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Routes of Administration and Dose Optimization of Soluble Antigen Arrays in Mice with Experimental Autoimmune Encephalomyelitis

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Abstract

Soluble Antigen Arrays (SAgAs) were developed for treating mice with experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. SAgAs are composed of hyaluronan with grafted EAE antigen and LABL peptide (a ligand of ICAM-1). SAgA dose was tested by varying injection volume, SAgA concentration, and administration schedule. Routes of administration were explored to determine the efficacy of SAgAs when injected intramuscularly, subcutaneously, intraperitoneally, intravenously, or instilled into lungs. Injections proximal to the central nervous system (CNS) were compared to distal injection sites. Intravenous dosing was included to determine if SAgA efficiency results from systemic exposure. Pulmonary instillation was included since reports suggest T cells are licensed in the lungs before moving onto the CNS[1,2]. Decreasing the volume of injection or SAgA dose reduced treatment efficacy. Treating mice with a single injection on day 4, 7, or 10 also reduced efficacy compared to injecting on all three days. Surprisingly, changing the injection site did not lead to a significant difference in efficacy. Intravenous administration showed efficacy similar to other routes, suggesting SAgAs act systemically. When SAgAs were delivered via pulmonary instillation, however, EAE mice failed to develop any symptoms, suggesting a unique lung mechanism to ameliorate EAE in mice.

Keywords

hyaluronan; experimental autoimmune encephalomyelitis; SJL mice; HPLC; facilitated diffusion/transport; peptides; immunology; biomaterials; polymeric drug delivery system

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INTRODUCTION

Multiple sclerosis (MS), an autoimmune disease that destroys the myelin sheath, impacts the patient both physically and mentally[3]. Loss of vision, varying degrees of paralysis, loss of balance, and lack of coordination constitute the most common physical symptoms. Mental symptoms include slurring of speech and cognitive difficulties. The onset of disease typically occurs in young adults, but the disease continues into adulthood. Current therapeutic interventions can mitigate symptoms but none address the underlying immune response specific to offending myelin autoantigens.

MS can be classified into the following types: (1) secondary progressive, (2) primary progressive, (3) progressive relapsing, and (4) relapsing remitting. Because relapsing remitting MS (RRMS) affects 80% of patients with MS, current treatments have focused on this form of the disease[4]. In RRMS, the symptoms manifest periodically. In a period of relapse, the patient is acutely aware of the symptoms, but feels them to a lesser degree during periods of remission. Over time, symptoms are continually perceived even during periods of remission, a development indicative of progressive RRMS.

Not much is known about the cause of MS in humans. One theory asserts that MS is a systemic disease in its early stages, and in the later stages MS is localized to the lymph nodes located close to the spinal cord{Weller, 2010 #21}[5]. As a result, designing a treatment that can be active at the lymph nodes may be important. Another theory suggests that T cells are licensed (programmed with a tropism for the cervical lymph nodes) in the lungs before being sent to the CNS, alluding that an important site of intervention may be in the lungs, instead of the cervical lymph nodes[1,6].

The animal model typically used to mimic MS is EAE, where young mice are given adjuvant along with specific epitopes of PLP (proteolipid protein) or other antigens from proteins that make up myelin sheath[7–9]. In this PLP-induced EAE model, one cycle of MS-like symptoms is followed by remission over a 25-day period. Clinical scores are assigned by the degree of paralysis and balance. Other symptoms usually present in MS, such as cognitive ability, are difficult to measure in mice. The severity of EAE is measured by the clinical scores given to each mouse, with an increase in score indicating an increasing degree of paralysis, ascending from the tail to the hind-limbs and then the fore-limbs as the disease worsens.

The available treatments for MS are known to slow the disease progression and to manage specific symptoms. In early stages of the illness, disease-modifying treatments like interferon-β, glatiramer acetate, mitoxantrone, and natalizumab are prescribed. Natalizumab, also known as Tysabri®, inhibits transit of lymphocytes across the blood-brain barrier. In the later stages of the disease, very low dose cancer chemotherapeutics may be used to suppress the immune system while minimizing systemic toxicity[10]. Another approved treatment, fingolimod (Gilenya®), sequesters lymphocytes within lymph nodes, thereby restricting further destruction of the myelin sheath[11]. This leaves the patient open to opportunistic infections, however, as is the case with global immunosuppressors. Designing therapies that specifically target lymphocytes associated with MS or that induce tolerance to autoantigens

by inhibiting costimulation during autoantigen recognition may further stymie the progression of the disease.

Our lab has developed Soluble Antigen Arrays (SAgAs), composed of a hyaluronic acid (HA) backbone with grafted autoantigen (e.g. PLP epitope) and a second peptide, such as an ICAM-1 ligand (LABL) (Table 1)[12]. The size of SAgAs can be designed to drain with interstitial fluid and fit through the pores of lymphatic vessels, which range between 10 nm and 100 nm[13]. At least three factors affect SAgA drainage and, ultimately, absorption: the site of injection, diffusion of the SAgA, and local vascular and lymphatic network. After subcutaneous injection, for example, HA can drain to lymph nodes and certain molecular weights of HA can have a long retention time[14]. By varying the route of administration (site of injection and the type of injection), we investigated which route offers the best efficacy. SAgA treatment alleviates the clinical symptoms of EAE, yet the mechanism of action is still unclear[12]. Here, variations in dose schedule, SAgA dose, injection volume, and different routes of administration were tested to improve understanding of the clinical mechanism. Intramuscular (IM), subcutaneous (SC), intraperitoneal (IP), intravenous (IV), and pulmonary instillation (PI) routes of administration were studied.

