Vaxfectin Adjuvant Improves Antibody Responses of Juvenile Rhesus Macaques to a DNA Vaccine Encoding the Measles Virus Hemagglutinin and Fusion Proteins

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DNA vaccines formulated with the cationic lipid-based adjuvant Vaxfectin induce protective immunity in macaques after intradermal (i.d.) or intramuscular (i.m.) delivery of 0.5 to 1 mg of codon-optimized DNA encoding the hemagglutinin (H) and fusion (F) proteins of measles virus (MeV). To characterize the effect of Vaxfectin at lower doses of H+F DNA, rhesus macaques were vaccinated twice with 20 μg of DNA plus Vaxfectin i.d., 100 μg of DNA plus Vaxfectin i.d., 100 μg of DNA plus Vaxfectin i.m. or 100 μg of DNA plus phosphate-buffered saline (PBS) i.m. using a needleless Biojector device. The levels of neutralizing (P = 0.036) and binding (P = 0.0001) antibodies were higher after 20 or 100 μg of DNA plus Vaxfectin than after 100 μg of DNA plus PBS. Gamma interferon (IFN-γ)-producing T cells were induced more rapidly than antibody, but were not improved with Vaxfectin. At 18 months after vaccination, monkeys were challenged with wild-type MeV. None developed rash or viremia, but all showed evidence of infection. Antibody levels increased, and IFN-γ- and interleukin-17-producing T cells, including cells specific for the nucleoprotein absent from the vaccine, were induced. At 3 months after challenge, MeV RNA was detected in the leukocytes of two monkeys. The levels of antibody peaked 2 to 4 weeks after challenge and then declined in vaccinated animals reflecting low numbers of bone marrow-resident plasma cells. Therefore, Vaxfectin was dose sparing and substantially improved the antibody response to the H+F DNA vaccine. This immune response led to protection from disease (rash/viremia) but not from infection. Antibody responses after challenge were more transient in vaccinated animals than in an unvaccinated animal.

Measles remains an important cause of child morbidity and mortality in developing countries despite the availability of a safe and effective live attenuated virus vaccine (1–3). Recent efforts to reduce mortality through increased routine vaccination combined with supplemental immunization activities have improved measles control but have been difficult to sustain (4–6). One impediment is the inability to reliably immunize infants younger than 9 months of age due to immaturity of the immune system and the interference of maternal antibody (7, 8). In high-transmission settings, this leads to a window of susceptibility, and many infants, particularly those born to HIV-infected mothers, acquire measles during the first year of life (9–11).

A measles vaccine for infants under the age of 6 months could improve measles control by allowing delivery with other infant vaccines. DNA vaccines are attractive candidates for development because they are safe, are relatively inexpensive to produce, may not require a cold chain, induce strong cellular immune responses, and can be delivered without the use of a syringe and needle (12). However, DNA vaccines have often been disappointing when tested in humans and nonhuman primates because of the relatively poor induction of antibody (13). Approaches to improving responses have included increasing the amount of DNA given, microparticle formulation, plasmid redesign, altered delivery methods, and use of adjuvants (14–18).

One safe and easily manufactured adjuvant class consists of cationic lipids (19, 20). Vaxfectin, an equimolar mixture of the cationic lipid GAP-DMORIE [(±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(cis-9-tetradecenyloxy)-1-propanaminium bromide] and a neutral colipid DPyPE (1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine) (21, 22) is dose sparing and enhances antigen-specific antibody production in small animals (21–31). Limited evaluation in humans and nonhuman primates (21, 32–35) has also indicated that it is well tolerated.

Measles virus (MeV) encodes two surface glycoproteins, hemagglutinin (H) and fusion (F), involved in attachment and entry. MeV uses at least three cellular receptors for entry of different target cells: membrane cofactor protein or CD46, a complement regulatory protein present on all nucleated cells (36, 37); signaling lymphocytic activation molecule (SLAM) or CD150, present on activated immune cells (38); and poliovirus receptor–related 4 (PVRL4) or nectin 4, present on epithelial cells (39, 40). Both vaccine and wild-type (WT) strains can use SLAM as a receptor, and most H proteins can bind both CD46 and SLAM, but receptor affinity and efficiency of entry differ, and most WT viruses cannot use CD46 as an entry receptor (41–48). Antibodies that inhibit MeV infection in neutralization assays are directed primarily
against the H protein, which also contains important CD8+ T cell epitopes (49). Vaccine-induced antibody that does not neutralize WT virus can lead to enhanced disease (50, 51).

