Comparative immunogenicity of *Na*-GST-1 human hookworm vaccine with synthetic glucopyranosyl lipid adjuvant (GLA) in BALB/c mice.

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Introduction

More than 740 million people worldwide are infected with Hookworm. Hookworm infection is most prevalent in the poorest of the poor populations of the world, and has serious health effects. Hookworm infection causes blood loss leading to iron deficiency anemia and protein energy malnutrition, which results in a compromised immune response. Consequently, the target human population suffers from an increased susceptibility to infectious diseases including hookworm infection. We have developed recombinant adult hookworm vaccines against hookworm infection to break this vicious cycle. *Necator americanus* glutathione-S-transferase-1 (*Na*-GST-1) is a 24-kDa protein from *N*. americanus has peroxidase activity and catalyzes the conjugation of reduced glutathione to a variety of electrophiles. Recombinant Vaccine antigens like *Na*-GST-1, require adjuvants to enhance the strength and duration of the immune response to these antigens which may be weakly immunogenic on their own[1,2,3]. Aluminum salts such as aluminum hydroxide and aluminum phosphate (alum) were the only adjuvants in approved human vaccines in the USA. Although, alum is effective in boosting antibody responses, achieving these responses requires repeated administration. Adjuvants like CpGs, MPL, GLA-AF etc in combination with alum adjuvanted vaccines are known to boost immune response in healthy and immunocompromised individuals. A non-toxic derivative of LPS, monophosphoryl lipid A (MPL) a TLR-4 agonist with alum has been used in two vaccines; the human papilloma virus (Cervarix ®) and hepatitis B virus vaccine (Fendrix ®). For *Na*-GST-1 vaccine, we use a Synthetic Glucopyranosyl Lipid Adjuvant- Aqueous Formulation (GLA-AF) which is a pure synthetic hexaacylated lipid A derivative when compared to MPL which is naturally-derived and more heterogenous[4,5,6]., We believe that co-injecting (GLA-AF) a novel TLR-4 agonist with adult hookworm *Na*-GST-1 + Alhydrogel® vaccine will produce a robust and sustainable immune response in this target human population. Here, we discuss the study design and the results of the pre-clinical comparative immunogenicity study of the Human Hookworm *Na*-GST-1 + Alhydrogel® Vaccine in BALB/c mice with and without GLA-AF.

Na-GST-1 Hookworm Vaccine[6]

Necator americanus glutathione-S-transferase-1 (*Na*-GST-1) is a 24-kDa protein from N. americanus that has peroxidase activity and catalyzes the conjugation of reduced glutathione to a variety of electrophiles. Also, *Na*-GST-1 exhibits a high affinity for heme in vitro. Hookworm GSTs may bind and detoxify heme and hematin byproducts generated during the blood degradation process. Antibodies induced by *Na*-GST-1 vaccine will neutralize the activity of *Na*-GST-1, leading to accumulation of heme and hematin. Both heme and hematin contains oxidative iron, which can generate toxic reactive oxygen species that can damage parasite structures leading to reduce fecundity and worm burden. The recombinant polypeptide *Na*-GST-1 was expressed in Pichia pastoris. *Na*-GST-1 Hookworm Vaccine Drug Product was formulated at 0.1 mg/ml of *Na*-GST-1 with 0.8 mg/ml of Alhydrogel® in imidazole, glucose and phosphate buffer. Drug Substance was formulated at 2 mg/ml of *Na*-GST-1.

Synthetic Glucopyranosyl Lipid Adjuvant (GLA)

GLA-AF a pure synthetic hexaacylated lipid A derivative is a novel, clinical-stage, human toll-like receptor-4 (TLR-4) agonist[8]. GLA activates human TLR-4 receptors, induces Th1 CD4 helper cells and elicits broad humoral immunity. GLA was also shown to be safe and well-tolerated in humans subjects in a Phase I clinical study in combination with Fluzone® (inactivated influenza virus vaccine)

Methods

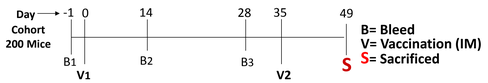
The study was comprised of 200 BALB/c Mice (see Table 1 for experimental design and dosing information). Ten BALB/c mice per group were immunized twice intramuscularly (i.m.) at 5-week intervals with 5.6 µg *Na*-GST-1/44.8 µg Alhydrogel® alone (high dose formulation) or with 5.6 µg *Na*-GST-1/44.8 µg Alhydrogel® mixed with varied doses of GLA-AF. Similarly, Ten Balb/c mice per group were immunized twice intramuscularly (i.m.) at 5-week intervals with 1.9 µg *Na*-GST-1/14.9 µg Alhydrogel® alone (low dose formulation) or with 1.9 µg *Na*-GST-1/14.9 µg Alhydrogel® mixed with varied doses of GLA-AF. Table 1 shows the doses and groups in detail. Aeras Manufacturing Record MFG-FM-257, Lot 09-69D-001 *Na*-GST-1 Drug Substance was used to generate freshly formulated vaccine.

