IMMUNOLOGY

The adjuvant GLA-AF enhances human intradermal vaccine responses

Darrick Carter[1,2]*, Neal van Hoeven[1], Susan Baldwin[1], Yotam Levin[3], Efrat Kochba[3], Al Magill[4†], Nathalie Charland[5], Nathalie Landry[5], Khin Nu[1], Aude Frevol[1], Jill Ashman[1], Zachary K. Sagawa[1], Anna Marie Beckmann[1], Steven G. Reed[1]

Adjuvants are key to shaping the immune response to vaccination, but to date, no adjuvant suitable for human use has been developed for intradermal vaccines. These vaccines could be self-administered and sent through the mail as they do not require long needles or technical expertise in immunization. In the event of a pandemic outbreak, this approach could alleviate the congregation of patients in health centers and thus reduce the potential of these centers to enhance the spread of lethal infection. A reliable and potent vaccine system for self-administration would provide an effective countermeasure for delivery through existing product distribution infrastructure. We report results from preclinical and clinical trials that demonstrate the feasibility of an adjuvanted, intradermal vaccine that induced single shot protection in ferrets and seroprotection in humans against one of the more lethal strains of pandemic flu, Indonesia H5N1. In the human trial, the vaccine was safe and clinical responses were above approvable endpoints for a protective flu vaccine. Inclusion of a modern TLR4 (Toll-like receptor 4) agonist–based adjuvant was critical to the development of the response in the intradermal groups. In humans, this is the first report of a safe and effective intradermal adjuvant, GLA-AF (aqueous formulation of glucopyranosyl lipid adjuvant), and provides a future path for developing a vaccine-device combination for distribution by mail and self-administration in case of a pandemic.

INTRODUCTION

An effective response to an emerging pandemic on a global scale will require a combination of technologies to enable sufficient vaccine supply, distribution, and effectiveness. Modern manufacturing approaches rely on producing defined antigens in recombinant systems including mammalian cells (*1*, *2*), *Escherichia coli* (*3*), baculovirus (*4*), and plants (*5*, *6*).

Influenza virus–like particles (VLPs)—noninfectious particles resembling the influenza virus—represent a promising alternative to inactivated and split-influenza virions as antigens. These VLPs are expressed using the exact hemagglutinin (HA) sequences of the recommended wild-type influenza viruses. The multi-array arrangement of HA antigens in particle-based vaccines allows for better antigen presentation and uptake by antigen-presenting cells. These antigens can thus induce a more potent immune response through both humoral and cellular components of the immune system (*7*).

Medicago has developed a plant-based transient influenza VLP manufacturing platform capable of producing VLPs with unprecedented speed, with the ability to deliver the first vaccine doses 19 days after a new strain sequence is identified (*8*). This platform thus has the potential to respond to strain mismatch. Immune responses generated by influenza VLP vaccines can also provide cross-protection against strains different from those included in the vaccine, another advantage of the product for both seasonal and pandemic flu (*9*, *10*).

Moreover, adding to the challenges of rapid production and distribution in the general population, vaccines against influenza strains with pandemic potential, such as those from avian origin, generally elicit poor antibody responses compared to vaccines against strains that cause seasonal flu. To compensate for this limitation, the inclusion of adjuvants and alternative delivery routes are being investigated to reduce the required dose of vaccine, broaden the immune response, and increase vaccine effectiveness (*11*–*14*).

Intradermal (ID) vaccination has been at the forefront of vaccine improvement (*15*). It has primarily been explored for its ability to generate equivalent antibody responses at lower doses ("dose-sparing") (*16*), which is especially important in addressing high-surge situations, such as flu pandemics, and in global health problems, such as polio, where antigen prices limit population-wide coverage (*17*, *18*). ID vaccination holds promise to trigger enhanced immune responses compared to other routes of immunization when the same dose is given—as has been demonstrated in influenza and other viruses (*11*, *19*). Using microneedles to harness skin immunity will allow the development of self-administration devices. Since these devices are deemed painless and could not penetrate deep tissue or blood vessels, user error is eliminated or reduced. In addition, microneedle patches can be made with dried antigen and adjuvant, providing further stability benefits (*20*).

Adjuvants provide signals to innate immune cells to generate an appropriate milieu for the rapid maturation of an adaptive response capable of protecting the host organism from disease (*21*). Inflammatory signals induced by the inclusion of the widely used alum salts (*22*, *23*) and the more modern squalene-based emulsions can boost the magnitude of the response (*24*), and their combination with specific stimulators of the innate immune system such as Toll-like receptor (TLR) agonists has been used to enhance adaptive antibody maturation and diversification (*14*, *25*, *26*), but certain formulations may not be suitable for ID vaccination because of potential reactogenicity (*27*). Because some TLRs are highly expressed by dermal dendritic cells and because of their responsiveness to TLR4 agonists

[1]Infectious Diseases Research Institute, Seattle, WA 98102, USA. [2]PAI Life Sciences Inc., Seattle, WA 98102, USA. [3]NanoPass Technologies Ltd., Nes Ziona, Israel. [4]Defense Advanced Research Projects Agency, Arlington, VA 22203, USA. [5]Medicago, Québec City, Québec, Canada.
*Corresponding author. Email: dcarter@idri.org
† Deceased.