Because protection from measles correlates best with the quality and quantity of neutralizing antibodies at the time of exposure (52, 53), most experimental vaccines have used H alone or H and F for induction of MeV protective immunity (52, 54–56). We have previously shown that juvenile and infant macaques are protected from rash and viremia after intradermal (i.d.; 500 μg) or intramuscular (i.m.; 1 mg) vaccination with two doses of Vaxfectin-formulated plasmid DNA encoding codon-optimized H and F (32). In the present study, we have directly compared the immune responses and protective efficacy in rhesus macaques of low doses of Vaxfectin-formulated and naked MeV H+F DNA delivered i.d. (100 and 20 μg) and i.m. (100 μg) with a needle-free Biojector device.

MATERIALS AND METHODS

Animals. Thirteen 1-year-old juvenile rhesus macaques (Macaca mulatta) born to measles naive mothers were obtained from the Johns Hopkins Primate Breeding Facility. Monkeys were anesthetized with ketamine (10 to 15 mg/kg) during procedures. All animals were maintained within the guidelines, and studies were performed in accordance with experimental protocols approved by the Animal Care and Use Committee for Johns Hopkins University.

Vaccine, vaccination, and challenge. As previously described (32), codon-optimized DNA encoding MeV Moraten strain H and F proteins were used to produce plasmids VR7302 (H) and VR7303 (F). Prior to vaccination DNA was formulated with Vaxfectin (22) or with phosphate-buffered saline (PBS) and delivered intramuscularly (i.m.) or intradermally (i.d.) with a Biojector 2000 needle-free injection system (Bioject, Inc., Tualatin, OR). Groups of three monkeys were vaccinated with either 100 μg of Vaxfectin-formulated H+F i.d. (14V, 30V, and 33V), 20 μg of Vaxfectin-formulated H+F i.d. (32V, 37V, and 39V), 100 μg of PBS-formulated H+F i.m. (20V, 22V, and 28V), or 100 μg of Vaxfectin-formulated H+F i.m. (24V, 26V, and 31V) in a volume of 100 μl on days 0 and 28. Monkeys were bled at 1- to 2-week intervals to monitor vaccine-induced immune responses. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by density gradient centrifugation using Lympholyte Mammal (Cedarlane Laboratories). Plasma was collected and stored at −20°C. Monkey 32V was euthanized 11 months after vaccination for reasons unrelated to the present study.

All vaccinated monkeys and an unvaccinated measles-naïve monkey (55V) were challenged intratracheally with 106 tissue culture infective doses (TCID50) of the WT Bihoven strain of MeV (A. Osterhaus, Erasmus University, Rotterdam, Netherlands) 18 months after vaccination. Monkeys were shaved, examined for rash, and bled at regular intervals to monitor viremia and immune responses. Plasma from three monkeys (46U, 55U, and 67U) that were similarly challenged was used for comparison of MeV neutralization.

Virus assays. Viremia was assessed by cocultivation in triplicate of serial dilutions of PBMCs with Vero cells expressing human CD150 (Vero/hSLAM cells) (47) in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Wells were scored at 120 h for MeV-positive syncytia. The data are reported as numbers of infected cells/105 PBMCs. MeV RNA was measured by quantitative reverse transcription-PCR (RT-PCR) for the nucleocapsid (N) gene as previously described (57).

Antibody assays. Neutralizing antibodies were measured by the ability of serially diluted plasma to reduce plaque formation by the Chicago-1 strain of MeV on Vero cells by 50% (i.e., plaque reduction neutralization [PRN]). Plasma from 48 weeks after immunization was tested for neutralization of Bihoven WT MeV infection of Vero/hSLAM cells, as well as for neutralization of Chicago-1 MeV infection of Vero cells. The data are expressed as geometric means of the reciprocal titer.

