Microgel particulate adjuvant: characterisation and mechanisms of action.

R. Vialle\textsuperscript{a}, L. Dupuis\textsuperscript{b}, S. Deville\textsuperscript{b}, F. Bertrand\textsuperscript{b}, J. Gaucheron\textsuperscript{c} and J. Aucouturier\textsuperscript{b}

\textsuperscript{a}120 Lower Delta Road, 13-11 Cendex Centre Singapore 169208
\textsuperscript{b}SEPPIC, 22 Terrasse Bellini, 92800 Puteaux, France.
\textsuperscript{c}SEPPIC, R&D center, 81100 Castres, France.

Abstract

Polymers and self aggregating molecules have been widely tested as vaccine adjuvants. For this type of adjuvant, electrostatic potential can be managed through the selection of chemical synthesis routes and monomer types in order to control adsorption phenomena of amphiphilic charged macromolecules according to isoelectric points at different pH. Thanks to an original polymerisation process, compatible with injectable polymers development, calibrated spheric micronic gel particles of sodium polyacrylate have been synthesised. These aggregates measuring about one micrometer in diameter are made of a reticulated high molecular weight synthetic polymer. Adsorption properties (possibly leading to sustained release) persistence at the injection site and irritation phenomena are the main mechanism of action identified as responsible for immune response improvement. Synthetic cross linked macromolecules, highly chemically stable and used at low rate can persist at injection site. Microgel particles have been characterised with one model antigen to establish binding constants in various conditions. During development steps, safety profile of this innovative formula was assessed through Guinea pig histological studies after intramuscular injection and compared to aluminium salts based adjuvant.

Keywords: Adjuvant ; Montanide™ Gel 01 ; Veterinary adjuvant ; Mechanism of action ; Polymer ; Safety

1. Introduction

Adjuvants technologies dedicated to veterinary vaccines are mainly aluminium salts and oil based vaccines [Audibert and Lise, 1993; Cox and Coulter, 1997]. Oil based vaccines are well known as powerful adjuvant and are used in the field when a strong immune response is needed [Aucouturier \textit{et al}, 2001]. Aluminium salts are sometimes considered as a reference in term of safety and are used in most of the more sensitive species because of their safety profile [Oda K \textit{et al} 2006]. Nevertheless those two kinds of adjuvants are presenting drawbacks either in their safety profile [Oda K \textit{et al} 2006] or ability to induce
an efficacious immune response [Aucouturier et al., 2001]. Many physiochemical parameters and biological properties have been optimised to create a new adjuvant based on a dispersion of a high molecular weight polyacrylic polymer. This new adjuvant is a dispersion of highly stable calibrated spheric micronic gel particles of sodium polyacrylate in water. Montanide™ Gel 01 has already been used in several vaccine models with a promising safety and efficacy profile [Parker et al. 2008]. Nevertheless, sparse data can be found upon physico-chemical characterisation and very few is known upon mechanism of action. In this study, we investigated the Montanide™ Gel 01 physical characteristic and properties linked to the adjuvant mechanism of action: particle sizing and ability to fix antigenic proteins have been studied. Expected mechanisms of action are three: a depot effect, an arrangement of the antigen in a particulate form around the polymer and pro-inflammatory properties. Therefore, inflammatory profile of the Montanide™ Gel 01 has been illustrated in a laboratory animal model and microgel particles, globally negatively charged, have been characterised for their ability to adsorb antigens. An ovalbumine adsorbing model was studied in vitro by antigen specific ELISA methods.

2. Material and methods

2.1. Microgel particle aspect and size distribution

Polymer aspect was characterised by two distinct criteria: direct observation of Gel dispersion and particle sizing. Polymer particles were prepared by organic solvent washings followed by 3 hours drying. A suspension of the extracted polymer was then prepared in a solution of NaCl 2%. The collected particles were observed under white light microscope with 600 magnification rate.

Microparticle sizing was performed on a polymer suspension prepared as described for the direct microscopic observation. Polymer particle sizing was performed using a Malvern Mastersizer S equipped with a small volume sample dispersion unit. The dispersion is diluted in water and passed in a capillary system equipped with laser light and diffraction detectors. The size and volume of the particle is calculated according to the light diffraction and expressed in micrometers.

2.2. Antigen adsorption

Quantification of adsorption of a model antigen Albumin from chicken egg white (ref A55O3-5G, SIGMA) (OVA) onto Montanide GEL particles have been performed by ELISA. Constant concentration of OVA (100µg/ml) was blend with different concentrations of Montanide GEL 01 ranging from 0 to 5%, and left overnight at 4°C. ELISA method was used to detect and quantify the free protein in the antigenic phase after the gel adsorption activity. Montanide™ Gel 01 plus OVA mix was added in ELISA plate (Nunc Maxisorp, ref 055133) and incubated at 37°C for 2 hours. The plates were then washed with PBS / Montanox™ 20 (Polysorbate20, SEPPIC) 0.05%. After three washings, the plates were incubated with 200µl of blocking solution (5% swine gelatin, Prolabo), 0.05% Montanox™ 20 in PBS) for 30 min at 37°C. A mouse serum containing an IgG1 antibody high titre and diluted 1/1000 in blocking solution was added (100µl). Plates were incubated for 1h at 37°C and washed 3 times. Goat anti - mouse IgG1 HRPO (Invitrogen ref M32007), diluted 1/6000 in blocking solution was added (100µl) and the plates were incubated for 1h at 37°C. The peroxidase activity was visualized with TMB (100µl) (ZYMED), stopped with 50µl of H2SO4 (12.5%). The optical density (OD) is evaluated at 450nm. OD quantifies the amount of OVA in solution and not linked to polymer particles.