(*28*), we decided to explore the synergy of combining a novel TLR4 agonist–based adjuvant with ID delivery.

In our research, we evaluated the synergies of combining three technologies to demonstrate proof of concept for a system that could be rapidly deployed and profoundly affect the death toll in the case of a pandemic outbreak: (i) a hollow microneedle that can deliver vaccine to the immunologically rich dermis, resulting in the potential ability to self-administer and spare doses of antigen (*15*); (ii) recombinant flu VLPs that are being manufactured by Medicago, enabling rapid response to newly identified antigen sequences in drifted flu strains (*10*); and (iii) a platform adjuvant technology based on a synthetic TLR4 ligand that stimulates the immune system and boosts the effectiveness of recombinant vaccines, enabling dose-sparing and overcoming immune senescence (*29*, *30*). In these preclinical and clinical studies, our aim was to produce a vaccine candidate with the potential for dose-sparing and self-administration; thus, we focused on combining the VLP with adjuvant formulations suitable for ID use.

Previous studies had demonstrated that the Medicago plant-based production technology can produce a safe and effective VLP vaccine antigen (*10*, *31*) and that glucopyranosyl lipid adjuvant (GLA)–based adjuvants can provide powerful immune-stimulating effects in humans (*32*, *33*). Medicago produced the H5-VLP pandemic influenza vaccine antigen by transient expression of a recombinant protein in nontransgenic plants, using an agrobacterial expression vector. The A/Indonesia/5/05 strain was selected because it was recommended by the World Health Organization (WHO) as a candidate vaccine, in addition to it being one of the most virulent H5N1 strains, having a mortality rate of 80% with 141 confirmed cases in 2008 (*34*).