For enzyme immunoassays (EIAs), MeV-infected Vero cells lysate (Advanced Biotechnologies, Columbia, MD) was used (1.16 μg of protein/well) to coat 96-well Maxisorp plates (Nunc, Rochester, NY) and then incubated overnight at 4°C with plasma diluted 1:100 to 1:300, followed by alkaline phosphatase-conjugated rabbit antibody to monkey IgG (Bio-makor; Accurate Chemicals, Westbury, NY) or horseradish peroxidase (HRP)-conjugated goat antibody to monkey IgM (Nordic, Capistrano Beach, CA). The data are presented as optical density (OD) values.

To measure antibody-secretion cells (ASCs) in blood and bone marrow, mononuclear cells were isolated by density gradient centrifugation as described above. Cells were added to Multiscroll HTS HA Opaque ELISpot plates (Millipore) coated with MeV-infected Vero cell lysate or purified goat anti-mouse IgG, IgM, and IgA (H and L; Open Biosystems) for 6 h. To measure MeV-specific ASCs in bone marrow, 5×10⁷ cells were added to each of 8 replicate wells. To measure MeV-specific ASCs in PBMCs and total ASCs, 2-fold serial dilutions, starting at 5×10⁵ cells, were added to each of six wells. After incubation, bound immunoglobulin was detected with HRP-conjugated goat anti-mouse IgG, developed with stable diaminobenzidine solution (DAB; Invitrogen, Carlsbad, CA). Plates were read on an ImmunoSpot plate reader and analyzed using ImmunoSpot 5.0 software, both obtained from Cellular Technology, Cleveland, OH. The data on MeV-specific ASCs are presented as spot-forming cells (SFCs)/10⁶ PBMCs or 5×10⁷ bone marrow cells.

T cell ELISPOT assays. A total of 1×10⁵ to 5×10⁵ PBMCs was added to plates coated with antibody to human gamma interferon (IFN-γ; 2 μg/ml), interleukin-4 (IL-4; 5 μg/ml; BD Pharmingen), or IL-17 (2 μg/ml; eBioscience, clone eBio64cap17), along with 1 μg of pooled MeV H, F, or N peptides/ml, 5 μg of concanavalin A/ml, 5.8 μg of MeV-infected Vero cell lysate/ml, or medium alone. After 40 h at 37°C, the plates were washed and incubated with 1 μg of biotinylated antibody to IFN-γ (Mabtech)/ml, 2 μg of biotinylated antibody to IL-4 (Pharmingen)/ml, or 1 μg of biotinylated antibody to IL-17 (eBioscience, clone eBio64Dec17)/ml at 37°C for 2 h. After washing, 50 μl of HRP-conjugated avidin (Vector Laboratories) was added to each well, followed by incubation for 1 h at 37°C. The assays were developed with DAB. Wells were scanned in an ImmunoSpot reader and analyzed using ImmunoSpot 5.0 software. MeV-specific data are presented as SFCs/10⁶ PBMCs after subtraction of the medium control.

Statistical analysis. Student unpaired t test was used for comparisons between two groups, and one-way analysis of variance (ANOVA) was used for comparison between multiple groups of monkeys using Prism 4 software.

