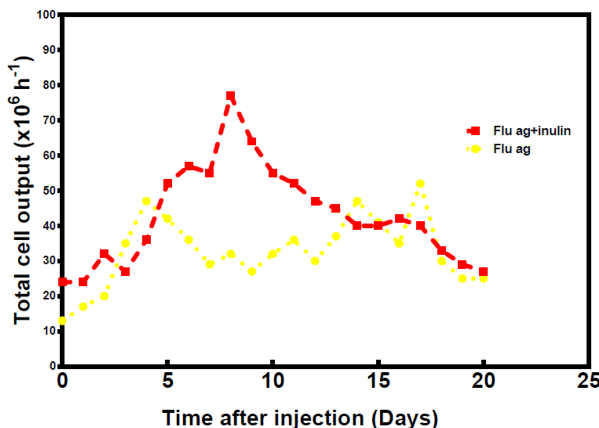
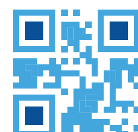


Figure1: Total cell outputs (x 10^6 h^{-1}) following injection of Flu ag+inulin and Flu ag.

The horizontal line indicates the time after injection per day.

DISCUSSION

In this study, efferent lymphatic duct cannulation model in sheep was performed to investigate the immunogenicity of new derived inulin adjuvant. This model allows collecting lymph fluid and lymphocytes trafficking out of the lymph node. Upon injection of adjuvant, local immune responses can be detected at the site of draining node. The results described here, confirmed that the new novel inulin derived adjuvant was able to increase the immunogenicity of Flu ag. Following injection of Flu ag+inulin, the sharp elevation of cells outputs that continue for several days and peaked up at 9 days (80 x10^6 h^{-1}) was observed, whereas Flu ag alone induced less marked cell output for short time up to 4 day (45 x10^6 h^{-1}). Injection of inulin alone also has shown an increase in lymphocytes output (data not shown). It has been suggested that antigens/adjutants enhanced recruitment of lymphocytes from blood into the node.[13,14] The increasing in recirculating lymphocytes improves the screening of low antigen-reactive naive cells by antigen presenting cells.[11,15] Inulin possibly enhanced lymphocyte recruitment through stimulating cytokines release that contribute to this activity. Many studies reported that pro-inflammatory cytokines may be contributed in lymphocyte migration. For example,



IFN-γ and IL-6 promotes the migration of lymphocytes.[16] Further study is required to investigate the influence of inulin on cytokine release.

Injection of sheep with Flu ag+inulin resulted in significantly elevated titres of total Ig antibodies when compared to those following administration of Flu ag alone. Flu ag+inulin derived adjuvant increased total Ig through a stimulating effect on IgG1 (Th2 isotype), IgG2 (Th1 isotype), IgA and IgM. Similarly, in mice AdvaxTM (a novel adjuvant based on delta inulin) enhanced both IgG1 (Th2) and IgG2 (Th1) isotypes. In guinea pigs where IgG1 also responsible for a Th2 isotype and IgG2 to a Th1 isotype.[17,18] Recently a study in human reported that Advax is safe, tolerable with good immunogenicity that is able to enhance humoral and cellular immunity to hepatitis B.[19] More interesting, by using efferent lymphatic cannulation model in sheep, it was enabled to detect that Flu ag+inulin enhanced production of IgA. Immunoglobulin A is related with mucosal immune response. Thus this model might be applicable to measure titre of mucosal IgA. Antibody responses were comparable for cellular proliferation responses in efferent lymph cells. Where, Flu ag+inulin induced higher proliferation responses than that for Flu ag alone.

CONCLUSION

Using efferent lymphatic duct cannulation model in sheep allows us to investigate the immunogenicity of inulin adjuvant. The results show that Flu ag mixed with the new derived inulin adjuvant provokes lymph node activation including lymphocyte recruitment and elevation of antibodies production.

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