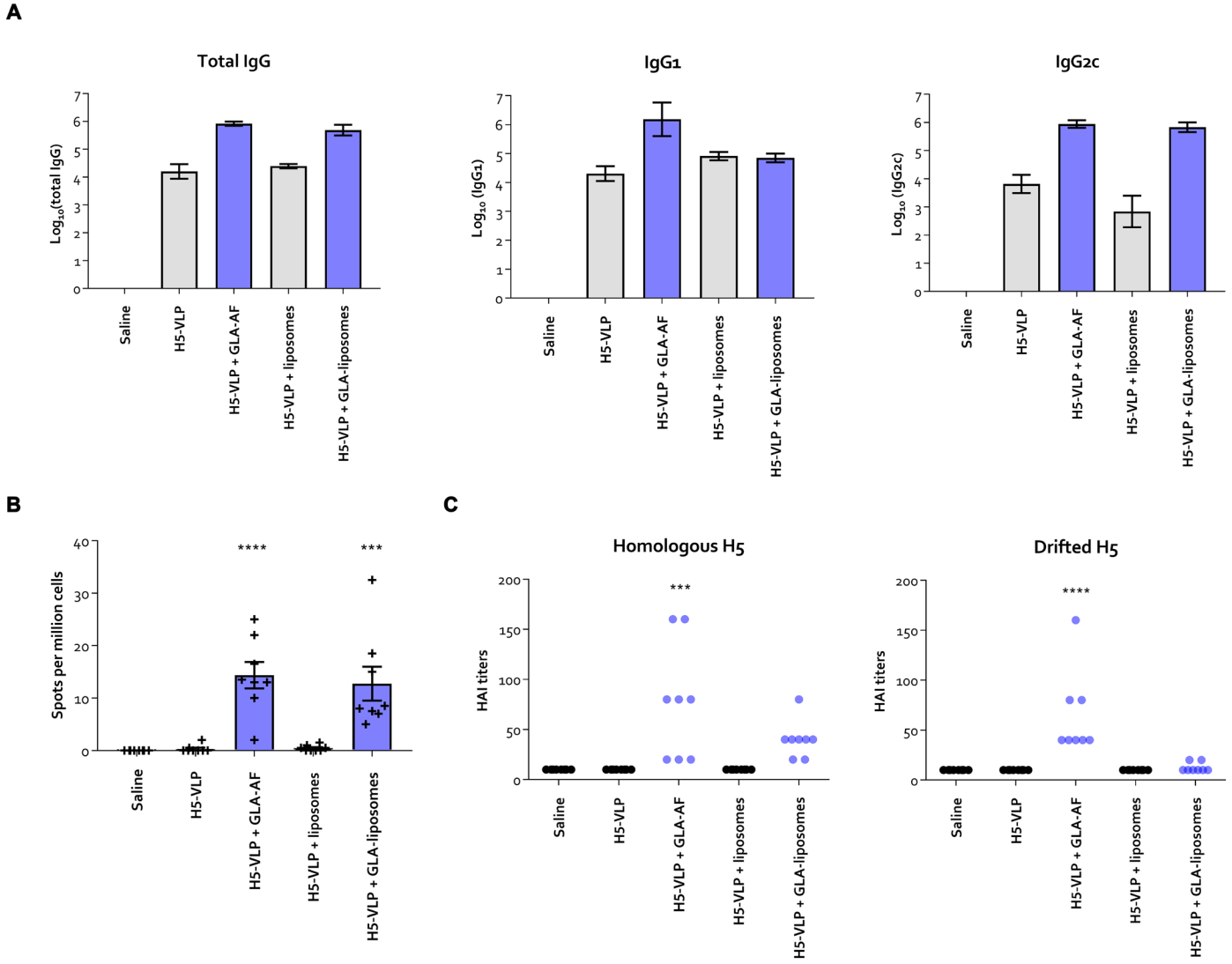

Fig. 1. Immunogenicity in mice. (**A**) Antibody endpoint titers. Following the boost immunization, the H5-specific total IgG, IgG1, and IgG2C titers were determined. As expected, the adjuvants enhanced titers across groups with high IgG2C titers, which are indicative of a T_H1-type immune response, observed in groups containing the TLR4 agonist GLA. (**B**) Bone marrow enzyme-linked immunosorbent spots (ELISPOTs). Similarly, H5-specific antibody–secreting plasma cells were increased with H5-VLP + GLA-AF. ****$P < 0.0001$ for H5-VLP + GLA-AF versus other groups. ***$P < 0.001$ for H5-VLP + GLA-liposomes versus other groups not containing GLA. (**C**) HAI titers. HAI titers are the standard measurement for functional antibody in flu. The A/Indonesia/5/2005 virus strain was not available for testing; however, two different clades, either the "homologous" clade 2.3.4 A/Anhui/1/2005 or the "drifted" clade 2.1 A/Duck/Hunan/795/2002, were tested to determine whether these adjuvants could induce cross-reactive titers. ****$P < 0.0001$ for H5-VLP + GLA-AF versus all other groups; ***$P < 0.001$ for H5-VLP + GLA AF versus all other groups.

RESULTS

Murine immunogenicity studies

Initial studies using the Medicago H5-VLP formulated in GLA-based adjuvants injected subcutaneously demonstrated potent immune responses in mice. Overall, antibody titers in the adjuvanted groups were markedly higher than those in the groups immunized in the absence of these agonists with almost three orders of magnitude enhancement of IgG2C titers, consistent with the T helper cell 1 (T_H1) biasing effect of these adjuvants (Fig. 1A). The agonists also enabled a durable immune response as bone marrow–derived plasma cells were only detected in the TLR4-containing vaccine groups (Fig. 1B). Finally, in terms of functional antibody, hemagglutination inhibition (HAI) titers were detected in the adjuvanted groups against the homologous virus and in the GLA-AF (aqueous formulation of GLA)–adjuvanted group not only against the homologous virus but also against a drifted strain (Fig. 1C).

Safety studies in guinea pigs

All H5-VLP vaccines were considered safe, as measured by skin reactions, temperatures, and weights. The skin reactogenicity was observed primarily with the H5-VLP vaccines combined with GLA-AF, SE (stable emulsion), or GLA-SE formulations; no adverse temperature spikes or decreases in weight were observed in any of the vaccine groups. GLA-SE had significantly higher erythema and edema scores than any of the other formulations (fig. S1); it was therefore decided to proceed with GLA-AF intradermally as the lead candidate.

Ferret challenge studies

Next, we tested protective efficacy in the ferret challenge model. Since the pandemic vaccine is intended for rapid response to a pandemic threat, efficacy was tested after a single administration of the vaccine, the desired scenario for the self-administered pandemic response. Groups of ferrets were given the H5-VLP intradermally or intramuscularly with and without GLA-AF, a formulation suitable for ID delivery, or an alum adjuvanted control formulation given intramuscularly. There were no adverse responses observed with any of the vaccines at the site of injection. Similarly, there was no increase in body temperature with any of the vaccines tested. In these studies, a single ID administration of the H5-VLP antigen formulated in GLA-AF completely protected ferrets against lethality after heterologous challenge with pandemic influenza (Fig. 2A). Notably, this level of protection was seen after a single vaccination with antigen derived from sequences of the A/Indonesia/5/05 virus strain and challenged with an A/Vietnam/1203/04 H5N1 virus—that is, a clade 2.1.3.2 immunization showing protection against a virus from clade 1. Other parameters were measured following challenge, including clinical scores and viral titers in nasal lavages (Fig. 2, B and C). While