MATERIALS AND METHODS

Peptide Synthesis

Peptides were made using an automated solid phase peptide synthesizer (Pioneer; Perceptive Biosystems, Framingham, MA). 9-florenylmethyloxycarbonyl-protected amino acids were used to synthesize both $PLP_{139-151}$ and LABL. In order to conjugate these peptides onto HA, an aminooxy (Ao) group was added to the N-terminus of PLP and LABL in the peptide synthesizer. Peptides were cleaved off of the resin using trifluoroacetic acid (TFA) and scavengers.

The peptides were then purified using a C18 semipreparative column (Higgins Analytical, Proto200, 5 μm, 200 Å, 250x20mm) with reversed-phase high performance liquid chromatography (HPLC). A gradient method was used to purify the peptides, with an aqueous mobile phase A (94.9% d.d. H_2O, 5% acetonitrile, and 0.1% TFA) and an organic mobile phase B (99.9% acetonitrile and 0.1% TFA). The purity of the peptides was analyzed using HPLC on a C18 analytical column (Higgins Analytical, Proto200, 5 μm, 200 Å, 250x4.6mm) with the same mobile phases and similar gradient program. The molecular weight of the peptides was identified using an electrospray ionization time-of-flight mass spectrometer (Supplemental Figure 1).

SAgA Synthesis

After the peptides were purified, they were then conjugated onto HA. A 2-mg/mL solution of HA (16.9 kDa, 16 kDa, Lifecore Biomedical, Chaska, MN) was made using 20 mM acetate buffer at pH 5.5. AoLABL and AoPLP were mixed together in equal molar proportions, then added to the 2-mg/mL HA solution at a ratio of 1 aminooxy-peptide to 2 monomers of HA. The reaction continued for 24 hours, and then the reaction was dialyzed

(MW 3500, 6000–8000) against purified water for 24 hours. Samples were frozen and dried using a lyophilizer.

SAgAs were further analyzed to check peptide content. The SAgAs were dissolved in 0.1-N HCl at pH 1 for at least 4 hours to cleave the peptides. Using a calibration curve of free AoLABL and AoPLP at various concentrations, the amount of peptides in SAgAs were determined via reversed-phase HPLC on a C18 analytical column.

Dynamic Light Scattering (DLS)

The size distribution of SAgAs were determined using a Brookhaven Zeta-PALS at various concentrations (0.5 – 10 mg/mL) in PBS after they were filtered through a 0.45 μm PVDF membrane. Light scattering was detected at 90° and using a laser operated at 658 nm. The hydrodynamic radius (R_h) was determined from the Stokes-Einstein equation: $R_h=k_bT/6\pi\eta D_T$, where k_b is the Boltzmann's constant, T is temperature (K), η is the solvent viscosity, and D_T is the translational diffusion coefficient. The particle size was derived using the Stokes-Einstein equation and the autocorrelation function using a non-negatively constrained least squares (NNLS) deconvolution algorithm.

EAE Model

The 6–8 week old female SJL/J mice for the Route of Administration study were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The 3-week old female SJL/J mice for the dosing study were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). The mice were housed in a pathogen-free facility at the University of Kansas approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures using live animals have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kansas.

On Day 0 of the study, the SJL/J mice were induced with EAE through four 50-μL injections of an emulsion containing Incomplete Freund's Adjuvant Oil (BD Difco Adjuvants, Franklin Lakes, NJ), *Mycobacterium tuberculosis* (BD Difco Adjuvants, Franklin Lakes, NJ), and 200 nMol of PLP per animal. The SC injections were given above each of the shoulder blades and the rear haunches of the mouse. Each animal was also given a 100-μL IP injection of 200 ng of pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) on Days 0 and 2. The mice were weighed on each day of the 25-day study and were given a clinical score ranging between 0 and 5 from Day 7 to the end of the study. The clinical score increases with disease progression. Scores increased in correlation with the level of paralysis, starting with the tail and advancing to the head. The following clinical score scale was used: 0 – no symptoms of disease were seen, 1 – limp tail and waddling gait, 2 – partial hind leg paralysis, 3 – paraplegia (complete hind leg paralysis), 4 – partial front leg paralysis, 5 – moribund or complete front leg paralysis. The mice were then treated on Days 4, 7, and 10 with 100 μL of SAgA (containing 200 nMol of PLP) on each treatment day, unless otherwise stated. There were 6 mice in each group, with three mice housed together per cage. All the statistical analysis was done on Prism GraphPad 5 software, using ANOVA analysis.

Changing the Dose Schedule, Volume, and Amount of SAgA Treatment

EAE mice were treated on only one of the three days mentioned (day 4, 7, or 10). Days 4 and 7 was chosen as a treatment day to monitor disease progression if treatment occurred prior to symptoms and day 10 was chosen to track the disease severity if treated after the symptoms were visible. SAgA was still given on a 200-nMol PLP basis using 100 uL per injection. Two other variables were changed in the study, the amount of SAgA given and the volume of the injection. One group of mice was treated on a 50-nMol PLP basis of SAgA (100 uL per injection) given on all three treatment days. Another group of mice was treated on a 200-nMol PLP basis of SAgA using 20 uL per injection given on all three treatment days. All of these injections were given in the Upper SC site. The same SAgA was used for each animal study and the PLP:LABL ratio (1:1) was kept the same in each study.

As a negative control, a group that only received phosphate buffered saline (PBS) as a treatment was included in each study. A group that received a SC SAgA injection in the upper back was also included as a positive control in each study since treatment route was used in previous[12,15].

Route of Administration EAE Study

The administration routes initially explored were IM, SC, and IP. More sites were then added to test whether clinical scores may improve if administering SAgAs near the upper lymph nodes, close to the spinal cord. As a result, the following injection sites were used: IP, Upper IM, Lower IM, Upper SC, and Lower SC (Figure 1).

