DNA vaccination of macaques by a full-genome simian/human immunodeficiency virus type 1 plasmid chimera that produces non-infectious virus particles

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A DNA vaccination regime was investigated previously in rhesus macaques using a full-genome human immunodeficiency virus type 1 (HIV-1) plasmid, which, due to mutations in the nucleocapsid (NC) proteins, produced only non-infectious HIV-1 particles (Akahata et al., Virology 275, 116–124, 2000). In that study, four monkeys were injected intramuscularly 14 times with the plasmid. All of them showed immunological responses against HIV-1 and partial protection from challenge with a simian immunodeficiency virus/HIV (SHIV) chimeric virus. To improve this DNA vaccination regime, the plasmid used for vaccination was changed. In the present study, four macaques were injected intramuscularly eight times with a full-genome SHIV plasmid that produces non-infectious SHIV particles. CTL activities were higher than those observed in monkeys vaccinated previously with the HIV-1 plasmid. In all macaques vaccinated, peak plasma virus loads after homologous challenge with SHIV were two to three orders of magnitude lower than those of the naive controls, and virus loads fell below the level of detection at 6 weeks post-challenge. This suggested that the vaccination regime in this study was partially effective and better than the previous regime.

INTRODUCTION

Live attenuated vaccines have been shown to be the most effective vaccine against AIDS in non-human primate models (Daniel et al., 1992; Shibata et al., 1997; Ui et al., 1999). The main advantage of using live attenuated vaccines is that they mimic natural infection in the host. However, the safety of attenuated vaccines for human use is questionable because they have been shown to be pathogenic in neonates and possibly even in adult monkeys (Baba et al., 1999).

Another candidate for an AIDS vaccine is DNA, which can elicit both humoral and cell-mediated immunity. This method consists of injection of DNA plasmids that encode antigenic proteins under a strong enhancer/promoter, such as the human cytomegalovirus immediate/early promoter. DNA vaccines are considered to be much safer than live attenuated vaccines and initial results have been promising. Recent reports employed DNA vaccination in combination with boosting of antigenic proteins (Boyer et al., 1997; Letvin et al., 1997), live virus vectors (Robinson et al., 1999) or cytokine augmentation (Barouch et al., 2000). None of the vaccination regimes using DNA alone have achieved complete protection, which is in contrast to the rather satisfactory results obtained with live attenuated vaccines.

Previously, we reported a DNA vaccination regime using a full-genome plasmid producing non-infectious whole particles of human immunodeficiency virus type 1 strain NL432 (HIV-1_{NL432}), transcribed under its original promoter, HIV-1 LTR (Akahata et al., 2000). The plasmid has mutations (Cys...Cys...His...Cys→Ser...Ser...His...Cys) in an N-terminal zinc-finger motif of the nucleocapsid protein (NC), located in the gag region of HIV-1. Zinc-finger motifs are believed to play an important role in packaging the viral...
genomic RNA. The mutant plasmid expresses whole virus component proteins to produce virus particles that do not possess genomic RNA (Mizuno et al., 1996). All monkeys that had been injected intramuscularly with the plasmid 14 times showed immunological responses against HIV-1: stable anti-HIV-1 Env antibodies were raised in two monkeys and CTL activity against HIV-1 was induced in the other monkeys. All monkeys showed partial protection against challenge with 100 TCID₅₀ of a simian immunodeficiency virus/HIV chimeric virus (SHIV), termed SHIV-NM-3rN (Kuwata et al., 1995). Namely, peak plasma virus loads of the challenge virus were two to three orders of magnitude lower than they were in naive controls. However, we repeated the DNA injections a total of 14 times because of the inefficient expression of the proteins encoded by the plasmid.

To improve the DNA vaccination regime described previously, we vaccinated animals with a novel SHIV plasmid, termed pSHIV-NM-3rN ZF1*. The mutant plasmid pSHIV-NM-3rN ZF1* produces non-infectious SHIV whole virus particles similar to those produced by pNL432 ZF1* due to the mutation in the NC proteins. We expected that the expression of pSHIV-NM-3rN ZF1*, which is driven by the SIV LTR promoter, might be better in monkeys than the expression of pNL432 ZF1*, which is driven by the HIV-1 LTR promoter.