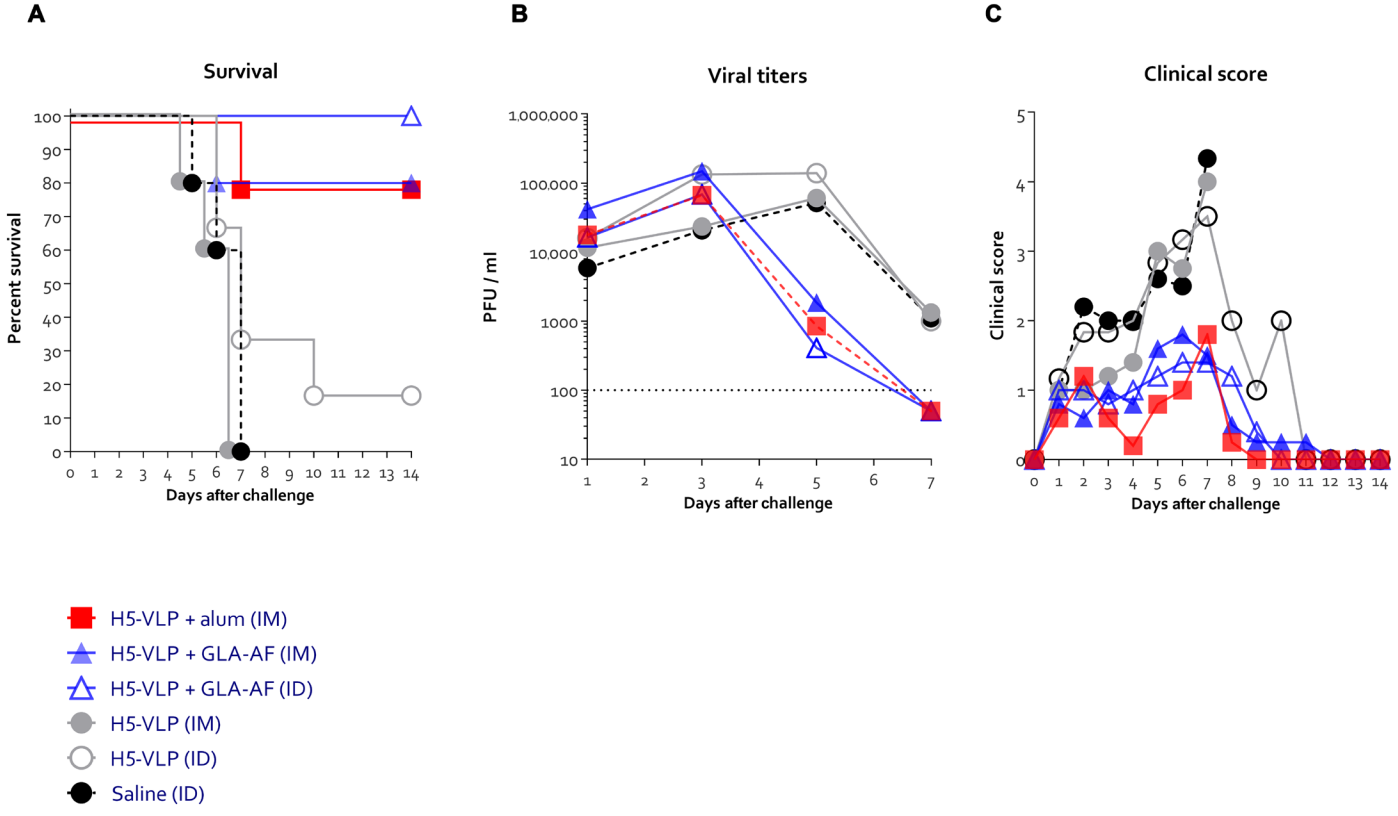

Fig. 2. Efficacy study in ferrets. Ferrets were immunized (either intradermally or intramuscularly, as indicated) once at day 0 and challenged with the heterologous A/Vietnam/1203/04 H5N1 strain of flu virus 3 weeks later. (**A**) Survival data. All ferrets that were immunized intradermally with H5-VLP/GLA-AF survived the heterologous challenge. All the ferrets succumbed to infection in the saline-treated group and in the group that received an intramuscular (IM) immunization with the unadjuvanted H5-VLP, whereas one ferret survived in the intradermally immunized group that received unadjuvanted H5-VLP. (**B**) Viral titers. Nasal swabs were performed to measure virus in the upper airways on the days indicated. Paralleling the survival data, the adjuvanted groups appeared to control the infection and rapidly demonstrated lower viral titers than those found in all the unadjuvanted groups. (**C**) Clinical scores. Clinical signs of morbidity including lethargy, body temperature, anorexia, and dyspnea were monitored daily. PFU, plaque-forming units.

Table 1. Treatment assignments. The table depicts the number of subjects in each group and the treatment regimen, dose, and dose timing for each group. One hundred subjects were enrolled across three study sites and randomly assigned to one of five treatment groups for a total goal of 20 subjects in each of the five groups. An additional five subjects were screened and enrolled to replace five subjects who withdrew from the study for reasons other than dose-limiting toxicity before day 21. A total of 105 subjects were enrolled, as shown in the table.

Group	Treatment assignment	Route	Volume	Timing of injections	$N = 100$*	$N = 105$†
1	20 μg of H5-VLP + 2.5 of μg GLA-AF	ID	0.2 ml	Days 0 and 21	20	20
2	20 μg of H5-VLP + 2.5 μg of GLA-AF	IM	0.2 ml	Days 0 and 21	20	23
3	20 μg of H5-VLP alone	ID	0.2 ml	Days 0 and 21	20	22
4	20 μg of H5-VLP + 1 mg of alhydrogel‡	IM	0.2 ml	Days 0 and 21	20	20
5	90 μg of influenza virus vaccine, H5N1 (Sanofi Pasteur)	IM	1.0 ml	Days 0 and 21	20	20

*Planned enrollment. †Total including replacements. ‡Al^{+3} content of 1 mg of alhydrogel = 0.5 mg.

