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Research Article

Respiratory Administration of Infliximab Dry Powder for Local Suppression of Inflammation

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Abstract. The airways are verified as a relevant route to improve antibody therapeutic index with superior lung concentration but limited passage into systemic blood stream. The current research aimed to process spray-dried (SD) powder of Infliximab to assess the feasibility of respiratory delivery of antibody for local suppression of lung-secreted tumor necrosis factor α (TNFα). Molecular and structural stability of powders were determined through size exclusion chromatography (SEC-HPLC) and Fourier transform infrared (FTIR) spectroscopy. Particle properties were characterized by laser light scattering, twin stage impinger (TSI), and scanning electron microscopy (SEM). *In vitro* biological activity was quantified applying L-929 cell line. Ovalbumin (OVA)-challenged balb/c mice were employed to evaluate the anti-TNFα activity of antibody formulation as *in vivo* experimental model. SD sample consisting of 36 mg trehalose, 12 mg cysteine, and 0.05% of Tween 20 was selected with minimum aggregation/fragmentation rate constants of 0.07 and 0.05 (1/month) based on 1 and 2 months of storage at 40°C and relative humidity of 75%. Fine particle fraction (FPF) value of this formulation was 67.75% with desired particle size and surface morphology for respiratory delivery. EC_{50} was 8.176 and 6.733 ng/ml for SD Infliximab and Remicade®, respectively. SD antibody reduced TNFα (26.56 pg/ml) secretion in mouse lung tissue, more than 2 orders of magnitudes comparing positive control group (TNFα, 68.34 pg/ml). The success of antibody inhalation mainly depended on the spray drying condition, formulation components, and stability of antibody within aerosolization. Inhaled Infliximab could be a potential drug for local inhibition of lung inflammation.

KEY WORDS: infliximab; spray drying; inflammation; respiratory delivery; bioactivity.

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Abbreviations *SD*, Spray-dried; *FD*, Freeze-dried; *TNFα*, Tumor necrosis factor alpha; *mAb*, Monoclonal antibody; *IL5*, Interleukin 5; *AHR*, Airway hyperresponsiveness; *MDIs*, Metered dose inhalers; *DPIs*, Dry powder inhalers; *IgG*, Immunoglobulin G; *IVIG*, Intravenous immunoglobulin; *SEC-HPLC*, Size exclusion high pressure liquid chromatography; *SDS-PAGE*, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; *FTIR*, Fourier transform infrared; *SEM*, Scanning electron microscopy; *MTT*, Methyl tetrazolium; *RPMI*, Roswell Park Memorial Institute; *ATCC*, American Type Culture Collection; *KBr*, Potassium bromide; *TSI*, Twin stage impinger; *ED*, Emitted dose; *FPD*, Fine particle dose; *FPF*, Fine particle fraction; *ADA*, Anti-drug antibody

INTRODUCTION

Asthma is the most prevalent chronic disease in the world and significant populations of patients are resistant to the systemic corticosteroids and high doses of bronchodilators (1). New approaches in the treatment of asthma are based on suppression of inflammatory pathways and prevention of structural changes in the airways (2,3).

Lung levels of pre-inflammatory cytokines like tumor necrosis factor α (TNFα) are increased in asthma. TNFα as a non-glycosylated protein is involved in pathogenesis of asthma, rheumatoid arthritis, multiple sclerosis, and other types of inflammatory disorders (4,5). The mRNA of TNFα is over-expressed in the airways of asthmatic patients. Therefore, inhibition of TNFα could be a reasonable approach in the treatment of asthma (6).

Infliximab, as a chimeric monoclonal antibody (mAb) against TNFα, was approved by FDA for the treatment of rheumatoid arthritis, Crohn's disease, and psoriatic arthritis (7). In patients with simultaneous asthma and rheumatoid arthritis, administration of Infliximab was encouraged in alleviation of respiratory symptoms (8); moreover, the

reduction of inflammatory markers as well as variations in peak expiratory flow rate and exacerbation were observed in asthmatic patients through application of Infliximab (9). Inhalation of therapeutic proteins, such as antibodies, can provide an encouraging opportunity for their needle-free delivery, high drug bioavailability, low required dose, and patient compliance especially in the case of chronic diseases (10).

Spray drying is regarded as a method of choice for embedding proteins, as labile molecules, in stabilizing additives like sugars, amino acids, and other types of carriers (11). Although incorporation of suitable excipients could enhance the efficacy of spray-dried (SD) protein powders, preparation of stable powder of mAbs for inhalation therapy still remains a bit challenging. Besides, limitations such as low efficiency in local delivery of exact dose and low repeatability of inhalation devices should be considered (12). Our previous studies on optimization of SD intravenous immunoglobulin (IVIG) and Trastuzmab focused on molecular stability, as well as particle behavior (13,14) of processed powders. Furthermore, screening the effect of different amino acids was done which suggested cysteine as the amino acid of choice in SD IgG formulations (15). Also experimental design was utilized to determine the best quantitative combination of IgG formulation in the presence of trehalose, cysteine, and Tween 20 (16).

The present study was designed to further evaluate the feasibility of respiratory delivery of Infliximab as a suitable alternative to its systemic administration. In order to assess the hypothesis, SD preparations were characterized considering biological activity *in vitro* and in a mouse model of inflammation.