RESULTS

Antibody response to vaccination. Groups of three monkeys were vaccinated with H+F DNA either Vaxfectin-formulated (100 or 20 μg i.d.), Vaxfectin-formulated (100 μg i.m.), or PBS-formulated (100 μg i.m.) on days 0 and 28. All animals developed neutralizing antibody titers predicted to be protective (>120) (52, 53) within 2 weeks after the boost with the highest titers in the group that received 100 μg of Vaxfectin-formulated DNA i.d. and the lowest titers in the group that received 100 μg of PBS-formulated DNA i.m. (Fig. 1A). Differences between i.d. and i.m. delivery were not significant (P = 0.52), while animals receiving Vaxfectin-formulated DNA i.m. had higher titers than animals receiving PBS-formulated DNA i.m. (P = 0.036). MeV-specific binding IgG measured by ELA was also induced (Fig. 1B). The lowest levels of ELA antibody were in the monkeys that received the unadjuvanted DNA while the groups of monkeys receiving Vaxfectin-adjuvanted DNA developed similar higher levels of antibody (P = 0.0001).
compared to those elicited by infection with WT virus. The anti-
neutralized Bilthoven 3- to 11-fold less well than Chicago.
from most (6/7) monkeys in the other immunization groups neu-
also observed after recovery from WT infection. However, plasma
responses to pooled H peptides (Fig. 2A and B) and pooled F pep-
weeks after vaccination (Fig. 3).
not significant. Very few IL-4-producing cells were generated at 2
Vaxfectin-formulated DNA either i.m. or i.d., but differences were
measured by 50% plaque reduction of Chicago-infection of Vero cells. The data are plotted as geometric means ±
the standard errors of the mean (SEM). A dashed line indicates the generally accepted protective levels. Comparison of 100 μg of DNA i.m. with or without Vaxfectin (P = 0.0356, Student t test). (B) Enzyme immunoassay of plasma (1:100) IgG binding to MeV lysate-coated wells. The data are expressed as optical
density + the SEM (P = 0.0001, one-way ANOVA). (C) Plasma taken 48 weeks after vaccination or 135 days after infection with Bilthoven compared for
neutralization of Chicago-1 infection of Vero cells (interaction with CD46; black bars) and neutralization of Bilthoven infection of Vero/hSLAM cells (interaction
with CD150; gray bars).

FIG 1 Antibody responses to vaccination. Monkeys were vaccinated on day 0 and boosted 4 weeks later with codon-optimized DNA plasmids expressing the MeV H and F proteins. The vaccine was delivered either intradermally (i.d.) or intramuscularly (i.m.) either naked (PBS) or formulated with Vaxfectin (Vax). (A) Reciprocal titers of neutralizing antibody as measured by 50% plaque reduction of Chicago-infection of Vero cells. The initial vaccine dose and in monkeys that received 100 μg of Vaxfectin-formulated DNA given i.d.
neutralized the Chicago and Bilthoven viruses equivalently, as was
also observed after recovery from WT infection. However, plasma
from most (6/7) monkeys in the other immunization groups neu-
tralized Bilthoven 3- to 11-fold less well than Chicago.

The Chicago strain of MeV used for neutralization assays can
use CD46 as a receptor and infects Vero cells efficiently. To determine the antibody of antibody to neutralize WT virus that uses
CD150/hSLAM as a receptor, plasma samples obtained 48 weeks after vaccination or after WT Bilthoven infection were simultane-
ously tested in PRN assays with Vero cells and Chicago as the challenge virus and in Vero/SALM cells with Bilthoven as the chal-
lenge virus (Fig. 1C). The responses of vaccinated animals were
compared to those elicited by infection with WT virus. The anti-
body induced by 100 μg of Vaxfectin-formulated DNA given i.d.
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IFN-γ and IL-4 T cell response to vaccination. T cell re-
sponses to pooled H peptides (Fig. 2A and B) and pooled F pep-
tides (Fig. 2C and D) were measured by IFN-γ ELISPOT assays. T
cell responses to both H and F were generally higher 2 weeks after
the initial vaccine dose and in monkeys that received 100 μg of
Vaxfectin-formulated DNA either i.m. or i.d., but differences were
not significant. Very few IL-4-producing cells were generated at 2
weeks after vaccination (Fig. 3).

Protection from infection and disease after challenge. Mon-
keys were challenged 18 months after vaccination with 10^4 TCID<sub>50</sub>
of Bilthoven WT MeV. All vaccinated animals were protected
from disease defined by presence of a rash or viremia, while the
unvaccinated naive monkey developed a rash and a viremia that
peaked at day 10 (Fig. 4). MeV RNA was not detected in PBMCs
from vaccinated monkeys during the first 3 weeks after challenge
but was detected at low levels in two monkeys (33V [100 μg i.d.,
Vaxfectin] and 28V [100 μg i.m., PBS]) at 88 days.