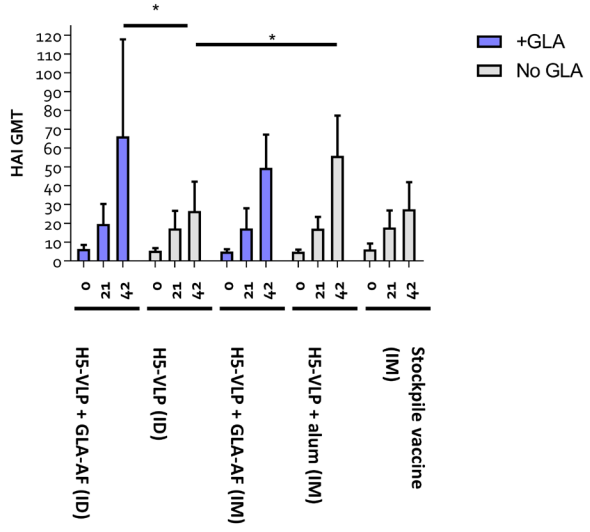

Fig. 3. HAI titers from a human clinical study. The immunogenicity of the vaccine was evaluated by comparing HAI antibody responses of subjects in each treatment group. GMTs of HAI antibody responses were evaluated on days 0, 21, and 42, as shown below the *x* axis. Each group of three bars corresponds to these timepoints. Error bars indicate 95% confidence intervals. *$P < 0.05$ between indicated groups. Statistically significant differences were only achieved at the day 42 timepoint.

all adjuvanted vaccine groups demonstrated at least 80% survival, the adjuvanted ID group had 100% survival and was not inferior to the IM groups. On the basis of these observations, it was determined that an intradermally administered, adjuvanted pandemic flu vaccine was feasible for human use and the H5-VLP + GLA-AF was selected for inclusion in a GLP (Good Laboratory Practices) rabbit toxicity study and for human clinical trials.

Human clinical study

A first-in-man, phase 1, multicenter, randomized, open-label trial of the pandemic influenza vaccine H5-VLP and GLA-AF was completed in more than 100 volunteers to evaluate the safety, tolerability, and immunogenicity of the adjuvanted H5-VLP vaccine following IM or ID administration in healthy adult subjects. The licensed H5N1 vaccine from the Strategic National Stockpile [influenza virus vaccine, H5N1 (Sanofi Pasteur)] was included as a control group (see Table 1 for groups and treatment assignments).

Safety

The vaccines were safe and well tolerated in this study, with the main notable difference—as expected—being the fact that transient erythema was seen in the ID groups. No serious adverse events (AEs) or AEs of special interest were reported. All study injection–related AEs occurred during period I and were of grade 1 or grade 2 severity. The most frequent study injection–related AEs were injection site tenderness, injection site pain, fatigue, headache, and injection site erythema/redness (tables S2 and S3).

Immunogenicity

HAI titers are a commonly used efficacy surrogate accepted by both U.S. and European regulatory bodies for influenza vaccines. High HAI titers have been observed to correlate with clinical protection. Individual subjects' HAI geometric mean titers (GMTs) were calculated from assay replicates and are summarized in Fig. 3. Seroconversion, seroprotection, and GMT fold increases were defined on the basis of CHMP (Committee for Medicinal Products for Human Use) criteria for seasonal flu re-licensure (*35*). While there were no statistically significant differences in seroprotection and seroconversion rates (Table 2), statistically significant differences were observed in GMTs. The group receiving H5-VLP + GLA-AF by the ID route had the highest HAI titers; the increased immunogenicity compared to the group receiving H5-VLP alone demonstrated the utility of the GLA-AF adjuvant when given intradermally. No statistically significant differences were observed when comparing the adjuvanted ID vaccine to the GLA or alum adjuvanted IM groups. Both groups demonstrated comparable HAI responses after one immunization with good rates of seroprotection at day 21 and were similar to the other control groups with a trend of higher responses in the ID group. To examine the breadth of vaccine responses, we tested HAI against

Table 2. Seroconversion and seroprotection rates by serum HAI response in humans. Seroconversion was defined as the proportion of subjects in a given treatment group with either a ≥4-fold increase in HAI titers or a rise of an undetectable HAI titer (that is, <8) before vaccination to an HAI titer of ≥32 after vaccination. Seroprotection was defined as the proportion of subjects in a given treatment group attaining an HAI titer of ≥32 after vaccination. Statistical differences were found among the treatment groups at day 42 ($P = 0.036$ for seroconversion and $P = 0.014$ for seroprotection), but pairwise comparisons with multiplicity adjustments failed to reach significance.