MATERIALS AND METHODS

All sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents, disodium hydrogen phosphate, sodium sulfate, trehalose dihydrate, actinomycin D, methyl tetrazolium (MTT) solution, ovalbumin, ketamin, xylazine, acetonitrile, alum, trypsin, potassium bromide (KBr) as well as dialysis tubing membrane were supplied by Sigma, UK. Polysorbate 20 (Tween 20) was obtained from Merck, Germany. Infliximab was provided by Jenssen Biotech, USA. Roswell Park Memorial Institute (RPMI)-FBS was supported by Gibco, USA. The cell line L-929, adherent fibroblast-cell line derived from subcutaneous connective tissue of mouse, was offered by American Type Culture Collection (ATCC), USA. Gelatin capsules of size 3 were kindly gifted by Cipla, India. Mouse-TNF α enzyme-link immunosorbent assay (ELISA) kit from Abnova was utilized for *in vivo* assay, assessment of TNF α level in a mouse model of inflammation. Data were measured and reported as means ± SD. One-way ANOVA, SPSS 16, was done to assess the significant differences between prepared formulations. The results were considered with a p value less than 0.05 as significant. The graphs were drawn by Graph Pad® Prism 5.

Preparation of Formulations and Spray Drying

Freeze-dried (FD) powder of Infliximab (Janssen Biotech, USA) was dissolved in sterile WFI and transferred into a dialysis tubing (cutoff, 8 kDa), shaking overnight at 4°C. A final pure antibody mount was 30 mg for each formulation. Based on the compositions presented in Table I, the F_1-F_{10} formulations were prepared, sterile filtered by 0.22 μm filters, and kept at 4°C prior to spray drying. Lab-scale spray dryer (Buchi 191, Switzerland) was applied to prepare dry powders of antibody. The standard operating conditions were as follows: drying air inlet temperature of 100°C, aspiration setting of 100%, and liquid feed rate of 10% (1.8 ml/min). This operational condition resulted in an average outlet temperature of 56°C. The recovered powders in the collection vessel were collected and stored at 4°C in sealed glass vials for further examinations.

Size Exclusion Chromatography (SEC-HPLC)

The percentage of antibody monomer and the presence of high molecular weight (aggregates) as well as low molecular weight species (fragments) were determined by SEC-HPLC analysis. SEC-HPLC column (TSK Gel G3000swxl, Silica gel column, Tosoh, Germany) was applied to inject 20 μl of 0.45-μm filtered samples at final concentration 2.5 mg/ml of antibody. The HPLC system (Jascow, Japan) was equipped with a pump and a UV detector at 214 nm. Mobile phase consisted of 0.1 M disodium hydrogen phosphate and 0.1 M sodium sulfate with pH adjusted at 6.8. The injections were performed in isocratic mode at running time of 35 min for each sample in triplicate. The Unicorn software (GE, Sweden) was used to calculate the area under the curve (AUC) of monomer peak to that of induced antibody aggregates and/or fragments after spray drying as well as upon 1 and 2 months of storage at 40°C and relative humidity of 75%. The percent of aggregates, fragments, and monomer were quantified through integrating each peak area as have been shown below:

Aggregates (%) = AUC aggregates (%) / AUC total peaks (%)

Fragments (%) = AUC fragments (%) / AUC total peaks (%)

Table I. Composition of SD Infliximab Formulations. Excluding F_1, All Samples Contained 0.05% of Tween 20

Formulation	Infliximab (mg)	Trehalose/antibody Mass ratio	Cysteine/antibody Mass ratio
F_1	30	–	–
F_2	30	1:1	1:5
F_3	30	1.2:1	1:5
F_4	30	1.4:1	1:5
F_5	30	1:1	1:3.33
F_6	30	1.2:1	1:3.33
F_7	30	1.4:1	1:3.33
F_8	30	1:1	1:2.5
F_9	30	1.2:1	1:2.5
F_{10}	30	1.4:1	1:2. 5

Natural logarithm of aggregates/fragments (%) was plotted *versus* storage time (1 and 2 months). The rate of fragmentation/aggregation was calculated considering first-order kinetic trough linear regression of the prepared plot.

FTIR Spectroscopy

Infrared spectra were measured applying a spectrometer (Nicolet Magna, USA). Two milligrams of SD powders was mixed with 200 mg KBr and pressed through 6–7 T of pressure (Carver press, USA). The analysis of amide I region (1600–1700 cm^{-1}) was performed to determine the secondary structure of Infliximab as a beta-dominant protein. Jasco Spectra Manager Software facilitated fitting through a mixed Gaussian/Lorentzian function. The position of peaks for IgG was described as follows: The peaks located at 1640 cm^{-1} as well as 1690–1695 cm^{-1} are attributed to β-sheet structures, peaks from 1660 to 1650 cm^{-1} are indicative of α helix, and turn structures are positioned from 1690 to 1665 cm^{-1} (17).

Electrophoretic Analysis (SDS-PAGE)

SDS-PAGE is an established technique to compare the integrity of the protein solution with the standard sample. In SDS-PAGE, proteins are resolved on polyacrylamide gel based on their overall molecular weight and banding pattern formed can be compared between reference and test solution to confirm the integrity of protein following spray drying procedure. Non-reducing SDS-PAGE (Bio-Rad, USA) was carried out preparing 12% gel. About 20 μl of prepared samples (containing 100 μg/ml of antibody) was loaded at voltage 220 V for 3 h. The resolving gel was stained with Coomassie blue for visualization of antibody related bands in SD Infliximab formulations and comparison with those of Remicade.