Antibody response to challenge. Changes in levels of MeV-
specific antibody after challenge were measured by EIA (Fig. 5A).
All vaccinated monkeys had an anamnestic response to challenge with an increase in IgG by 7 to 10 days compared to day 14 for the
naive monkey. However, antibody titers for vaccinated monkeys
declined over the next 3 to 4 months, while they continued to
increase for the unvaccinated monkey. To further characterize the
induction of antibody-secreting cells (ASCs) and the generation of
long-lived plasma cells after challenge, PBMCs and bone marrow
(BM) were examined for the presence of MeV-specific ASCs. Plas-
mblasts secreting antibody to MeV, indicating the stimulation of
memory B cells to become ASCs, were detectable in circulation 7
and 10 days after challenge in most vaccinated animals but not in the
unvaccinated animal (Fig. 5B). Bone marrow, the site of resi-
dence of most long-lived plasma cells that produce the antibody
found in plasma (58), was sampled at the time of challenge (17
months after the vaccine boost) and at 105 and 140 days after challenge (Fig. 5C). Small numbers of bone marrow plasma cells
(0 to 4/5 × 10<sup>6</sup> cells) were established after DNA vaccination and
present at the time of challenge. After challenge, the numbers of
ASCs in the bone marrow increased in all animals, but at 3 to 4
months were substantially higher in the unvaccinated animal than
in the previously vaccinated animals.

IFN-γ and IL-17 T cell responses to challenge. After chal-
lenge, PBMCs were cultured in the absence (Fig. 6A and B) or
presence of overlapping peptides from the H (Fig. 6C and D)
and F (Fig. 6E and F) proteins and assessed for the production

FIG 2 ELISPOT assays. T cell re-
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IFN-γ and IL-17 T cell responses to challenge. After chal-
lenge, PBMCs were cultured in the absence (Fig. 6A and B) or
presence of overlapping peptides from the H (Fig. 6C and D)
and F (Fig. 6E and F) proteins and assessed for the production
of IFN-γ. Substantial numbers of IFN-γ-secreting cells were detected without in vitro stimulation, indicating in vivo activation. These numbers were further increased after peptide stimulation in vitro. In vaccinated animals, maximum stimulated and unstimulated ex vivo IFN-γ responses were at 10 and 18 days after challenge and at 18 days in the unvaccinated monkey reflecting vaccine-induced priming of the T cell responses to H and F. Primary T cell IFN-γ responses to overlapping peptides from N, which was not present in the vaccine, were also induced in all of the vaccinated animals, as well as the naive monkey (Fig. 6G and H).

**DISCUSSION**

This study has shown that Vaxfectin is an effective adjuvant for increasing antibody responses to measles DNA vaccination. MeV-specific neutralizing and EIA antibody increased after the second dose of vaccine, and the levels were significantly improved with Vaxfectin as an adjuvant. The levels of antibody after two doses of either 20 or 100 μg of Vaxfectin-formulated DNA were higher than levels after 100 μg of DNA without Vaxfectin indicative of a dose-sparing effect. MeV-specific IFN-γ-producing T cell numbers were not significantly im-
proved by Vaxfectin formulation. Animals in all vaccine groups were protected from disease, as manifested by rash and viremia, but not from infection, as evidenced by the late appearance of MeV RNA in PBMCs and the induction of an immune response to the N protein. After challenge, anamnestic MeV-specific B cell responses resulted in the appearance of ASCs in blood at 7 to 10 days and rapid increases in the levels of antibody. The amount of antibody produced after the challenge of vaccinated animals was similar to that of an unvaccinated animal at 2 to 4 weeks but was less durable, with decreasing levels of antibody in plasma and few MeV-specific long-lived plasma cells resident in the bone marrow at 2 to 4 months. Prior DNA vaccination had little effect on the induction of MeV-specific IFN-γ and IL-17-producing T cells after challenge.

As has been observed in some prior studies of DNA vaccinated nonhuman primates, T cell responses were induced more readily than antibody (32, 59). MeV-specific T cells were abundant in circulation 10 to 14 days after the first dose of vaccine and were minimally boosted by the second dose. The T cell response was characteristic of a Th1-type response with production of IFN-γ rather than IL-4. No differences related to dose of DNA, route of injection, or Vaxfectin adjuvant were noted.