Timepoint	H5-VLP + GLA-AF (ID)	H5-VLP + GLA-AF (IM)	H5-VLP (ID)	H5-VLP + alum (IM)	Control vaccine
Seroconversion					
Day 21	30.0%	19.0%	30.0%	20.0%	21.1%
Day 42	65.0%	84.2%	52.6%	83.3%	43.8%
Seroprotection					
Day 21	35.0%	23.8%	30.0%	25.0%	26.3%
Day 42	70.0%	89.5%	52.6%	88.9%	50.0%

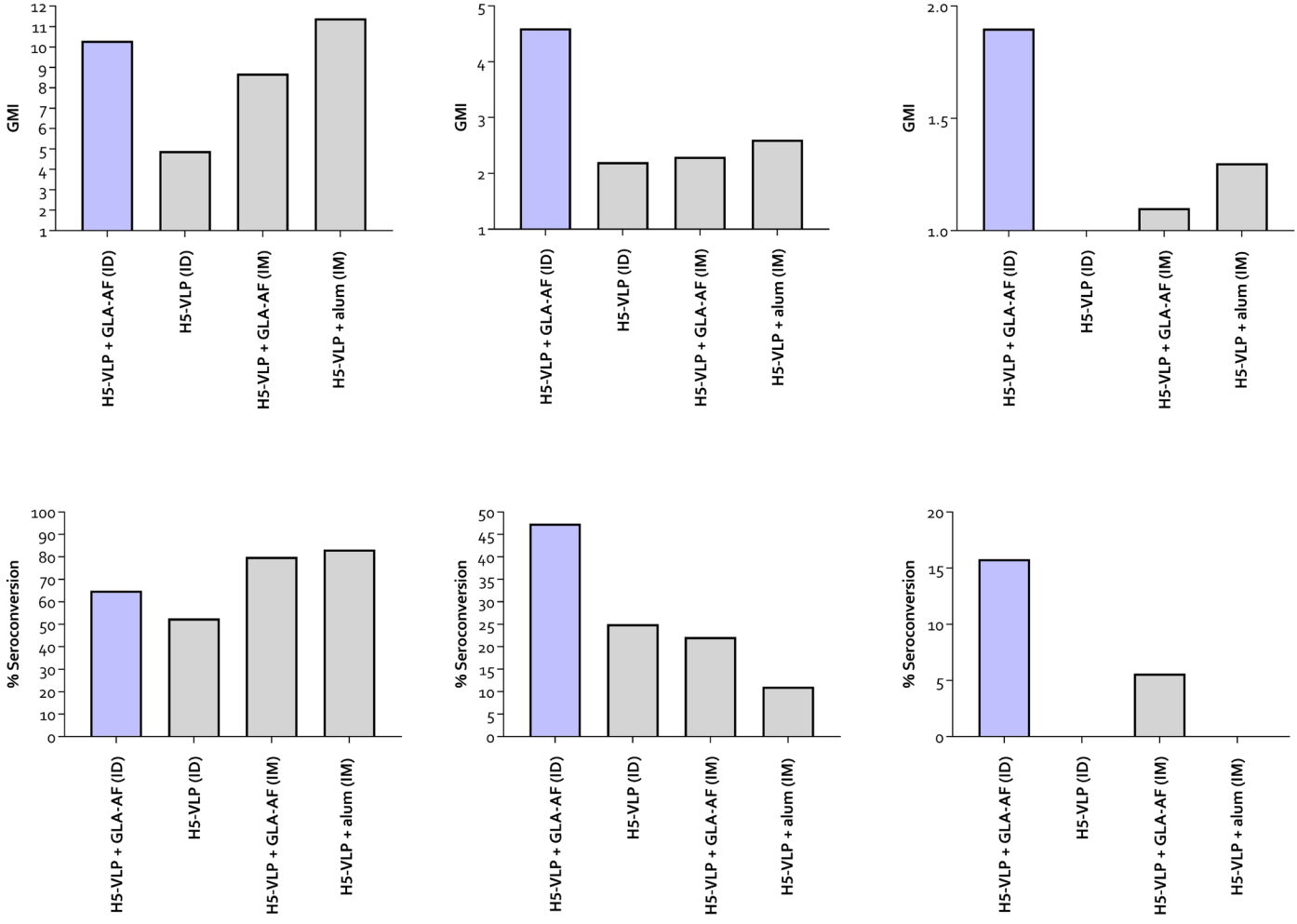

Fig. 4. Diversity of the immune response. HAI antibody responses of subjects in each treatment group were determined against a homologous virus, A/Indonesia/5/2005 (H5N1); a drifted virus within the homologous clade, A/Anhui/1/2005 (H5N1); and a drifted virus outside the clade, A/Vietnam/1203/2004 (H5N1). GMTs of HAI antibody responses were compared between days 0 and 42 to calculate geometric mean increases (GMI, top row), and the percentage of subjects seroconverting was plotted below. Adjuvanted injection groups are drawn in blue.

different strains: a clade 1 virus (Vietnam), a clade 2.1 virus (Indonesia), and a clade 2.3.4 virus (Anhui). The Indonesia A/Indonesia/5/05 strain is homologous to the Medicago H5-VLP antigen. This strain shares 98.9% similarity with the in-clade Anhui strain and has 97.9% similarity to the clade 1 Vietnam strain. The Vietnam and Anhui strains are 97.7% similar to each other. When looking at antibody responses that could recognize drifted strains, the adjuvanted ID groups looked more potent at providing human immune responses to strains not present in the vaccine preparation (Fig. 4). In the GLA ID groups, more than 40% (the approvable endpoint for flu vaccines) of subjects seroconverted to the more Indonesia H5–related Anhui virus, and this group also had a significant portion of subjects who also recognized an even further drifted Vietnam H5 virus, demonstrating the ability of the adjuvanted vaccine to provide broadened protection when given intradermally.