Particle Size Analysis *Via* Laser Light Scattering

Laser light scattering (Malvern Mastersizer, UK) was employed to calculate particle sizes. Five milligrams of SD samples was suspended in 10 ml of acetonitrile and sonicated trough water-bath sonicator (Starsonic, Italy) for 5 min to be fully homogenized. Obscuration from 0.15 to 0.2 was applied to measure particle sizes in triplicate for each sample.

Inhalation Performance of SD Infliximab Formulation

Aerosolization efficacy of SD powders was characterized through TSI (Apparatus A, 2000, Copley, UK). Approximately, 15 mg of selected powders (F_1 and F_{10}) was filled in HPMC capsule of size 3 and was placed in a Cyclohaler®. The capsules (three capsules for each formulation) were emptied from the Inhalator at 60 l/min for 5 s using vacuum pump (P 504, Cole Parmer, UK). The upper and lower stages of TSI were rinsed with 7 and 30 ml of purified water, respectively, and the amount of Infliximab was quantified by spectroscopy method, at 280 nm. Temperature and relative humidity were controlled at 25°C (± 1°C) and 40–45% respectively for all experiments. The deposition profile of selected formulations was characterized through calculation of fine particle fraction (FPF) as well as emitted dose (ED). The ED is regarded as the total amount of collected powder at all stages. Fine particle dose (FPD) is the total amount of powders with particle size < 6.4 μm which deposited in the lower stage of TSI. FPF is obtained through division of FPD to the ED (18).

Scanning Electron Microscopy

The surface morphology of selected formulations was investigated utilizing scanning electron microscopy (SEM) system (XL30, The Netherlands). Powder samples were mounted on top of an aluminum stub and sputter coated (BAL-TEC, Switzerland) with gold at 100 Pa (75 mTorr), for 90 s at room temperature. The images were scanned at accelerating voltage of 20 kV and magnifications of ×1000 and ×15,000.

In Vitro Biological Activity of SD Infliximab *Versus* Remicade®

SD Infliximab bioactivity was quantified by L929 cells. TNFα causes lysis of L929 cells after sensitization with actinomycin D. Antibodies bound to human TNFα neutralized the effect, resulting in the rescue of L929 cells from cell lysis. Briefly, cells were seeded on sterile 96-well plates (2.5×10^4 cells/well) in 50 μl of DMEM F12 supplemented with 10% FBS. One hundred microliters of different concentrations of SD Infliximab or Remicade (0.1–100 ng/ml) was added to the cells. Afterward, 50 μl of TNFα solution (50 pg/ml) was added to each well. In the negative control sample, no TNFα was present. Cells were incubated for 72 h at 37°C in the CO_2 incubator (Shel lab, USA). The optical density of the well (at 620 nm) is directly proportional to the concentration of live cells in each well with the aid of MTT assay. The cell viability was plotted against the monoclonal antibody concentration to assess comparability of biological activity between F_9 and Remicade.

Animals, Induction of Airway Sensitization, and Challenge

Female balb/c mice (6–8 weeks old) were donated by Pasteur Institute of Iran. All the animal experiments were conducted under a protocol approved by committee of medical ethics at Tehran University of Medical Sciences. The protocol was in accordance with National Institutes of Health guide for animals (NIH Publications No. 8023, revised 1978). Mice were categorized into three groups and each group consisted of five mice (group 1, negative control; group 2, positive control; and group 3, test). The experimental protocol was described in Table II. All groups were generally sensitized through intraperitoneal (i.p.) injection of 50 μg ovalbumin on days 0, 7, and 14. Groups 2 and 3 received 500 μg, intratracheal (i.t.) ovalbumin on days 21–27. Twenty micrograms/day of SD Infliximab powder was administered *via* trachea and with the aid of insufflator, 3 h before challenge test in group 3, on days 21–28. Prior to i.t. injections, mice were anesthetized by 100 μl, i.p. injection of ketamin/xylazine (10:1). For i.t. injection, mice were fixed on special boards, the tongue of the mice was brought out by sterile forceps, the insufflator was put precisely in the trachea

Table II. Experimental Protocol for Sensitization, Challenge, and Antibody Administration

Group	Day 0	Day 7	Day 14	Days 21–28	Day 29
1 (−)	OVA sensitization (50 μg, i.p.)	OVA sensitization (50 μg, i.p.)	OVA sensitization (50 μg, i.p.)	Challenge: no Treatment: no	BA lavage
2 (+)	OVA sensitization (50 μg, i.p.)	OVA sensitization (50 μg, i.p.)	OVA sensitization (50 μg, i.p.)	OVA challenge (500 μg, i.t.) Treatment: no	BA lavage
3 (T)	OVA sensitization (50 μg, i.p.)	OVA sensitization (50 μg, i.p.)	OVA sensitization (50 μg, i.p.)	OVA challenge (500 μg, i.t.) Treatment: 20 μg/day SD Infliximab (i.t.) 3 h before challenge test	BA lavage

OVA ovalbumin, *BA* bronchoalveolar, *SD* spray-dried

of the mice, and finally the powder of the drug was placed in the trachea with the aid of insufflators.