In a second component of the study, IV and pulmonary routes were also compared to the Upper SC delivery of SAgAs. A tail vein IV injection site was chosen to measure what the effect systemic delivery of SAgA would produce. SAgAs were given at a 200-nMol PLP basis at an injection volume of 100 μL. For PI, SAgAs were given at a 200 nMol PLP basis, but the injection volume was decreased to 50 μL due to the limitation in volume that can be safely delivered to lungs. Administration positive and negative controls were included for both studies as mentioned above.

For PI administration, each animal was anesthetized with inhaled isoflurane in an induction chamber. After the mouse was fully anesthetized, the mouse was positioned with its back on a dosing board at approximately a 60° angle to a supine position using a thin wire to suspend them by their incisor teeth with a nose cone being used to maintain anesthesia. The mouth was opened and the tongue was gently pulled out and to the side of the mouth. A laryngoscope was then positioned to depress the tongue and see the cords at the top of the trachea and 50 uL of solution was administered at the top of the trachea. The tongue was withheld for 3 breaths at which time the mouse was maintained under anesthesia for an additional 3 minutes on the dosing board. The mouse was then removed from the dosing board and held vertically until she began to recover from the anesthesia.

RESULTS

Peptide Conjugation Efficiency

Before any of the SAgAs were injected into animals, the amount of peptide per SAgA was confirmed. For the first EAE study exploring the dosing schedule, amount and volume, approximately 9 LABL molecules and 8 PLP molecules were in SAgAs. Table 1 reports the characteristics of SAgAs that were used for the remaining two routes of administration studies. Using these data, a 200-nMol PLP dose was calculated for each SAgA treatment. The average molecular weight of the SAgA, the approximate number of PLP and LABL molecules on one 16 kDa HA chain, and the final ratio of PLP:LABL in SAgA were calculated using reverse phase HPLC data. (Table 1)

DLS

Figure 1 shows the hydrodynamic radius of SAgAs ranged between 3 – 10 nm. At concentrations at 1 mg/mL and lower, SAgA size ranged between 5 – 10 nm. SAgAs were injected at a maximum of 10 mg/mL, which falls in the size range of 3 – 10 nm. A range of sizes was expected for SAgAs since the starting HA exhibits a size distribution and the number of peptides associated with each HA strand may vary. According to studies tracking passive tissue drainage of HA, SAgAs at the higher end of the size distribution (~10 nm) would be expected to infiltrate the lymphatic system. At the lower size range (~3–5 nm), SAgAs would be more likely to enter the systemic circulation. The size of SAgAs is also small enough to bind immune cells and be actively transported to secondary lymphoid organs.

EAE Study of Dosing Schedule, Amount, and Volume

The SAgA dosing schedule has been determined from work by Siahaan et al. using their bifunctional peptide inhibitor (BPI), which is a small molecule version of SAgAs that contains PLP and LABL with a linker in between. BPI ameliorated disease best when given on days 4, 7, and 10, at a concentration of 100 nMol[16–19]. In early studies, SAgAs were injected on a 100-nMol PLP basis, but after a small dosing study, it was found that a 200-nMol PLP basis decreased clinical scores of EAE mice more than the 100-nMol dose[12,15].

Here, the treatment schedule was changed to a single SC injection on only one of the three treatment days (day 4, 7, or 10 of the study). Treating only on day 7 seemed to decrease the disease symptoms the most when compared to either the PBS-treated group or the group that received treatment on all three days. Differences in clinical score, however, were not significantly different (Figure 2A). The incidence of disease graph is another way to view the clinical scoring of animals. A score of 1.5 indicates that the animal experiences full paralysis of the tail with partial paralysis of hind-limbs. As a result, 1.5 is a critical score to determine if the disease affects mouse movement or not. The incidence of disease for variation of dosing schedule (Figure 2B) indicated, however, that only half of the group dosed on day 7 had a score of 1.5 or higher.

In another study, the dose was decreased from 200 nMol PLP to 50 nMol PLP. As mentioned above, the SAgA dose was calculated based on the amount of conjugated PLP

antigen administered. Decreasing the dose reduced efficacy, even if the SC treatment was dosed on all three days. The clinical scores (Figure 2C) and incidence of disease (Figure 2D) resembled that of the PBS control group more closely than the group that was treated at the 200-nMol dose on all three days.

Lastly, the total volume of the SC injection was reduced from 100 μL to 20 μL, while maintaining the 200-nMol PLP dose. The clinical score data (Figure 2E) showed no difference compared to either of the control groups (PBS or SAgA treatment using 100 μL injections). The incidence of disease (Figure 2F), however, suggested lowering the injection volume reduced efficacy in a majority of the animals, even when the dose injected did not change. The significant difference in clinical scores between the 20 μL and the 100 μL injection suggested a larger injection volume may improve SAgA exposure (e.g. local drainage or systemic absorption).

EAE Study of Routes of Administration

Next, the 200-nMol dose in 100 μL was injected into different sires: upper SC (as before), lower SC, upper IM, lower IM, and IP (Figure 3). In exploring the different routes of administration, each of the treatments was found to be statistically significant ($p<0.05$) from the PBS control group during the peak of disease, days 12–17 (Figure 4A). In this study, no difference was apparent between different SAgA routes of administration. Both the clinical score data and the weights of the animals revealed differences between the PBS control group and the various treatment groups. The incidence of disease graph (Figure 4B), however, suggested the upper IM group had the lowest number of animals with a disease score of 1 or higher.

A subsequent study compared upper SC, IV, and PI routes of administration. The SC and IV group scores (Figure 4C) and weights (Supplemental Figure 2E) were similar to one another and were, once again, significantly improved compared to the PBS group ($p<0.05$) during the peak of disease. The PI group maintained a clinical score of zero and showed the best maintenance of weight throughout the study. In the incidence of disease profiles (Figure 4D), no mice in the PI group had a clinical score of one or higher, further supporting pulmonary instillation of SAgAs provided the best amelioration of disease.