An advantage of a full-genome plasmid is that it is capable of expressing all of the viral antigenic components, including the Gag, Pol and Env proteins. Recently, some reports suggested that a single viral epitope-specific CTL response may not be sufficient to block infection with pathogenic SIV (Hanke et al., 1999; Yasutomi et al., 1995). Our DNA vaccine included all viral epitopes. In addition, this regime more closely resembles the use of a live attenuated vaccine than the foregoing DNA vaccination regimes. Live attenuated vaccines replicate in infected cells and viral antigenic components are presented by way of MHC molecules on the infected cell surface, thus eliciting a strong immunity against the virus in the host. Viral genes contained in the full-genome plasmid are expected to be expressed in a similar manner, leading to the assembly of virus components and budding of virus particles in the cells that took up the plasmid. This series of events is believed to be effective for acquisition of cellular and humoral immunity in the host.

**METHODS**

**Plasmid for DNA vaccination.** An infectious molecular clone of SHIV-NM-3rN (Kuwata et al., 1995) was used as a parent proviral DNA in this study (Fig. 1). The BamHI–PvuII fragment (nt 2106–2705), which includes a part of the gag open reading frame, was subcloned between the BamHI/HinCII sites of pUC119 and, using this plasmid as a template, site-directed mutagenesis of the zinc-finger motifs was performed by PCR (Fig. 2).

To generate the first finger-motif mutant (designated SHIV-NM-3rN ZF1*), in which the first two cysteine residues in the motif were replaced by serine residues, PCR was performed using the oligonucleotide 5'-TTGCGGCTCTGCAATTGCAGACTGGTCCCTTTCACAGATTCCAAGACTTAAATGGC-3' (nt 2535–2475) as a reverse primer and the F13 universal primer as a forward primer. The former oligonucleotide was designed to create an EcoRI site (indicated in boldface) in addition to altering specific nucleotides (underlined). The product amplified by PCR was electrophoretically separated on an agarose gel and purified using the GeneClean II kit (BIO 101). The fragment was then digested with BamHI (nt 2106) and BanII (nt 2533). Another fragment obtained from the above-mentioned plasmid pUC119 harbouring the BamHI–PvuII fragment of SHIV-NM-3rN (nt 2106–2705) was also digested with BanII/PvuII. These two fragments, BamHI–BanII (nt 2106–2533) and BanII–PvuII (nt 2533–2705), were subcloned as a consecutive form between the BamHI site and the HinCII site of pUC119. The plasmid generated was then digested with BamHI/PspMI (nt 2698). The BamHI–PspMI fragment obtained was reininserted into the corresponding position in the pUC119 plasmid harbouring the BamHI–Sse8387I fragment of **Fig. 1.** Genetic structure of HIV-1₄L₄₃₂, SIV₄₃₉₃₉, SHIV-NM-3rN, zinc-finger mutant SHIV-NM-3rN ZF1* and a challenge virus, SHIV-NM-3rN. Solid and open boxes represent sequences derived from HIV-1₄L₄₃₂ and SIV₄₃₉₃₉, respectively. '#', Mutated amino acids in SHIV-NM-3rN ZF1* zinc-finger motifs present in the NC. Mutated amino acid residues are underlined.
SHIV-NM-3rn (nt 2105–3402). The plasmid generated was then digested with BamHI/Sse8387I and the BamHI–Sse8387I fragment (nt 2105–3402) obtained was reinserted into the corresponding position in pSHIV-NM-3rn (Kuwata et al., 1995).

Cell culture and transfections. COS-1 cells were cultured in DMEM containing 10% heat-inactivated FBS (Gibco-BRL) and 20 mM L-glutamine. M8166 cells (a human CD4+ lymphoid cell line) were cultured in RPMI 1640 medium containing 10% FBS and 20 mM L-glutamine. Cells were transfected using the FuGENE-6 Transfection Reagent kit (Roche) with 20 µg of the viral plasmid DNAs, following the manufacturer’s recommendations.

Reverse transcriptase (RT) assay and ELISA. The RT assay was performed as described (Mizuno et al., 1996). The amount of p27CA in the culture supernatant was measured using the SIV Core Antigen ELISA kit (Coulter).