Bronchoalveolar Lavage and Sample Preparation for ELISA Test

Mice were sacrificed on day 29, after being anesthetized. The lungs were meticulously excised and washed with 1 ml PBS buffer three times. The lavage fluid was homogenized with ultrasonic hemogenizer (IKA T25, Germany) for 15 s and then centrifuged at 800 rpm for 10 min at 4°C. The supernatants were stored at − 20°C for further experiments.

Enzyme-Link Immunosorbent Assay

Mouse-TNFα ELISA kit (KA0257, Abnova, Taiwan) was applied to quantify level of TNFα in the lung tissue of three mice groups. Present TNFα in each sample was captured by the antibody immobilized to the wells and by the biotinylated TNFα-specific detection antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed. Following the second wash step, TMB substrate solution was added to the wells, resulting in color development proportional to the amount of bound TNFα. The Stop Solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm (BioSpectrometer, Eppendorf). The standard curve was generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis *versus* the corresponding TNFα concentration (pg/ml) on the horizontal (X) axis. TNFα was diluted in the range from 62.5 to 2000 pg/ml. The content of secreted TNFα in three groups was quantified by comparison with a standard curve.

RESULTS AND DISCUSSION

TNFα as a pro-inflammatory cytokine has been proved to be in charge of airway pathology in asthma (19). Anti-TNFα therapy in preliminary studies confirmed better lung function, lower airway hyperresponsiveness (AHR), and less frequency of exacerbation in patients with refractory asthma (20). In a clinical study composed of 38 patients with moderate asthma, intravenous infusion of Infliximab caused reduction in the number of exacerbated patients. Likewise, significant decrease in level of TNFα was inspected in sputum supernatants without any serious adverse reactions (21). In prospect of developing an inhalable form of Infliximab, for local inhibition of TNFα in inflammatory asthma, spray drying was investigated to process stable dry powder employing combination of stabilizers. In the subsequent parts, the characterization of samples has been discussed with respect to stability aspects, particle features, *in vitro* biological activity as well as *in vivo* suppression of TNFα in a mouse model.

Physicochemical Stability of SD Infliximab

SEC-HPLC was utilized to quantify the amount of induced aggregates/fragments of antibody in the formulations after spray drying process and at 1 and 2 months of storage at 40°C (Table III). The results were then engaged to calculate the rate constants of aggregation/fragmentation regarding the first-order kinetic of degradation (chromatograms of F_1, F_9, and Remicade were provided as Fig. 1AA, B, C supplement). Pure antibody was statistically less stabilized against aggregation and fragmentation comparing formulated samples (one-way ANOVA, SPSS 16, Table file 1 supplement), with p value < 0.05. F_1 showed the highest rate of fragmentation and aggregation with corresponding values of 0.14 ± 0.001 and 0.15 ± 0.004 (1/month), respectively. The least rate constant of fragmentation and aggregation was observed in F_7 (0.06 ± 0.002 and 0.07 ± 0.002, 1/month) and F_9 (0.05 ± 0.001 and 0.07 ± 0.002, 1/month). Considering the most value of statistical differences between F_9, with other samples, it was selected as the physically stable sample for further analysis (one-way ANOVA, SPSS 16, Table file 2 supplement). The superiority of F_9 was statistically proved in terms of aggregation comparing all powders excluding F_7 and F_{10} after process and 1 month storage, p value < 0.05; after 2 months of storage was similar to F_4 and F7. With regard to fragmentation, F_9 was better than F_1 after process and 1 month storage, p value < 0.05; after 2 months of storage F_9 was statistically preserved against aggregation better than F_1, F_2, and F_5, p value < 0.05. The overall conclusion was derived that F_9 was the most stable powder with respect to molecular stability profile.

To confirm the preserved integrity of antibody, SDS-PAGE analysis was performed under non-reducing condition (Fig. 1). The single band at 150 kDa appeared in all formulations without any remarkable bands at the upper or lower areas. Such observation implied the fact that under the