Table 1. Study Design

Groups	*Na*-GST-1 (µg)	Alhydrogel® (µg)	GLA-AF (µg)	Animals (n)
1	5.60	N/A	N/A	10
2	N/A	44.8	10	10
3	5.60	44.8	N/A	10
4	1.90	14.9	N/A	10
5	5.60	44.8	0.25	10
6	1.90	14.9	0.25	10
7	5.60	44.8	0.5	10
8	1.90	14.9	0.5	10
9	5.60	44.8	1	10
10	1.90	14.9	1	10
11	5.60	44.8	2	10
12	1.90	14.9	2	10
13	5.60	44.8	4	10
14	1.90	14.9	4	10
15	5.60	44.8	8	10
16	1.90	14.9	8	10
17	5.60	44.8	16	10
18	1.90	14.9	16	10
19	5.60	44.8	32	10
20	1.90	14.9	32	10

Mice were pre-bled, bled on D14 and D28 and Terminal bled on D49 2 weeks after the 2nd (boost) immunization. Figure 1. shows the immunization, bleeding and sacrifice schedule by study day

Figure 1. Bleed and Vaccination Schedule



The levels of IgG against *Na*-GST-1 was measured in the sera of all BALB/c mice by an indirect ELISA and expressed as Arbitrary Units (AU) of IgG *Na*-GST-1. Arbitrary Units were determined by interpolating the OD (492 nm) from test serum samples onto a 4 parameter logistic log modeled dose-response curve from high titer mice sera. We compared the anti-*Na*-GST-1-IgG levels between the vaccine (*Na*-GST-1 (µg) and Alhydrogel (µg)) only group and vaccine (*Na*-GST-1 (µg) and Alhydrogel (µg)) + GLA-AF group for each bleed. Fold rise in anti-*Na*-GST-1-IgG geometric mean units (GMU) was determine for each bleed. Also, we estimated an approximate amount of *Na*-GST-1 (µg) and Alhydrogel (µg) spared at serially diluted doses of GLA-AF (µg)

Results

Figure 2. Anti-*Na*-GST-1 IgG on Day 14 post prime vaccination.

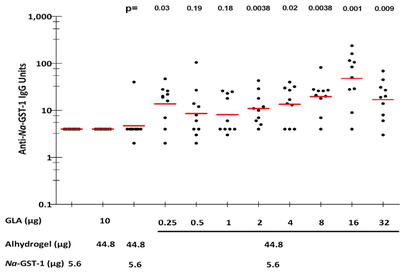


Figure 3. Anti-*Na*-GST-1 IgG on Day 28 post prime vaccination

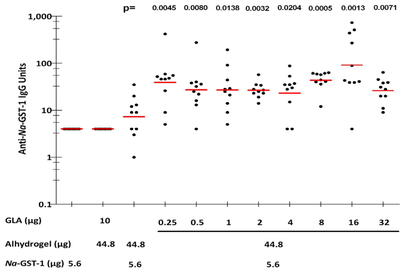
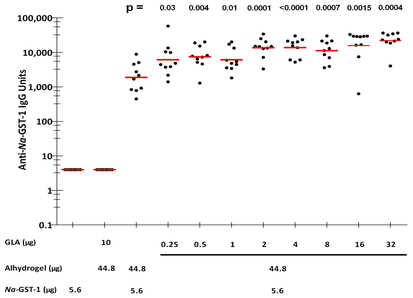


Figure 4. Anti-*Na*-GST-1 IgG on Day 49 post prime vaccination. (Day 14 post boost).



Black Dot (•) represents the anti-*Na*-GST-1 IgG Units in each mouse. p-value estimated using Student's t-test.

Results

Figure 5. Fold rise in anti-*Na*-GST-1 IgG in GMU (Geometric Mean Units)

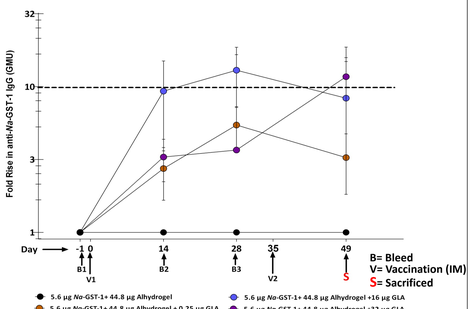


Figure 6. Dose Sparing Effect of GLA-AF on D49 post prime vaccination (Day 14 post boost)†.