Quantitative RT-PCR. To measure the genomic RNA in virus particles, we performed quantitative RT-PCR as follows. The transfected culture supernatant was filtered through a 0.45 µm pore-size filter and viral RNA was extracted using the QIAamp Viral RNA kit (Qiagen). To remove contaminating plasmid DNA, the precipitated RNA was digested with DNase I (Gibco-BRL) for 15 min at 37°C, followed by heat treatment (15 min at 70°C) to inactivate the DNase I. RT reactions and subsequent PCR conditions were performed using the Taqman RT-PCR kit (Perkin Elmer), according to the manufacturer’s recommendations. SIVII-909F (5′-GGAAA-TCTA-3′) and SIVII-784R (5′-TCTA-TCTATTTTACCAGGTGTTAT-3′) were used as primers, and a labelling probe, SIVII-731 (5′-Fam-TGTCACCTCAGGTGTTAT-3′) (Perkin Elmer), was also used.

Immunoblot analysis. Immunoblot analysis was performed as described (Mizuno et al., 1996). In brief, for an immunoblot analysis of transfected cells, the cells were washed three times and, at 3 days after transfection, were lysed in lysis buffer and subjected to analysis. Plasma from a SHIV-NM-3rn-infected monkey (Kuwata et al., 1995) was used to provide the first antibody. To analyse for virus particles, the transfected culture supernatant was filtered through a 0.45 µm pore-size filter. Each sample was adjusted to contain an equal amount of RT activity in the supernatant. The virus particles released were pelleted by centrifugation at 14,000 r.p.m. for 2 h at 4°C. The pelleted virions were then lysed in lysis buffer and subjected to immunoblot analysis.

Electron microscopy. The morphology of the virus particles produced by full-genome plasmids was examined under electron microscopy. Transfected COS-1 cells were prefixed for 2 h in 2% glutaraldehyde in PBS and washed thoroughly with PBS. The cells were then fixed with 1% osmium tetroxide in PBS, dehydrated and embedded in epoxy resin. Ultrathin sections of the cells were stained with uranyl acetate and lead citrate and then observed under a Hitachi H-7100 electron microscope, as described (Mizuno et al., 1996).

Vaccination protocol. Plasmids were amplified in Escherichia coli on a large scale and extracted from the cells by conventional alkaline lysis methodology followed by purification with ethanol and polyethylene glycol precipitation. Four mature male rhesus macaques (Macaca mulatta) (MM212, MM214, MM231 and MM234) were injected intramuscularly at two points on the right quadriceps and at two points on the calves with a solution containing 500 µg pSHIV-NM-3rn ZF1+ in 450 µl PBS and 450 µl bupivacaine (Fujisawa Pharmaceutical) per injection for a total of eight injections. We injected the plasmid in two sets of DNA immunization series. Each set comprised one injection per week for four consecutive weeks. We injected at 0–3 weeks post initial vaccination (p.v.) and, after an interval of 5 weeks, we gave the second set of DNA immunizations, at 10–13 p.v.

Animals. All macaques used in this study were serologically negative for SIV and simian T cell lymphotropic virus type 1 before vaccination. They were housed throughout the experimental period and autopsies were carried out in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University, Japan.