Table III. Molecular and Structural Stability of SD Infliximab Formulations

Sample	Fragmentation (%)			Aggregation (%)			Rate constant fragmentation (1/month)	Rate constant of aggregation (1/month)	Beta sheet content (%)
	T = 0	T = 1 month[1]	T = 2 months[2]	T = 0	T = 1 month[1]	T = 2 months[2]			
F_1	0.95 ± 0.02*	1.18 ± 0.01*	1.85 ± 0.01*	1.69 ± 0.02*	2.35 ± 0.01*	3.37 ± 0.01*	0.14 ± 0.001*	0.15 ± 0.004*	68.71 ± 1.34*
F_2	0.31 ± 0.05	0.38 ± 0.01	0.46 ± 0.08	0.87 ± 0.03	1.07 ± 0.01	1.36 ± 0.03	0.09 ± 0.002	0.10 ± 0.002	69.25 ± 1.45
F_3	0.21 ± 0.01	0.25 ± 0.05	0.30 ± 0.03	0.63 ± 0.03	0.75 ± 0.01	0.98 ± 0.03	0.08 ± 0.002	0.10 ± 0.002	69.44 ± 1.23
F_4	0.20 ± 0.03	0.23 ± 0.04	0.27 ± 0.09	0.47 ± 0.05	0.55 ± 0.03	0.70 ± 0.02	0.07 ± 0.001	0.09 ± 0.003	70.23 ± 2.31
F_5	0.29 ± 0.05	0.34 ± 0.02	0.40 ± 0.04	0.71 ± 0.02	0.85 ± 0.02	1.14 ± 0.02	0.07 ± 0.002	0.10 ± 0.001	69.37 ± 2.14
F_6	0.19 ± 0.01	0.23 ± 0.02	0.26 ± 0.02	0.59 ± 0.02	0.70 ± 0.02	0.83 ± 0.02	0.07 ± 0.003	0.07 ± 0.002	70.52 ± 1.34
F_7	0.16 ± 0.03	0.18 ± 0.05	0.21 ± 0.06	0.17 ± 0.01	0.20 ± 0.02	0.24 ± 0.03	0.06 ± 0.002	0.07 ± 0.002	70.54 ± 2.12
F_8	0.24 ± 0.02	0.28 ± 0.05	0.33 ± 0.02	0.55 ± 0.02	0.64 ± 0.01	0.86 ± 0.02	0.07 ± 0.001	0.10 ± 0.001	70.12 ± 1.24
F_9	0.12 ± 0.01*	0.13 ± 0.05*	0.15 ± 0.05*	0.08 ± 0.01*	0.09 ± 0.01*	0.11 ± 0.02*	0.05 ± 0.001*	0.07 ± 0.002*	70.61 ± 1.67
F_{10}	0.19 ± 0.01	0.22 ± 0.02	0.26 ± 0.07	0.10 ± 0.01	0.12 ± 0.01	0.15 ± 0.02	0.07 ± 0.001	0.09 ± 0.002	70.95 ± 1.89

p value less than 0.05 is considered as statistically significant. *: F_1 is statistically different to other samples with *p* value < 0.05. *: F_9 for aggregation is significantly different to other formulations excluding F_7 with *p* value < 0.05. For fragmentation, F_9 was different to F_1, F_2, and F_5 with *p* value < 0.05. For rate constants of degradation, F_9 meaningfully differs from F_1 with *p* value < 0.05

[1,2] 45 degree centigrade

selected conditions, processing of Infliximab formulations protected protein against fragmentation within spray drying.

Thermodynamic Properties of SD Antibody

FTIR spectroscopy was conducted to evaluate the structural changes in secondary conformation of SD Infliximab. A mixed Gaussian-Lorentzian curve-fitting analysis was employed to record the alterations in amide I region. In the amide I region, peaks were present at 1690, 1670, 1640, and 1620 cm^{-1} (22). Based on the second-derivative spectrum achieved from F_1 (Fig. 2), pure Infliximab has major β-sheet attributed bands at 1640, 1620, and 1690 cm^{-1} and a turn-specific band at 1670 cm^{-1} which is typical for a monoclonal antibody (23). The FTIR analysis of F_1–F_{10} demonstrated that the solid formulations of Infliximab retained properly in the native-like structure with predominantly β-sheet content ranging from 68.71 ± 1.34 to 70.95 ± 1.89% (Table III). Conclusively, there was no significant difference in the conformation of F_1 with that of F_2–F_{10} regarding β-sheet percent.

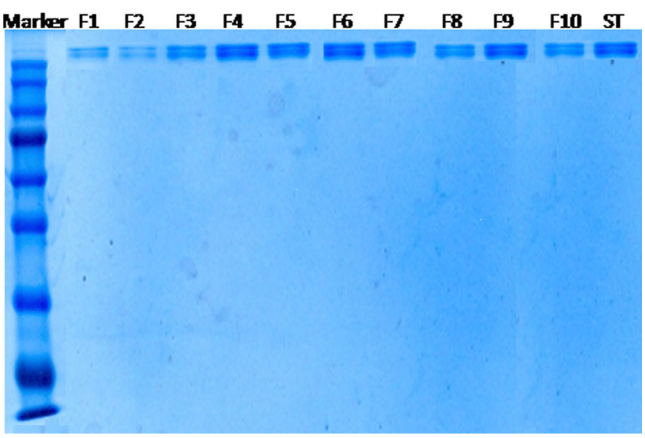

Fig. 1. Non-reducing SDS-PAGE of Infliximab formulations. From right to left: lane 1, Remicade; lane 2, F_1; lane 3, F_2; lane 2, F_3; lane 5, F_4; lane 6, F_5; lane 7, F_6; lane 8, F_7; lane 9, F_8; lane 10, F_9; lane 11, F_{10}; and lane 12, standard marker (11, 17, 25, 35, 48, 63, 75, 100, 135, and 180 kDa)

Similar to the findings of our previous publication, the mass ratio of amino acid/antibody, 0.4:1, and sugar/antibody, 1.2:1, produced the best stabilized composition through the properly sufficient interaction with antibody molecules within process and during storage at high temperatures (16).