Dose used for vaccinating animals with GLA-AF			Anti *Na*-GST-1 IgG (GMU)		Equivalent vaccine dose without GLA-AF	
***Na*-GST-1 (µg)**	**Alhydrogel® (µg)**	**GLA-AF (µg)**			***Na*-GST-1 (µg)**	**Alhydrogel® (µg)**
5.6	44.8	0.25	6,143	≈	7.79	62.95
5.6	44.8	0.5	7,479	≈	9.48	76.63
5.6	44.8	1	6,115	≈	7.75	62.66
5.6	44.8	2	13,702	≈	17.37	140.40
5.6	44.8	4	13,721	≈	17.40	140.59
5.6	44.8	8	11,250	≈	14.27	115.28
5.6	44.8	16	15,690	≈	19.90	160.78
5.6	44.8	32	22,015	≈	27.92	225.59

Figure 7. Dose Sparing Effect of GLA-AF on D49 post prime vaccination (Day 14 post boost)†.

GLA -AF (µg)		Dose Spared	
		***Na*-GST-1 (µg)**	**Alhydrogel® (µg)**
0.25	=	2.19	18.15
0.5	=	3.88	31.83
1	=	2.15	17.86
2	=	11.77	95.60
4	=	11.80	95.79
8	=	8.67	70.48
16	=	14.30	115.98
32	=	22.32	180.79

†Note- BALB/c mice when immunized twice intramuscularly (i.m.) at 5-week interval with 5.6 µg *Na*-GST-1/44.8µg Alhydrogel® (high dose) induced an 1.5 fold rise in anti-*Na*-GST-1 IgG (GMU) on Day 49 post prime immunization (D14 post boost) when compared to anti-*Na*-GST-1 IgG (GMU) in mice immunized with 1.9 µg *Na*-GST-1/14.9µg Alhydrogel® (low dose). The above estimates in tables 6 & 7 were determine using this fold rise in anti-*Na*-GST-1 IgG (GMU).

Results

➢Mice in the *Na*-GST-1 high and low dose formulation developed elevated levels of IgG to *Na*-GST-1 after prime and post boost vaccination. Figure 2, 3 & 4 shows elevated levels of GMU of IgG to *Na*-GST-1 on D14, D28 and D49 (D14 post boost) post-prime vaccination in groups receiving 5.6 µg *Na*-GST-1 + 44.8 µg Alhydrogel® (with or without GLA-AF).

➢Except for few groups, co-administration of different doses of GLA-AF to a high dose of vaccine (5.6 µg *Na*-GST-1 + 44.8 µg Alhydrogel®) induced a statistically significant increase in anti-*Na*-GST-1 IgG (GMU) on study day 14 and 28 when compared to anti-*Na*-GST-1 IgG (GMU) levels in mice vaccinated with high dose of vaccine alone (5.6 µg Na-GST-1 + 44.8 µg Alhydrogel®) (Figure 2 & 3).

➢A boost IgG response was observed in all *Na*-GST-1 + Alhydrogel groups after the second vaccination. Fig 4 shows that *Na*-GST-1/Alhydrogel co-administered with GLA-AF (all groups) had statistically significantly elevated levels of IgG (GMU) to *Na*-GST-1 compared to animals receiving only *Na*-GST-1 + Alhydrogel on days D49 post-prime (D14 post boost) vaccination for both the high and low dose vaccine groups (Data not shown for the low dose groups).

➢ Fig 5 shows that the co-administration of 16 µg GLA-AF with the high dose *Na*-GST-1/Alhydrogel® vaccine formulation induced a 9.32 and 12.96 fold rise in anti-*Na*-GST-1 IgG (GMU) on D14 & D28 post prime vaccination respectively, when compared to anti-*Na*-GST-1 IgG (GMU) in mice vaccinated with only the high vaccine dose formulation. On the contrary, co-administration of 32 µg GLA-AF induced only 3.29 and 3.67 fold rise in anti-*Na*-GST-1 IgG (GMU) on D14 & D28 post prime vaccination respectively.

➢A dose dependent, antigen (*Na*-GST-1) dose sparing effect of GLA-AF was observed, when GLA-AF was co-administered with *Na*-GST-1/Alhydrogel® vaccine in BALB/C (Fig 6 & 7).

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