DISCUSSION

Various strategies have been developed to date to bolster and broaden immunogenicity, including increasing the dose, adjuvantation with alum or modern adjuvants, and applying the vaccine intradermally, although there has been no report in humans of a successful combination of these approaches. The lack of success to date may be due to the lack of adjuvants suitable for ID administration that could enable defined vaccine antigens to elicit a potent immune response when given intradermally. We present here GLA-AF as an adjuvant that safely enhances human immune responses to ID vaccination.

Developing an effective vaccine for an emerging pandemic presents numerous challenges: The protein sequence of the antigen is not known a priori; thus, stockpiling vaccine is an issue since the time between identification of the antigen and deployment of the vaccine is more limited than in any other setting. Attempts to switch from the current egg-based systems to a recombinant system where a rapid production response could be feasible have been made. However, the antigens produced in these systems have not been highly immunogenic and still require syringe/needle administration. Implementing a population-wide program at the peak of a pandemic will be problematic owing to the burden on health care workers who themselves may become infected. In addition, subjects congregating to receive vaccines could infect each other, accelerating the spread of the disease. An overburdened health care system could result in a devastating death toll if a highly contagious and lethal pathogen were to emerge.

Here, three technologies that complement each other enable a pandemic countermeasure that could be deployed using existing infrastructure. The first is a plant-based expression system, not reliant on genetically engineered plants, and that can initiate vaccine manufacturing in less than 3 weeks after identification of the pandemic strain and match its exact HA sequence. The second is an adjuvant system that has been specifically formulated to be administered intradermally and, as we show here, that boosts responses to a point where they are as good as or better than those achieved by IM administration. The third is a commercially registered hollow microneedle device for efficient and consistent ID injections. This noninferiority of the ID route is an important finding in that it would allow the development of a device for pandemic response that could be distributed by existing postal infrastructure and be self-administered.

Another important finding of the research presented is the ability of appropriately stimulated dermal cells to generate an immune response that has the ability to neutralize drifted viral strains, as was seen in the studies here with single shot protection in the ferret heterologous challenge. A difficult problem with pandemic preparedness is the inability to predict exactly which strain of pathogen may cause a pandemic outbreak. Notably, the influenza H5 stockpile consists of a flu vaccine based on the Vietnam H5 virus, but if a drifted viral strain were to emerge, then it would be difficult to predict whether the stockpile would be able to provide satisfactory protection against the emerging pathogen. Each of the platform technologies combined herein has demonstrated some ability to broaden protection, and we believe that combining these together would be a prominent step toward a much broader protection of pandemic strains. The ability of the adjuvanted ID vaccine to provide both noninferior protection to the IM injection and broader protection against other strains of virus makes this route particularly interesting as a pandemic countermeasure. The ability of dermal cell populations like Langerhans cells to present to $CD4^+$ T cells and induce T follicular helper cells has been recently investigated, and these interactions are probably critical for efficient germinal cell formation and hypermutation, leading to the broadening of protection (*36*, *37*).

We have developed a prototype approach that combines ID administration, an adjuvant for ID use, and plant-produced, recombinant VLPs. The study presented here shows the feasibility of ID immunization, and while the technology will need further optimization to protect against pandemic flu, future studies can move this platform toward human implementation. The technologies will synergize to produce a potent immune response in humans with protection against drifted pathogens in an embodiment that allows self-administration. The GLA-AF adjuvant powers this approach by enhancing intradermally administered vaccines and may enable government countermeasures that are delivered to users to eliminate congregation at clinical sites in case of a pandemic.

MATERIALS AND METHODS

All murine and guinea pig experiments and protocols used in this study were approved by the Infectious Disease Research Institute's Institutional Animal Care and Use Committee (IACUC). The ferret experimental protocols used in this study were approved by the Colorado State University IACUC.

Murine experiments

Female C57BL/6 mice (purchased from Charles River Laboratories) were maintained under specific pathogen-free conditions and in accordance with animal procedures approved by the Infectious Disease Research Institute's IACUC. Mice entered experiments at 6 to 8 weeks of age and were immunized by subcutaneous injection at the base of the tail. Mice were injected a total of two times in a 3-week interval. Prebleed samples were taken at day −6, with immunization occurring at day 0. At days 14 and 40, sera were collected for HAI and enzyme-linked immunosorbent assay. Bone marrow was harvested for detection of long-lived plasma cells. Antigen-specific mouse bone marrow plasma cells were quantified by ELISPOT as previously described, 3 weeks after the second immunization (*38*). A purified rH5 antigen (Indonesia strain) purchased from Protein Sciences was used in the ELISPOT. HAIs were performed by MRIGlobal.