Physical Properties and Aerodynamic Behavior of Microparticles

After each spray drying run, collected samples were weighed for calculation of process yield (Fig. 3a). The achieved data from the process yield was in good agreement with the hydrophobicity of powders. F_1 had the lowest powder recovery amount of 20.33 ± 2.31%. The highest value of yield was attained in F_8 (38.7 ± 1.24%) which was composed of 12 mg cysteine and 30 mg trehalose, besides 0.05% of Tween 20. As general evidence and in accordance with previously developed experimental design (16), higher amounts of cysteine (as a hydrophobic component) and lower amounts of trehalose (as a hydrophilic excipient) were accounted for efficient powder recovery. Formation of hydrophobic particles with lower adhesiveness to the cyclone chamber aided powder fluidization and therefore, increased the amounts of collected powder.

The deposition of inhaled particles at various levels of respiratory system is dependent on the particulate characteristics namely particle size and morphology which affect the aerosolization properties of SD powders (24). To obtain enhanced drug deposition and residence time, particles must have aerodynamic diameters less than 6.4 μm (25). Laser light scattering data revealed that the geometric sizes of all particles (F_2–F_{10}) were in the acceptable range, from 4.03 ± 0.22 to 5.22 ± 0.54 μm, Fig. 3b).

Two basic conclusions could be derived. First, increasing the amount of cysteine to 12 mg decreased the particle sizes. Cysteine as a hydrophobic amino acid reduced the inter-particle forces and

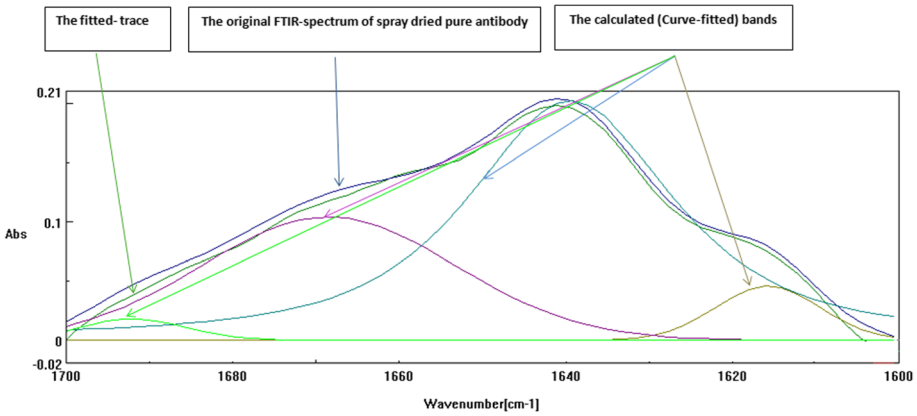

Fig. 2. FTIR spectrum of SD pure Infliximab powder (F_1). The fitted-trace, the original spectrum of SD pure antibody, and the calculated (curve-fitted) bands have been colorfully distinguished

prevented the formation of cohesive and larger particles. Secondly, in each amount of cysteine (6, 9, and 12 mg), increasing the trehalose amounts from 30 to 42 mg led to the formation of larger particles as a result of the hydrophilic nature of trehalose and creation of sticky particles. Creation of agglomerated particle was previously reported in SD protein containing trehalose (26); however, incorporation of a surfactant (*e.g.*, Tween 20) in addition to hydrophobic amino acid (cysteine) prevented the powders from agglomeration and particle sizes in all preparations were less than 6 μm. Comparing F_1 with aerodynamic diameter of 7.81 ± 0.71 μm, F_2–F_{10} showed lower sizes, acceptable for further respiratory delivery.

TSI was employed as an *in vitro* simulation device for evaluation of aerosol performance of SD powders. The ED and FPF of formulations have been compared in Fig. 3a. All samples exhibited better aerosol performance in comparison to F_1. The amounts of ED varied from 70.46 ± 1.43 to 93.21 ± 1.24%. Apart from F_1–F_4, all preparations demonstrated FPFs higher than 60%. As a general finding, formulations containing the highest levels of cysteine and the lowest amounts of trehalose showed maximum FPFs which is surely due to hydrophobic nature of cysteine. The best deposition was observed for F_8 and F_9 with respective FPF values of 68.65 ± 1.34 and 67.75 ± 1.29%.

Both the device and powder formulation must provide high FPF of the powder to be delivered to the respiratory system. The bulk combination of additives should successfully facilitate the actuation of powder during administration.

Similar findings were reported through incorporation of trileucine in SD powders of human growth hormone and salmon calcitonin (27). Low aqueous solubility of hydrophobic amino acids (*e.g.*, trileucine) facilitated the formation of corrugated particles with low density which promoted superior aerosolization of powder. In agreement with the present data, highly wrinkled, raisin-like particles conferred improved particle size and FPF, while spherical ones with smooth surface were not desirable respecting aerosolization efficacy (28).

The SEM photograph of F_9, as selected powder, is presented comparing F_1 (Fig. 4a, b). Pure SD Infliximab formed coherent and smooth surface particles (F_1), while in the presence of cysteine raisin-like particles were produced (F_9). High surface area created by cysteine was reasonably accounted for the corresponding high deposition performance (FPF value of 67.75 ± 1.29%).

In Vitro Biological Activity of Antibody

Since the alteration in mAb higher order structure leads to loss of bioactivity, assessment of biological activity is regarded as the key parameter in formulation development. The comparable bioassay could imply preservation of secondary, tertiary, and quaternary structures of related protein as well (29). Infliximab mechanism of action is attributed to TNFα binding, cell apotosis, induction of ADCC, and activation of complement system (30); however, its biological activity mainly depends on the ability to target TNFα. An overall reduction in inflammation occurs as a result of TNFα neutralization.