DISCUSSION

In vaccine treatments, IM injections are usually given to produce an immune response. In contrast, SC injections are typically used for hyposensitization of patients (e.g. allergy shots). Subcutaneous injections are more likely to diffuse from the injection site, where as intramuscular injections may form a depot, depending on the properties of the injected material. Since transport to the lymph nodes was thought to be vital for SAgAs, the SC route was chosen as it allows for both active transport and passive diffusion to the lymph nodes. Bagby et al. have found that injecting HA subcutaneously in a mouse footpad results in HA draining to lymph nodes[14]. Since SAgAs contain HA and are delivered subcutaneously, it is possible that they can also be transported to the lymph nodes, either by passively draining with interstitial fluid or by active transport by binding immune cells. Additionally, SC

injection of SAgA was previously shown to decrease EAE scores, so dose schedule, amount, and volume were explored by this route first[12,15].

EAE Study of Dosing Schedule, Amount, and Volume

Treating on one of the three treatment days or injecting SAgA at a lower concentration generally caused the disease symptoms to increase compared to treating on days 4, 7, and 10 with 200 nMol PLP conjugated to SAgA. Treatment on day 4 did not allow for the disease to fully manifest, since 7 – 10 days are required for the immune response to occur[20]. Treatment on day 10 allowed for the symptoms to be apparent, which means that the immune system has already damaged the CNS (e.g., spinal cord). The incidence of disease graph having clinical score greater than 1.5 supports the argument that day 7 had the lowest incidence of disease, however clinical scores were similar for all days.

Arguably, day 10 would be the most similar to a clinical setting with a human subject. While considering the incidence of disease trends, administering treatment after symptoms have persisted for a few days seems not as effective as treating earlier. For full effectiveness, it may be important to treat before the symptoms are felt, with enough time for the body to process an immunological response. For example, natalizumab treatment regimen is given on a monthly schedule, prior to symptom presentation, which would be similar to early treatment using SAgA[21–24]. These similar mechanisms suggest SAgAs may be interacting with immune cells and somehow inhibiting trafficking, sequestering these cells in lymphoid tissue, or triggering anergy.

SAgAs were not as effective when the overall dose was decreased from 200 nMol to 50 nMol PLP. All six of the mice had a clinical score greater than 1.5 just one day after the PBS-treated group reached that level. While the onset of disease was slightly delayed, symptoms progressed very quickly once present. Going forward, a 200-nMol PLP dose was deemed necessary for SC delivery to be effective.

To test the last dosing variable, SAgA concentration was increased to deliver 200 nMol of PLP in 20 μL instead of 100 μL. The injected material was hypothesized to remain more local and not spread under the skin to the same extent as in the typical 100 μL injection groups. While the onset of disease was delayed, the majority of the animals did get sick. The smaller volume injected did decrease the clinical score, but it was not as effective as the larger volume injection even though the dose was the same. In conclusion, the 100 μL SC injection of SAgA allowed for better amelioration of disease perhaps because of improved local distribution and exposure, compared to the lower volume injection. The larger volume injection was observed to spread under the skin to a greater extent than the smaller volume injection. As a result, the injection is much more likely to spread itself over a large surface area, giving SAgAs an increased likelihood of absorption or binding to peripheral immune cells for active transport. It is reasonable to suspect the higher volume injection may have even hydrostatically perfused more SAgA into lymphatic ducts, since lymph node clearance is driven by a pressure differential between the lymphatic ducts and tissue space.

EAE Study of Route of Administration

Since changes in dose and injection volume changed response, the route of administration was hypothesized to have an important effect. EAE mice were treated using the most effective regimen (200 nMol PLP, 100 μL injection days 4, 7, 10) by injecting upper SC, lower SC, upper IM, lower IM, or IP. All of the routes of administration significantly improved the disease state compared to the PBS group, but the treatment groups did not differ from each other (Figure 4A). Comparing the severity of disease in individual animals showed no animals in the upper IM group had a disease score of 1.5 or higher, whereas 5 out of 6 animals in the upper SC group had a score of 1.5 or higher at the peak of disease (Figure 4B). Additionally, all of the mice in the PBS control group had a score of 1.5 or higher by day 13.

SAgAs were originally designed to transport to the lymph nodes, through either passive drainage or active transport. Passive drainage allows SAgAs to diffuse with interstitial fluid to the lymph vessels, then drain to the appropriate lymph nodes. During active transport, peripheral antigen presenting cells such as macrophages or dendritic cells may carry SAgAs to the lymph nodes (e.g., after recognizing PLP antigen[25]). Intravenous delivery of SAgAs was used to compare results from upper and lower SC, upper and lower IM, and IP injections. Similar efficacy via different routes of administration chosen for this study would suggest SAgAs may act systemically to ameliorate EAE. This is supported by DLS data that indicates the size distribution of SAgAs would include some SAgA molecules that may be small enough to enter the circulation and some SAgA molecules large enough to favor passive drainage via the lymph vessels.

IV administration using the same SAgA dose injection volume and schedule was compared to upper SC administration. The IV route provided similar disease amelioration and this group provided a key benchmark for comparing other routes of administration (Figure 4C). Since there was no difference in scores between the IV and the SC groups, it is probable that the SC injection is also delivering SAgAs systemically. Small, twenty-gram mice were injected with 100 μL, perhaps increasing the likelihood that SAgAs absorb into circulation instead of creating a depot. In the incidence of disease graph (Figure 4D), the SC and IV treatment groups both had over half of the mice that were affected by the disease. This complements the clinical score data for the SC and IV treatment groups, supporting the notion that subcutaneous injection may lead to systemic delivery of SAgA. SAgAs may transport to the spleen when delivered systemically, which may allow for the SAgAs to interact with B and T cells. Further studies would be needed to support or disprove this hypothesis.