The production of detectable levels of MeV-specific antibody occurred more slowly than the T cell response and not until after the second dose. Previous studies using 500 μg of DNA elicited detectable antibody after a single dose, with a further increase after the second dose (32). DNA vaccines are often used for priming the immune response, followed by another type of vaccine as a boost to increase antibody responses (18, 60). In the present study, both the prime and the boost were with DNA. Vaxfectin significantly increased the antibody response to the boosting dose compared to the same vaccine given without the adjuvant that may serve to stimulate innate responses. The route of delivery did not appear to be an important variable, because similar responses were induced after i.m. and i.d. needleless injection.

After challenge, both antibody and T cells showed anamnestic responses. As previously observed with other immunizations (59), ASCs derived from memory B cells appeared in circulation 7 to 10 days after challenge, indicating some level of infection. However, few of these cells became resident in the bone marrow to increase the numbers of long-lived plasma cells necessary for maintaining high levels of antibody in plasma. Accordingly, levels of antibody waned quickly in previously vaccinated animals, while primary infection in the unvaccinated monkey led to a slower response, but many more ASCs in bone marrow and sustained plasma antibody levels. Plasma cell longevity is dependent on cell cycle exit, expression of appropriate regulatory factors and receptors, and migration to and occupancy of survival niches in the bone marrow or sites of inflammation (61–63). How the ASCs produced by DNA vaccine-primed animals differ from those produced by naive animals is a topic worthy of further study. It is possible that the low level of virus replication after challenge in previously vaccinated animals programmed responding B cells for short lives as plasma cells (64).

The vaccine-induced immune response to H and F prevented the appearance of infected lymphocytes in circulation early after infection but did not prevent infection. In two animals MeV RNA was detected in PBMCs 3 months after challenge and may have been present and missed in other animals due to infrequent sampling at late times after infection. Be-

FIG 5 B cell responses after challenge. Plasma, PBMCs, and bone marrow (BM) cells were collected from vaccinated and unvaccinated monkeys after challenge with WT MeV. (A) MeV-specific IgG in plasma as determined by enzyme immunoassay. The data are presented as the mean of OD values + the SEM. (B) Numbers of plasmablasts producing MeV-specific antibody present in circulation 7 and 10 days after challenge. ASCs, antibody-secreting cells. (C) Numbers of plasma cells in bone marrow producing MeV-specific antibody before (d0, 17 months after vaccine boost) and after (105 and 140 days) challenge.
cause the amount of antigen present in the challenge is insufficient to efficiently stimulate the immune system without replication, the induction of a T cell response to N, not present in the vaccine, in all of the vaccinated animals further indicated that the challenge virus caused infection. Evidence of immune stimulation by challenge has also been observed in lymphoid tissue even after vaccination with LAV, suggesting that some level of infection is common in vaccinated animals after exposure to WT MeV (65). The site of replication is unclear, but both the respiratory tract and local lymphoid tissues are possibilities, and the late appearance of MeV RNA in PBMCs has been observed in previous studies of other MeV vaccines that protect from initial viremia (66).

Challenge also induced the appearance of PBMCs producing IL-17 in all groups. In contrast to the appearance of IFN-γ-producing cells, MeV-specific IL-17-producing cells had a biphasic pattern after challenge. In mice, IL-17 can be produced by CD4+ T cells, CD8+ T cells, γδ T cells, lymphoid tissue inducer-like cells, and invariant NKT cells associated with both adaptive and innate immune responses to infection (67–71). IL-17 induces release of an array of immune signaling molecules and has a recognized role in defense against bacterial infections, maintenance of epithelial integrity, and autoimmune diseases (67, 72–76). The role of IL-17 in the pathogenesis of acute viral infections is only beginning to be explored. Circulating Th17 cells are increased during enterovirus 71 infection, but the timing and duration of the increase has not been examined (77). IL-17 has not been previously described as a part of the response to MeV infection of humans or nonhuman primates. Biphasic appearance after infection with MeV may reflect activation of different populations of IL-17-producing cells, changes in lymphocyte trafficking or different phases of memory T cell generation in response to continued presence of MeV RNA (78). Infections with human and simian immunodeficiency viruses are associated with an early decrease in Th17, but not Tc17, cells in blood and mucosal tissues that is
predictive of chronic immune activation and disease progression (79–82). Further characterization of MeV-specific IL-17-producing cells will be of interest as in vitro studies show that IL-17 enhances virus-induced inflammatory cytokines (83).

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