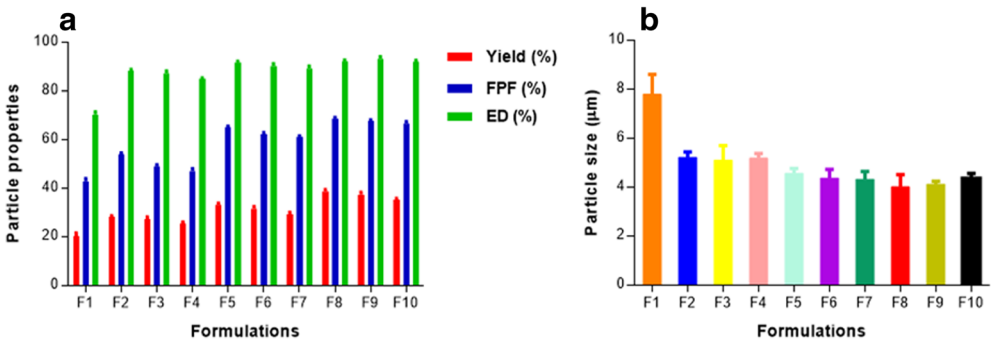

Fig. 3. Particle properties of SD Infliximab powders. **a** Particle size (μm). **b** Yield, FPF and ED (%)

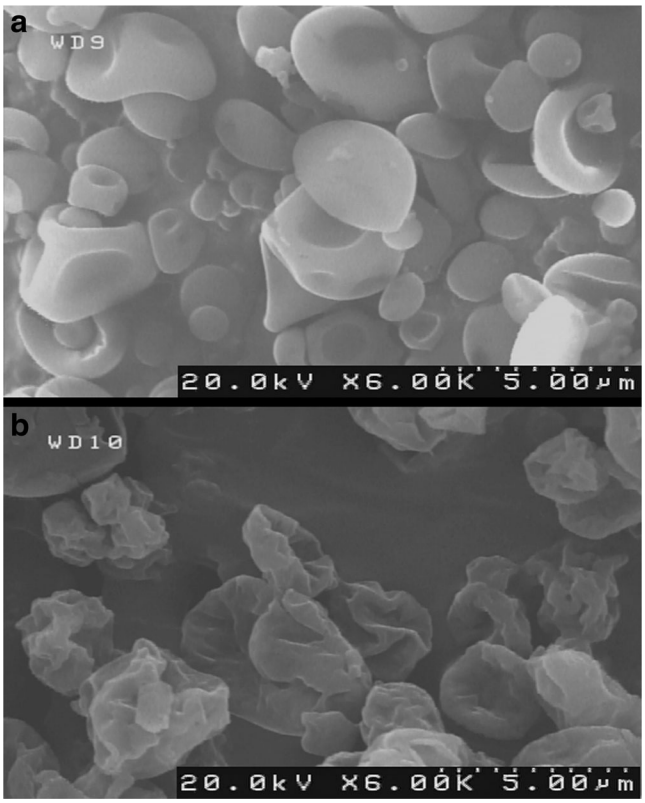

Fig. 4. SEM images of **a** SD pure Infliximab powder (F_1), and **b** F_9 (composed of 30 mg Infliximab, 36 mg trehalose, 12 mg cysteine, and 0.05% Tween 20)

In order to compare the processed formulation with reference standard, the measurement of cell viability was performed in the presence of dilution series of F_9 and Remicade®. Various antibody concentrations were added to the culture of L-929 cell line and following incubation, the cell viability was calculated applying calorimetric MTT assay. The absorbance of plate containing Remicade® and F_9 was measured at 620 nm. Both Remicade® and F_9 should neutralize the cytotoxic effect of TNFα on L-929 cell line. Increasing the ratio of antibody enhanced cell viability in both formulations. The cell viability was 99.8 and 92%, at 1000 ng/ml for Remicade and F_9. Based on the data presented in Fig. 5 (non-linear regression, Graph pad prism software, version 6.01), EC_{50} was 8.176 and 6.733 ng/ml for F_9 and Remicade®, respectively. To further assess the efficacy of formulation components on biological activity of Infliximab powder, EC_{50} of F_1 additionally calculated which was 11.20 ng/ml.

The comparison of formulations indicated the acceptable preservation of structure and efficacy of dry powder formulation of Infliximab. F_9 was maintained at high TNFα binding affinity in comparison to Remicade®. This study confirmed that spray drying stresses and especially high temperature could be desirably controlled in the presence of efficient excipients so that the structural stability and biological activity of antibody could be maintained more than 82% of Remicade®. Similar to the current results, spray drying was reported as a successful method for processing Infliximab powder with retained stability and successful TNFα binding (31). Also in a same procedure of spray drying, Trastuzumab affinity to HER_2 antigen was compared with that of Herceptin employing ELISA method. Preserved affinity of antibody to antigen was 86.5% in a SD sample comparing 100% affinity in Herceptin (32). Lower biological activity of F_9 *versus* SD Trastuzumab might be attributed to the sensitivity and instability of chimeric monoclonal antibody when compared to humanized ones.