Finally, another route of administration was explored in this study, pulmonary instillation. In the ‘Hub and Spoke’ model of the immune system, T cells are licensed in the lung before they are distributed throughout the body[2]. In the case of multiple sclerosis, the lung is the hub and the CNS tissue is the spoke. Odoardi et al also determined that T cells acquire the properties needed to migrate to other parts of the body in the lung[1]. Delivering SAgAs to the lungs may allow multiple sclerosis-specific T cells to interact with SAgAs. An exact mechanism between T cells and SAgAs is still being explored, but delivering SAgAs to the lungs proved to significantly decrease EAE disease symptoms. After PI of SAgAs, mice

showed disease scores of zero throughout the course of the 25-day study. In comparison to other routes of delivery (e.g., SC and IV), pulmonary delivery provided the lowest clinical scores, the best maintenance of weight, and no animals were affected by disease (Figures 4C and 4D).

The increased efficacy of PI of SAgAs compared to subcutaneous or intravenous injection may also be explained by some of the physiological differences in transport. Several different transport processes may occur after PI. Passive transport routes include diffusion of SAgAs through the lung epithelial tissue, followed by absorption into the lymphatic network or into circulation. Xie et al, determined that pulmonary instillation of an HA conjugate allowed the majority of the drug to stay within the lung, where it could be targeting the surrounding lymph nodes[26]. It is possible that the effect of SAgAs after PI administration is more pronounced than SC or IM because of the shorter pathways from the lung to the lymph node, thus leading to higher dose or better timing during the pre-symptomatic stage of immune response.

Another possibility to consider is that SAgA degradation and diffusion of components is possible, but our previous studies have shown neither free peptides nor HA (alone or in combination) have a therapeutic effect[12]. Active processes of mucocillary clearance and macrophage clearance may also affect SAgA transport. Mucous is transported by cilia located in the conducting airways and is deposited in the oropharynx and swallowed. The potency of SAgAs administered PI suggest minimal mucociliary clearance. Macrophage clearance typically occurs in the lower airways including terminal bronchioles and alveoli. Macrophages then transport phagocytosed cargo to the lymph nodes for processing. Lymph nodes are key to the efficacy of immune therapies, since that is a primary site of immune cell priming.

Observations from SC and IV routes of administration do not support a systemic mechanism of SAgA efficacy of after PI. Although, it is possible increased PI efficacy is linked to avoiding fist pass metabolism, which could diminish SAgA effect after IV or SC administration. Furthermore, it is plausible the lung may act as a SAgA depot after PI, since macrophage clearance can take days, providing an extended duration of action compared to the other administration routes. Perhaps the most plausible explanation is that pulmonary instillation may offer a direct avenue to the lymphatic system, either passively or via active transport or may somehow bind and inhibit T cells migrating through the lungs to be licensed for trafficking to the CNS. Additional studies will be required to elucidate these possible mechanisms.

CONCLUSION

SAgA treatment schedule, dose, injection volume, and routes of administration were systemically explored to treat EAE. Timing the SAgAs dose was important, suggesting it must follow the schedule of a typical natalizumab dosing, before symptoms are fully realized. Decreasing SAgA dose to 50 nMol (i.e. 50 nMol of PLP delivered) administered on days 4, 7, 10 was not as effective as 200 nMol. Decreasing the volume of the dose to 20 μL

meant increasing the concentration 5 fold and SAgAs were slightly less efficacious at the lower volume, suggesting the lower volume, higher concentration had limited exposure.

Exploring routes of administration provided new insight into possible mechanisms of action. The initial goal was to deliver SAgAs to lymph nodes. The first set of administration sites (upper and lower SC, upper and lower IM, and IP) was chosen with this goal in mind. Previous studies suggested a portion of SAgAs injected at the upper SC site would drain to the cervical lymph nodes, which drain the CNS and are implicated in EAE. Delivering SAgAs via the SC route was similar to delivering IV suggesting all peripheral injections may be acting via systemic exposure. The most surprising result was that the pulmonary delivery of SAgAs decreased the clinical scores with no mice exhibiting symptoms throughout the study. Applying the ‘Hub and Spoke’ theory to pulmonary delivery, it is possible that delivering SAgAs to the lungs allows maximized interaction with the immune system, either within the lung or via local lymphatics.