Assays for antibodies. The titres of anti-Env antibodies in the plasma of the monkeys vaccinated were determined using the Genscreen HIV-1/2 kit (Fujirebio), following the manufacturer’s recommendations. The kit is based on a sandwich ELISA and the wells of microtitre plates were coated with the recombinant proteins HIV-1 gp160 p25, and synthetic peptides HIV-1 gp41 and HIV-2 gp36. Particle agglutination was measured using the Serodia HIV-1/2 kit.
Assay for HIV-1 Env- and SIV Gag-specific killer cell activities. Specific killer cell activities for HIV-1 Env and SIV Gag were measured as described previously (Akahata et al., 2000; Yamamoto et al., 1990). Herpesvirus papio-transformed B lymphocyte cell lines (B-LCL) established from the respective monkey PBMCs were infected with an HIV-1 Env (HIV-1m) or SIV Gag (SIVmac239)-expressing recombinant vaccinia virus and used as target cells. The parental vaccinia virus-infected and non-infected cells were used as control targets. HIV-1 Env- and SIV Gag-specific killer cell activities were expressed as the percentage of specific lysis (% specific lysis) = (% lysis of the HIV-1 Env- or SIV Gag-expressing B-LCL)−(% lysis of the parental vaccinia virus-infected B-LCL), when the effector : target cell ratio was 50:1. (In some cases, slightly lower ratios were adopted due to a limited number of the available cells.) The respective monkey B-LCLs infected with HIV-1 Env- or SIV Gag-expressing vaccinia virus were killed by treatment with glutaraldehyde and were used as antigen-stimulator cells. The respective monkey PBMCs were stimulated by the HIV-1 Env- or SIV Gag-expressing B-LCLs and incubated for 5 days. Stimulated PBMCs were used as the effector cells. The values of per cent specific lysis were measured in triplicate and mean values are shown. A per cent specific lysis value of above 70% was considered as ‘specific killer cell activity’. HIV-1 Env- and SIV Gag-specific killer cell activities were measured in MM212 and MM214 at 16 and 24 weeks p.v. and in control, PBMCs were cultured in the presence of 1×10^6 PBMCs and the primer pair HnefF1 (5′-ACAGG-GCITGGAAAGGATITTTGCTA-3′, nt 9350–9374) and SnaF1 (5′-CCCGTAAACCTCCCTTGGAAGATGCCC-3′, nt 10178–10206). The challenge virus, SHIV-NM-3rN, was detected as a 859 bp band and the vaccination plasmid, pSHIV-NM-3rN ZF1*, was detected as a 680 bp band. To determine whether the vaccination plasmid (which has an Xhol site) was present, the PCR products were incubated with Xhol. Cleavage at this site would create bands of 580 and 100 bp in size. The regions of the challenge viruses amplified do not have an Xhol site. Thus, if the PCR products were not digested with Xhol, then they would have been derived only from the challenge virus.

RESULTS

Vaccinated plasmid

We used the plasmid pSHIV-NM-3rN ZF1* for DNA vaccination (Fig. 1). The plasmid is derived from an infectious molecular clone SHIV-NM-3rN (Kuwata et al., 1995), which is a SHIV comprising LTR, gag, pol, vif and vpx from SIVmac239 (Kestler et al., 1990) and vpr, tat, rev, vpu, env and nef from HIV-1NL432 (Adachi et al., 1986). The plasmid pSHIV-NM-3rN ZF1* has mutations (Cys→Ser…Ser→His…Cys) in an N-terminal zinc-finger motif of the NC protein in the gag region of SHIV-NM-3rN.

Zinc-finger mutants of HIV-1 and Moloney murine leukemia virus have been reported to be non-infectious (Gorelick et al., 1990; Meric & Goff, 1989; Mizuno et al., 1996). We next determined whether the pSHIV-NM-3rN ZF1* plasmid produced infectious virus. CD4+ M8166 cells were infected with the supernatants of transfected COS-1 cells containing equal amounts of viral RNAs, as measured by quantitative RT-PCR. Conspicuous syncytium formation was observed in M8166 cells infected with the supernatant from the SHIV-NM-3rN plasmid. The culture supernatant of these cells accumulated RT activity. In contrast, there was no indication of infectivity in the cells infected with the supernatants from pSHIV-NM-3rN ZF1* during the observation period of more than 1 month. Thus, the alteration in the N-terminal zinc finger resulted in the total loss of virus infectivity.

Viruses particles produced by the mutant DNA had almost the same amount of p27CA, p17MA and unprocessed Gag precursor proteins as the wild-type. An immunoblot analysis together with electron microscopic observation showed that the plasmid pSHIV-NM-3rN ZF1* produced non-infectious virus particles with an average diameter of...
Antibody response in DNA-vaccinated macaques

Four macaques (MM212, MM214, MM231 and MM234) were vaccinated using a DNA vaccination regime with the SHIV full-genome plasmid, pSHIV-NM-3rn ZF1*. The vaccination schedule is shown in Fig. 4.