2.3. Safety study in laboratory animal model.

Safety profile of Montanide™ Gel 01 was defined in a guinea pig model. Placebo formulation was used to define the intrinsic inflammatory properties of the adjuvant without any potential synergic effect of an antigen. For this study, placebo formulations studied were containing either 10% of Montanide™ Gel 01
or 1% of Aluminium hydroxide (Superfoss). Placebo vaccines were injected intramuscularly at day 0 and 28 in the hind leg under 100\(\mu\)l volume. The injected muscle was sampled after at day 14, 56 and 90 for histological study of the vaccine born reactions in the injection site. All biopsy were analyzed through Hemalun Eosin Safran coloration (HES), this work was performed by IDEXX Alfort laboratories (Alfortville, France). This specific coloration of samples allows the identification in the injection site of all cell population present as well as necrosis, fibrosis and vaccine remnants.

3. Results

3.1. Microgel particle aspect and size distribution

Optical observation of particles gives a semi-quantitative assessment of homogeneity (fig 1).

![Microscopic observation of Montanide GEL 01 microparticles.](image1)

Figure 2 presents the particle size repartition observed in water: 50% (in volume) of the particles are smaller than 0.5 \(\mu\)m and 90% of the particles are smaller than 1.2 \(\mu\)m in diameter.

![Particle size distribution of microgel particles in water measure by laser light diffraction.](image2)

3.2. Antigen adsorption

A constant amount of 100\(\mu\)g of antigens induces decreasing OD according to a range of Montanide\textsuperscript{TM} Gel concentration from 0 to 5%. The blank without adjuvant in the mix give an OD measured at 1.6; 0.5%
of adjuvant in the mix give a decrease of 50% of OD. The addition of 5% of Montanide™ Gel in the mix reduces the OD to the negative control level. Results indicate an immediate quenching of OD corresponding to a strong antigen adsorption for OVA on adjuvant concentration under 1%.

**Fig 3:** OD detected by ELISA after fixing 100µg of OVA on Montanide™ Gel 01 from 0 to 5%.

3.3. Safety study in laboratory animal model

Figure 4 presents the Hemalun Eosin Safran coloration results of the injection site observed in the guinea pig muscles. All pictures present at short term after injection an intense reaction in the injected muscle: strong inflammation with large infiltration of muscle by immune cells and even necrosis for the aluminium salts based adjuvant. These salts are inducing such intense reaction even at long term after injection: 90 days after first injection (meaning 62 days after booster) some granulomatous injection and adjuvant remnants are still found in the injection site. The Montanide™ Gel 01 based vaccines is presenting a different profile as no reactions could be observed in the muscle sampled at day 56 or 90.

**Fig 4:** Local reactions observed in subcutaneous tissue biopsies performed in guinea pigs 14, 56 and 90 days after first injection of several vaccines. a: normal tissue, b: granulomas, c: necrotic tissues, d: fibrosis, e: vaccine remnants.

4. Discussion

Direct observation of the polymer particles indicates homogeneous small individual particles. These polymeric individual particles have been characterised as having a smaller diameter than 1.2µm for 90% of the volume they occupy in the solution. This particle size, close to 1µm, is optimum for an efficacious phagocytosis [Lindblad 2004]. ELISA protocol used to characterize adjuvant/antigen interaction is still studied: on going test will validate that the presence of the adjuvant is not leading to unspecific perturbation of ELISA coating step. Nevertheless, the optical density measured by OVA specific ELISA demonstrated that in presence of Montanide™ Gel 01 the antigen is no longer able to coat the plastic walls of the ELISA plate wells. In the meantime, Montanide™ Gel 01 has been demonstrated to induce a short term strong inflammatory response needed to trigger an efficacious immune response: as observed 14 days after injection, a strong infiltration of monocytes and macrophages is induced by injection of Montanide™
Gel based placebo in muscle tissues. Compared to aluminium salts adjuvant formulation, the inflammatory process is shorter avoiding chronic inflammatory response that can be responsible for vaccine born side effects [Day et al, 2007]. One of the Montanide™ Gel 01 mechanisms of action of adjuvant is therefore linked to its safety profile. Indeed, the inflammatory response observed at short term after injection of vaccine based on Montanide™ Gel 01 insures the recruitment in the injected area of monocytes and macrophages: phagocyting cells of the innate immune systems. At last, we demonstrated that the polymer from Montanide™ Gel 01 presents a strong ability to fix antigenic proteins probably to its surface. Therefore, we can assume that Montanide™ Gel 01 based vaccine injection will induce the immune response against an antigen through three main ways:

- Antigen sustained release the injection site.
- Recruitment of the innate immune system by a pro-inflammatory profile in the injected muscle.
- Enhanced phagocytosis of the antigen complex with the polymer therefore raising the activity of antigen presenting cells.

Innate immune system will then trigger the adaptive immune system to build a highly specific immune response. At last, these results are consistent with data collected in other animal models such as dog and pigs [Deville et al, 2008; Dupuis et al, 2008; Parker et al 2008].

5. Conclusion

Few mechanisms of action of adjuvant technologies are described in the literature. Polymers have been widely tested and sometimes used as vaccine adjuvants. This work is presenting data upon the mode of action of one specific sodium polyacrylate based polymer adjuvant linking chemical interaction of antigen and adjuvant to the inflammatory process attracting the immune cells in the injected area. Control of synthesis parameters such as microgel particle size and surfacial electrostatic charge added possibility to design easily adjuvants for specific antigens.

6. Bibliography


Procedia in Vaccinology, Volume 1, Issue 1, 2009, Pages 140-147.