To further explore mechanisms of SAgA efficacy, the dosing schedule, amount, and volume studies could be repeated via the pulmonary instillation route of administration. Further studies that track the distribution of SAgA throughout the mouse after pulmonary instillation would provide useful insight to the trafficking of SAgA molecules. Additionally, in vitro work is under way to determine target cells by screening SAgAs in EAE splenocytes and measuring cytokine responses to help explain the efficacy observed in EAE mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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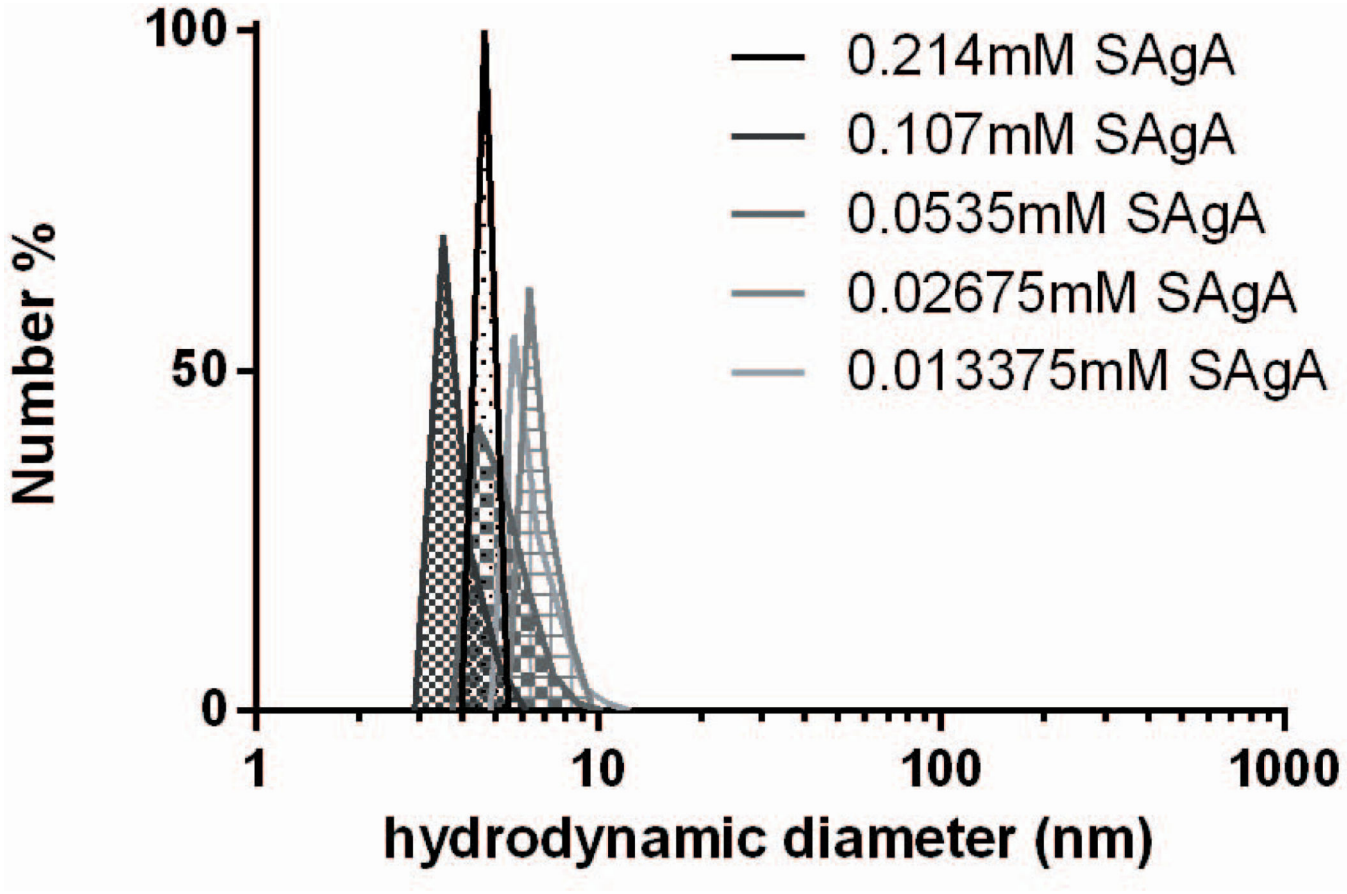


Figure 1.
DLS measurements show various concentrations of SAgAs to measure between 3 – 10 nm in hydrodynamic radius.