Anti-Inflammatory Effect of Processed Antibody in an Allergen-Induced Mouse Model

To further confirm the influence of F_9, *in vivo* evaluation was designed in a mouse model of inflammation. The characteristics of three groups as well as applied regimen are presented in Table II. TNFα titer in the lung tissue of killed mice was quantified by ELISA method. The standard curve was drawn based on average absorbance at 450 nm *versus* TNFα concentration (Fig. 2 supplement). The absorbance of the test, positive control, and negative control groups was determined according to the standard curve at 450 nm (Fig. 6).

As it is evident, comparing positive control group (with TNFα secretion concentration of 68.34 pg/ml), in the test group, lung TNFα secretion was significantly reduced (26.56 pg/ml) which proved the efficacy of F_9 in inhibition of cytokine secretion and subsequent induction of inflammation (one-way ANOVA, SPSS 16, Table file 3 supplement).

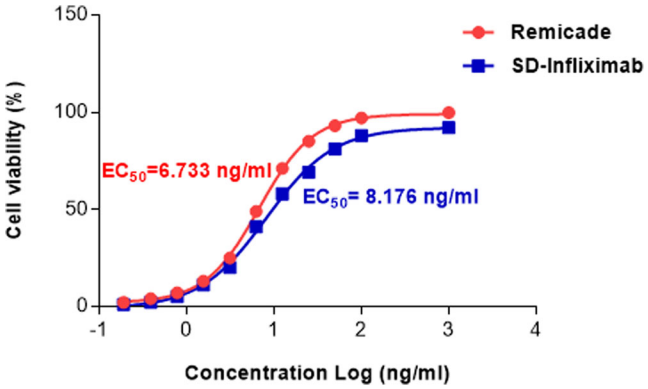

Fig. 5. EC_{50} (μg/ml) of Remicade and F_9 (composed of 30 mg Infliximab, 36 mg trehalose, 12 mg cysteine, and 0.05% Tween 20); log concentration is plotted *versus* cell viability to calculate EC_{50}

The lung serves as a local target for treatment of lung diseases including tuberculosis, lung cancer, cystic fibrosis, and asthma. Rapid onset of action along with favorable bioavailability caused drug molecules to be favorably administered *via* inhalation for lung target therapies (33). Lung delivery of monoclonal antibodies has presented satisfactory results in some previous investigations (34). Previously, administration of single dose (1 mg, i.p.) of Infliximab was reported to reduce the mouse allergic rhinitis following almost similar challenge protocol (35).

Furthermore, aerosolized inhaled mAbs against T cell receptor could successfully manipulate T cell activity in the airways and ameliorate AHR and inflammation in mice (36). Anti-CD49d antibody was administered both through i.p. (800 μg) and i.n. (300 μg) to compare the efficacy of systemic and local drug delivery. It was concluded that i.n. delivery blocked CD49d receptors in the airways, suppressed inflammation, and reduced IL-4 and IL-5 secretion. On the opposite site, eosinophilia was observed through systemic administration of drug (37).

Regarding the prospects of mAb pulmonary delivery for the treatment of respiratory dysfunction, inhaled Omalizumab (anti-IgE mAb) failed to subside the reaction of asthmatic patients to induced allergy (38). Lack of functionality of the drug was likely attributed to low systemic drug concentration in the blood stream for inhibition of secreted-IgE (39).

Considering the pulmonary delivery of Infliximab as a feasible method for local treatment of asthma, some challenges remain yet to be circumvented such as anti-drug antibodies (ADA) as a measure of immunogenicity. The formation of immune complexes between ADA and monoclonal antibody can cause adverse effects including hypersensitivity reactions and neutralization of drug effect (40); therefore, the formation of human anti-chimeric antibodies may promote immune responses in patients with asthma, as a chronic inflammatory disease, and should be further evaluated in human clinical examinations.

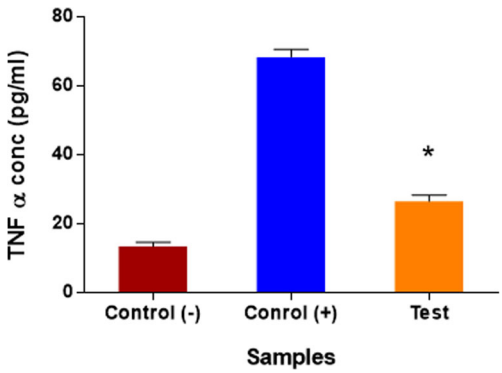

Fig. 6. TNFα levels in the test sample (challenged and received treatment), negative control sample (no challenge and no treatment), and positive control group (challenged and received no treatment). *p* values less than 0.05 are defined as statistically significant. Asterisk is statistically different to positive and negative control with *p* values < 0.05

CONCLUSION

Interest in local delivery of mAbs to the lung is growing as a potential means of boosting the efficacy and limiting the systemic adverse effects of drug. This study confirmed the feasibility for the pulmonary delivery of Infliximab. Remarkably, the spray drying process with the applied excipients preserved the antibody from gross aggregation and fragmentation. Additionally, antibody bioactivity was well-retained and functional binding with TNFα occurred. Although F_9 could prevent high TNFα levels following challenge test, elimination mechanisms of antibodies from the lung namely macrophages and mucociliary routes are still unavoidable which should be resolved in further experiments.

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