None of the monkeys showed an antibody response against HIV-1 Env up until 24 weeks p.v., as measured by ELISA. Antibody titres against HIV-1/2 were also measured by particle agglutination, which gave results that were the same as those obtained by ELISA. No detectable anti-HIV-1/2 antibodies were observed up until the day of challenge. We also employed an immunoblotting assay to see any antibody response, but the sera of all of the vaccinated monkeys at 8, 14, 16 and 24 weeks p.v. did not show any specific antibody by an HIV-2 Western blot kit that can detect anti-SIV Gag antibodies.

HIV-1 Env- and Gag-specific killer cell activities

Table 1 summarizes HIV-1 Env- and Gag-specific killer cell activities in the monkeys between 14 and 24 weeks p.v. All monkeys vaccinated showed HIV-1 Env- or SIV Gag-specific killer cell activities. MM234 showed HIV-1 Env- and SIV Gag-specific killer cell activities at 14 weeks p.v. (against Env at 14 and 19 weeks p.v. and against Gag at 14 weeks p.v.). MM212, MM214 and MM231 showed Gag-specific killer cell activities at 24 weeks p.v., at 16 and 24 weeks p.v. and at 19 weeks p.v., respectively.

Lymphocyte proliferation

Lymphocyte proliferation was measured by determining the ratio of incorporated BrdU by PBMCs in the presence of HIV-1 rgp160. SI values increased in all monkeys vaccinated, indicating the presence of antigen-specific helper T cell lymphocyte memory (Fig. 4). SI values in all monkeys before vaccination were below 2. However, values increased to above 3 after the eighth injection in MM212 and after the fourth injection in the other three monkeys.

Table 1. HIV-1 Env- or SIV Gag-specific killer cell activities in macaques vaccinated with DNA

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Weeks p.v.</th>
<th>% specific lysis</th>
</tr>
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<tbody>
<tr>
<td>MM212</td>
<td>16</td>
<td>3-4</td>
</tr>
<tr>
<td>MM214</td>
<td>16</td>
<td>3-5</td>
</tr>
<tr>
<td>MM231</td>
<td>14</td>
<td>6-3</td>
</tr>
<tr>
<td>MM234</td>
<td>14</td>
<td>7-0*</td>
</tr>
<tr>
<td>MM212</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td>MM214</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td>MM231</td>
<td>19</td>
<td>0</td>
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<tr>
<td>MM234</td>
<td>19</td>
<td>7-2</td>
</tr>
</tbody>
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*E:T ratio of 30:1.
†E:T ratio of 25:1.
ND, Not done.
Virus loads of challenge virus in plasma

Vaccinated monkeys were intravenously challenged with a 100 TCID$_{50}$ dose of SHIV-NM-3rN at 24 weeks p.v. Two naive macaques, MM125 and MM126, were also inoculated as unvaccinated controls.

Plasma virus loads were monitored by quantitative RT-PCR (Fig. 5). Viral RNA was not detected in any of the macaque sera before challenge with the infectious virus. Virus loads in the two naive monkeys (MM126 and MM125) at 1 week p.c. were approximately $2.7 \times 10^3$ and $3 \times 10^5$ copies ml$^{-1}$, respectively. Then, at 2 weeks p.c., the virus loads of these two monkeys reached their peak values (both approximately $10^7$ copies ml$^{-1}$). The virus loads of these monkeys remained above the detection limit until 12 weeks p.c. On the other hand, at 1 week p.c., the virus load of MM234 was below $5 \times 10^3$ copies ml$^{-1}$ and the virus loads of the other vaccinated monkeys were below the detectable level. Peak virus loads at 2 weeks p.c. of all vaccinated monkeys were two to three orders of magnitude lower than those of the naive. It is noteworthy that it took only 4 weeks for the virus loads to decrease below the detectable level in the three monkeys vaccinated.

Detection of recombination between the vaccinated plasmid and the challenge virus

No infectious virus was recovered from MM214, MM231 or MM234 at 2 and 4 weeks p.c., while infectious virus was recovered from MM212, but only at 2 weeks p.c. The nef region of the virus recovered from MM212 was amplified by PCR. It was found that the PCR product was not cut by XhoI. Since only the vaccination plasmid has an XhoI site, this indicated that only the challenge virus was present, suggesting that there was no recombination between the vaccination plasmid and the challenge virus. Although we extracted the PBMCs of the other animals and screened for recombination events, we could not amplify the proviral DNA from the stored, frozen PBMCs, probably due to the very low copy numbers of the proviral DNA.