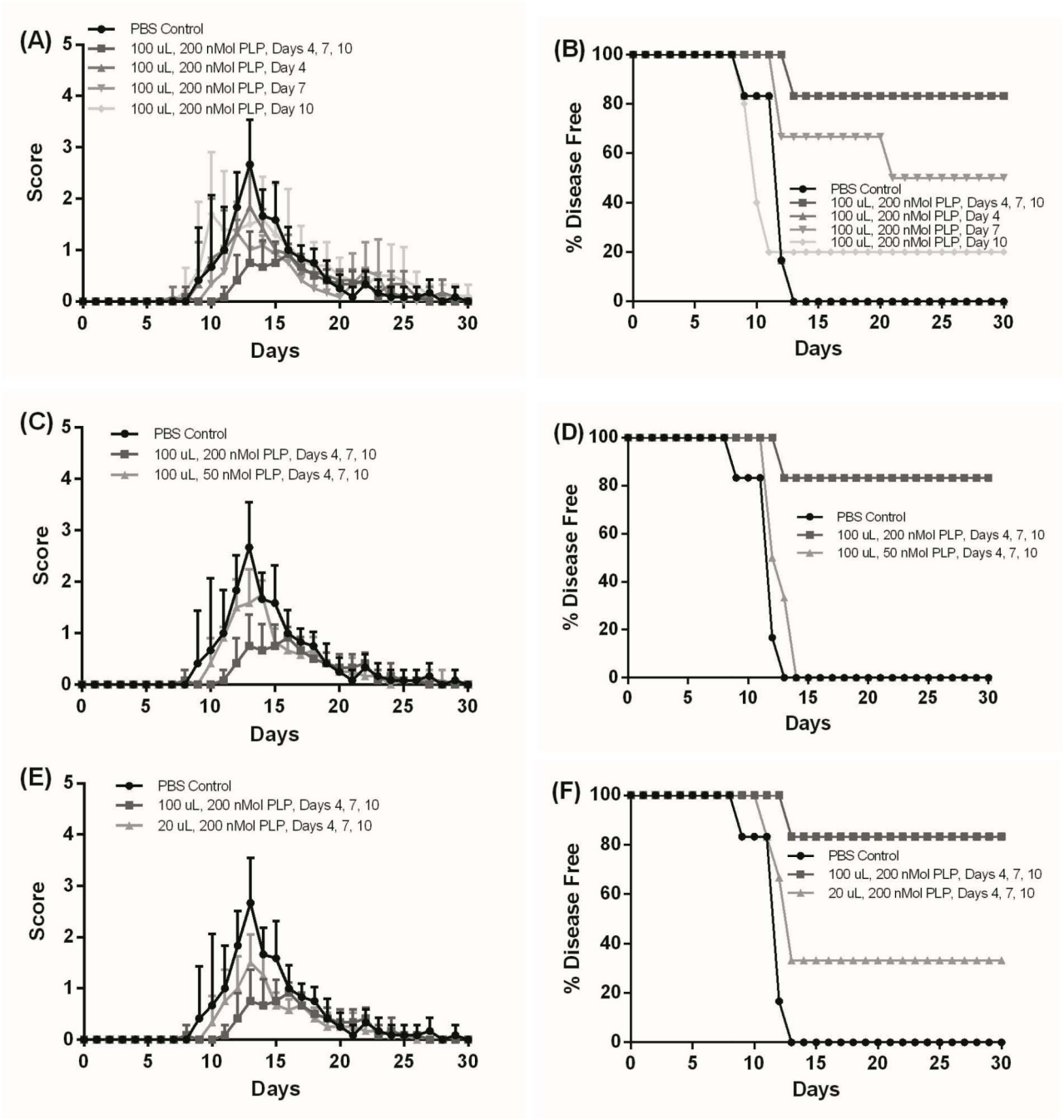


Figure 2.

Clinical score and incidence of disease data for dosing schedule, dosing amount and dosing volume. For the incidence of disease data, a disease score of 1.5 or higher shows that the animal is affected by the disease. (A) Dosing schedule was changed to one of the three treatment days. There is significance ($p<0.05$) on Days 13 and 15 of the study between the PBS control group and the group that was treated only on Day 7. (B) The incidence of disease for the dosing schedule study shows that treating on Day 7 results in the lowest incidence of disease. (C) Dosing amount clinical scores indicated that decreasing the dose

decreases the effectiveness of the treatment. (D) The incidence of disease for the dosing amount study showed that decreasing the dose increased the number of animals affected by the disease. (E) The dosing volume clinical scores showed that the lower volume injection is not as effective in comparison to the 100 uL injection. (F) The incidence of disease for dosing volume shows that the lower injection volume had a 50% greater incidence of disease.

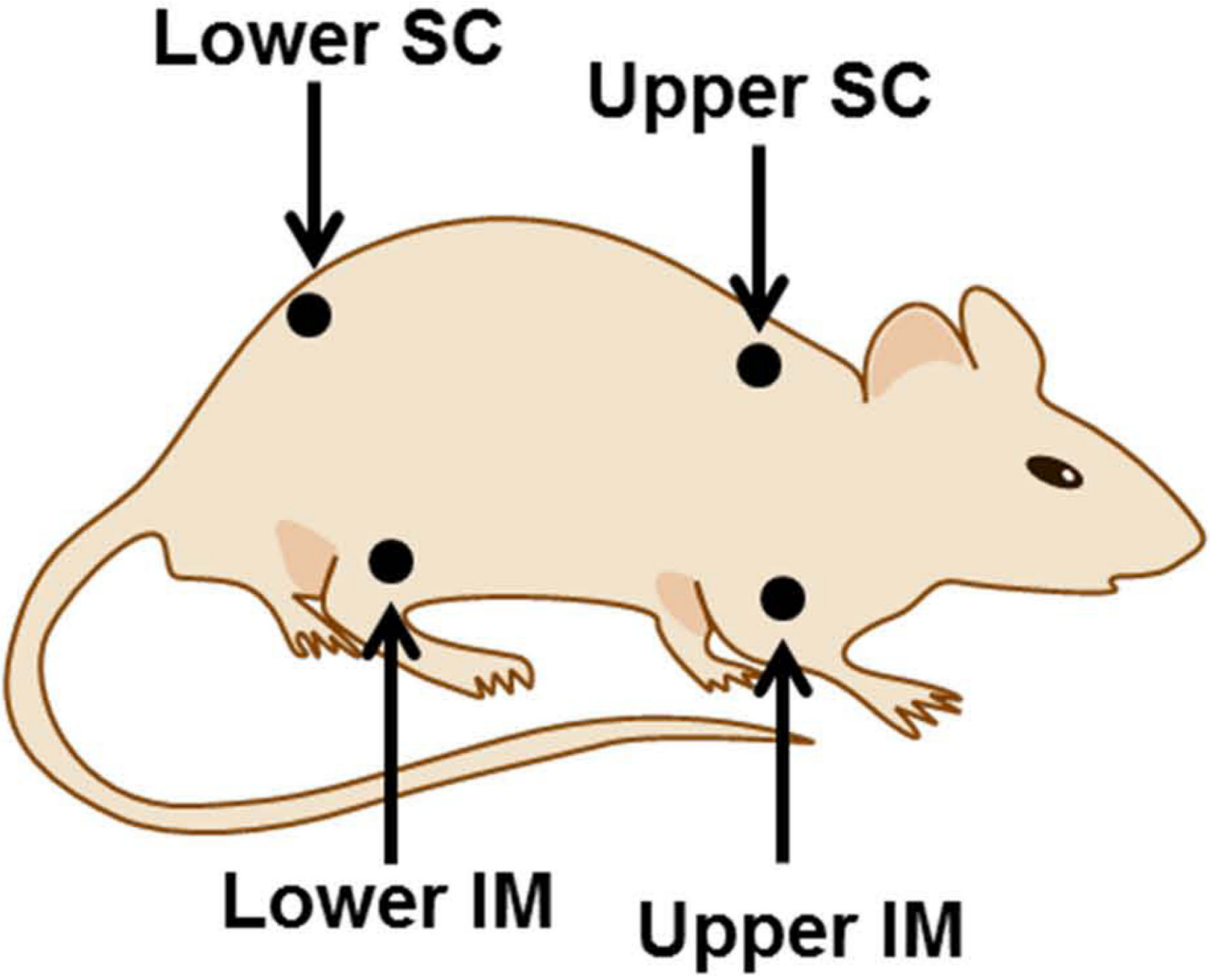


Figure 3.
Injection sites for route of administration studies.