Guinea pig studies

The objective of this experiment was to test dose-sparing and reactogenicity of the formulations using ID delivery. Five female Hartley

guinea pigs (purchased from Charles River Laboratories) were used per group. ID delivery was achieved using NanoPass MicronJet microneedles, as described below. Guinea pigs were immunized two times, 3 weeks apart (day 0 and day 21), and injection sites were scored for erythema and edema at 4, 24, 48, and 72 hours after injection. Body weights and temperatures were also monitored during the study. All experimental procedures were carried out using approved anesthesia protocols.

Ferret studies

Ferrets were immunized once on day 0 (either intradermally or intramuscularly, as indicated) and bled on days 0, 14, and 21 before challenge. HAI titers were performed on the serum timepoints. QuantiGene assays were performed at prebleed and at 4 and 24 hours after injection on whole blood for innate cytokine message analysis. Draize scoring was performed to determine reactogenicity at 0, 4, 24, 48, 72 hours after injection. Ferrets were challenged 3 weeks after immunization (day 21) with A/Vietnam/1203/04 H5N1. Clinical signs of morbidity, including weight loss, lethargy, temperature elevation using temperature chips (Bio Medic Data Systems), anorexia, and dyspnea, were collected daily. Nasal swabs were taken to measure virus in the upper airways on days 1, 3, 5, 7, 9, and 11. Ferrets were euthanized 14 days after challenge, and lungs, heart, brain, kidneys, and spleen were harvested for viral titers. Histology was performed on lungs, heart, brain, kidneys, and spleen.

Clinical trial

This study was conducted at Covance Clinical Research Unit centers in Madison, WI, Dallas, TX, and Daytona Beach, FL (ClinicalTrials.gov identifier: NCT01657929). H5-VLP, GLA-AF, and the stockpiled vaccine (Vietnam strain) were administered at 20, 2.5, and 90 μg, respectively. Males and females between 18 and ≤49 years of age were included in the study and immunized in groups, as shown in Table 1. The flowchart and table in fig. S2 and table S1 provide details on assessments and timing of immunizations. Subjects had to be in good general health as confirmed by a medical history and physical exam, vital signs, and screening laboratories conducted no more than 30 days before study injection administration and had to have a negative serum pregnancy test at screening. A further description of the study population is provided in the Supplementary Materials.

Vaccine preparation

The H5-VLP pandemic influenza vaccine antigen was produced by Medicago by transient expression of a recombinant protein, the HA from the A/Indonesia/5/05, clade 2.1, H5N1 strain, in nontransgenic plants, using an agrobacterial expression vector. The A/Indonesia/5/05 strain was selected because it was recommended by the WHO as a candidate vaccine, in addition to it being one of the most virulent H5N1 strains, having a mortality rate of 80% with 141 confirmed cases in 2008 (*34*). Previous studies had demonstrated that the Medicago plant-based production technology can produce a safe and effective vaccine antigen (*10*, *31*) and that GLA-based adjuvants can provide powerful immune-stimulating effects in humans (*32*, *33*).

GLA-based adjuvants

GLA is a synthetic TLR4 agonist that was synthesized at Corden Pharma. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids. GLA-AF was manufactured by mixing DPPC and the TLR4 ligand at a 2:1 DPPC/GLA molar ratio in chloroform, which was then evaporated. Ultrapure water was added to the resulting dried film, and the mixture was sonicated in a VWR 75D or Crest Powersonic CP230D sonicating water bath at ~60°C until the formulation was translucent.

ID delivery

ID injection was done using the NanoPass MicronJet600 device (NanoPass), which is a disposable three-pronged, 0.6-mm hollow microneedle device that attaches to any standard Luer lock or Luer tip syringe.

Immunogenicity analyses

Blood samples were obtained from subjects for HAI, which was performed as described in (*10*) with determinations on days 0, 21, and 42. Peripheral blood mononuclear cells were collected on days 0, 10, and 31 (10 days after vaccination).

Statistical/bioinformatic analyses

Statistically significant differences between groups in the animal experiments were determined by pairwise analysis of variance (ANOVA) comparisons with Tukey's multicomparison test in GraphPad Prism version 7.00 for Windows, GraphPad Software (www.graphpad.com). Bioinformatic sequence alignments were performed at the EMBL (European Molecular Biology Laboratory) website using the EMBOSS (European Molecular Biology Open Software Suite) needle algorithm (*39*).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/9/eaas9930/DC1

Section S1. Inclusion criteria

Section S2. Exclusion criteria

Fig. S1. Safety assessments in the guinea pig.

Fig. S2. Assessment schedule.

Table S1. Schedule of study visits and procedures.

Table S2. Subjects with adverse events by study period, injection interval, severity, and relatedness to study injection (safety population).

Table S3. Subjects with AEs occurring in ≥5 subjects in study period I (safety population).

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