DISCUSSION

In a previous study, we injected four monkeys 14 times with the HIV-1 plasmid pNL432 ZF1* (Akahata et al., 2000). In this study, we injected four monkeys with the plasmid pSHIV-NM-3rn ZF1*, expecting that the expression of this plasmid, which is driven by the SIV LTR promoter, would be better than that of the pNL432 ZF1*, which is driven by the HIV-1 LTR promoter, in monkeys. This is because HIV-1 has a narrow host range and cannot infect non-human primates such as rhesus macaques (Macaca mulatta). Although the determinants of macaque cell tropism are unknown, a SHIV comprising LTR and gag from SIV$_{mac239}$ and env from HIV-1 can infect macaques but a SHIV comprising LTR and gag from HIV-1 and env from SIV$_{mac239}$ cannot (Shibata et al., 1991). In this study, none of the monkeys vaccinated showed any anti-HIV-1 Env antibodies. The three of the monkeys showed no anti-SIV Gag antibodies and the fourth had only weak anti-SIV Gag antibodies. On the other hand, two of the monkeys vaccinated in the previous study (Akahata et al., 2000) showed anti-HIV-1 Env and Gag antibodies. In this regard, the pSHIV-NM-3rn ZF1* plasmid did not achieve higher expression than the previous plasmid. However, all monkeys vaccinated in the present study showed CTL responses, while only two of four monkeys vaccinated in the previous study showed CTL responses. In addition, CTL responses in the previous study were lower than those in the present study. These observations might be due to the higher expression achieved by the SIV LTR promoter. The challenge virus was given at the same dose that SHIV-NM-3rN was given in the previous study. After challenge with SHIV-NM-3rN, peak plasma virus loads were two to three orders of magnitude lower than those of the naive control monkeys in the present and previous study. However, the viral RNA load in all monkeys vaccinated in the present study was lower. The viral RNA load was detected in only one monkey at 4 weeks p.c. and it was not detected in any of the monkeys at 6 weeks p.c. and thereafter. On the other hand, in the previous study, the virus was detected for a longer period; it was present in two monkeys at 4 and 6 weeks p.c., even though the infections were much more frequent than those in the present study. Overall, these results suggested that the vaccination plasmid pSHIV-NM-3rn ZF1* was better than plasmid pNL432 ZF1* in monkeys.

Recently, some studies have reported DNA vaccination regimes that use a replication-defective full-genome plasmid for cats or non-human primates. The cats vaccinated with a full-genome plasmid showed a CTL response but did not show any antibody response (Hosie et al., 1998). In the 11 monkeys vaccinated with the full SIV genome, only three
monkeys showed antibody responses (Gorelick et al., 2000). Our previous report (Akahata et al., 2000) showed that two of four monkeys vaccinated with the full genome of HIV-1NL432 had antibody responses. In the present study, all monkeys vaccinated showed a CTL response. However, three monkeys did not show any antibody response and one monkey showed only a weak anti-Gag antibody response. These recent studies (Hosie et al., 1998; Gorelick et al., 2000; Akahata et al., 2000) reported that vaccinations that induced little or no antibody response still conferred moderate protection against challenge viruses. It seemed to be more important to induce a CTL response than an antibody response. DNA vaccinations with a full-genome plasmid tend to induce cell-mediated immunity but also tend to be not so effective at inducing humoral immunity during the period soon after the vaccination. Overexposure to the antigens may shift the immunity from a Th1-type immunity to a Th2-type immunity (Fuller & Haynes, 1994; Haynes et al., 1994). The difficulty of inducing antibody responses with the full-genome DNA vaccination could be due to insufficient antigen expression shifting to Th2-type immunity, as we suggested previously (Akahata et al., 2000).

In this study, we showed that a DNA vaccination regime using a full-genome plasmid was potentially effective without acting in combination with any other booster. Further studies are needed to determine whether modification of this regime, such as by using the plasmid in combination with protein or recombinant virus vector boosters, can achieve better protection.

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