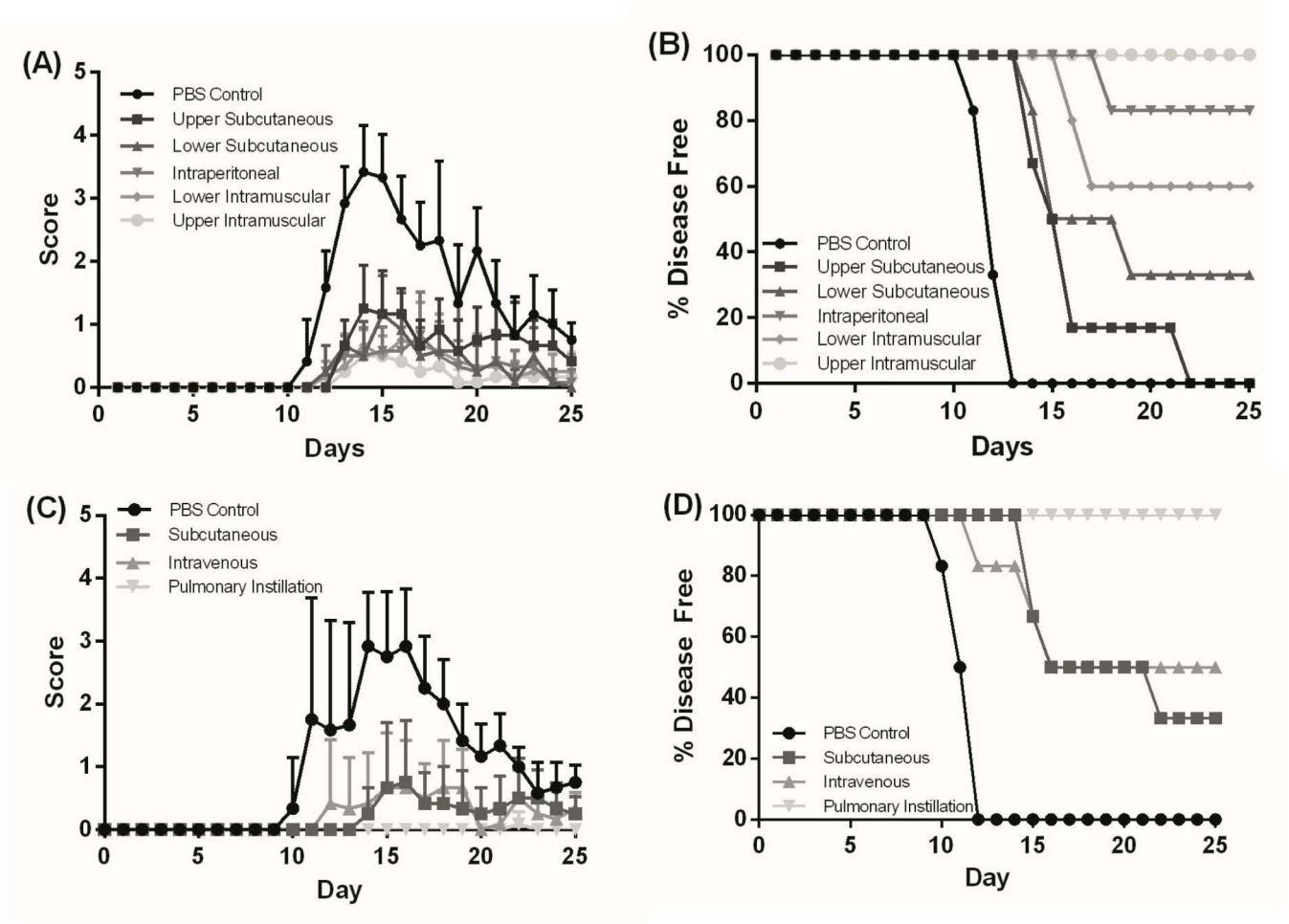
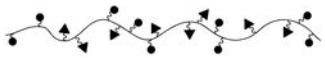


Figure 4.

Route of administration study clinical score (A) and incidence of disease (B) data. (A) There is no difference between different routes of administration, but there is a significant difference ($p<0.05$) between the PBS-treated group and all other SAgA-treated groups on Days 12–18. (B) Incidence of disease plots show animals with a disease score of at least 1.5 affected by the disease. The upper IM group was the least affected and the PBS-treated and upper SC groups were the most affected. **(C)** The pulmonary instillation group had disease scores of 0 throughout the peak of disease. **(D)** No animals in the pulmonary instillation group had a score of 1.5 or higher.

Table 1

Using pH 1.0 solution, the peptides were hydrolyzed from HA to indirectly quantify the amount of AoPLP (circle) and AoLABL (triangle) grafted to SAgA.

$SAgA_{PLP:LABL}$ used in EAE Studies	Calculated MW	Number of PLP Peptides per Scaffold	Number of LABL Peptides per Scaffold	Final Ratio (PLP:LABL)
 Dosing Schedule, Amount, Volume	39605	8.84	8.27	1.069:1
 Route of Administration (Upper and Lower SC, Upper and Lower IM, IP)	34269	6.42	6.85	0.94:1
 Route of Administration (SC, IV, PI)	34788	8.19	5.53	1.48:1