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The 3′ CCACCA Sequence of tRNA^{Ala}(UGC) Is the Motif That Is Important in Inducing Th1-Like Immune Response, and This Motif Can Be Recognized by Toll-Like Receptor 3

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In this article, the immunogenicity of tRNA and the recognition of tRNA by Toll-like receptors (TLRs) are analyzed. Analyses of the effects of different tRNA^{Ala}(UGC) fragments (tRNA^{Ala}1-76 [corresponding to positions 1 through 76], tRNA^{Ala}26-76, tRNA^{Ala}40-76, tRNA^{Ala}62-76, tRNA^{Ala}1-70, tRNA^{Ala}26-70, tRNA^{Ala}40-70, and tRNA^{Ala}62-70) on the immune responses of hepatitis B surface antigen (HBsAg) were performed with BALB/c mice. Results show that tRNA^{Ala}1-76, tRNA^{Ala}26-76, tRNA^{Ala}40-76, and tRNA^{Ala}62-76 adjuvants not only induced stronger T helper (Th) 1 immune responses but also cytotoxic-T-lymphocyte (CTL) responses relative to tRNA^{Ala}1-70, tRNA^{Ala}26-70, tRNA^{Ala}40-70, and tRNA^{Ala}62-70 adjuvants in HBsAg immunization. A deletion of the D loop (tRNA^{Ala}26-76), anticodon loop (tRNA^{Ala}40-76), or TψC (tRNA^{Ala}62-76) loop of tRNA^{Ala}(UGC) does not significantly decrease the adjuvant characteristic of tRNA^{Ala}(UGC). However a deletion of the 3′-end CCACCA sequence (tRNA^{Ala}1-70, tRNA^{Ala}26-70, tRNA^{Ala}40-70, and tRNA^{Ala}62-70) of tRNA^{Ala}(UGC) significantly decreased the adjuvant characteristic in Th1 and CTL immune responses. Moreover, the recognitions of different tRNA^{Ala}(UGC) fragments by TLR3, TLR7, TLR8, and TLR9 were analyzed. Results show that a deletion of the 3′ CCACCA sequence of tRNA^{Ala}(UGC) significantly decreased the recognition by TLR3. We concluded that the 3′ CCACCA sequence of tRNA^{Ala}(UGC) is the important motif to induce Th1 and CTL responses and this motif can be effectively recognized by TLR3.

RNAs are important in the immune response of viral infection, modulation of cytokines, differentiation of T helper 1 (Th1) cells, and stimulation of cross-priming and antiviral effects. Recently, different kinds of RNAs have been studied in order to stimulate immune response or analyze recognition by Toll-like receptors (TLRs). For example, (i) eukaryotic cell RNA (30), (ii) RNA from bacteria (20), (iii) small interfering RNA (17), (iv) mRNA (18), (v) synthetic double-stranded RNA (dsRNA) (7, 13, 35, 36), (vi) synthetic single-stranded RNA (ssRNA) (8, 14, 39), and (vii) fungal tRNA (3) have been studied.

In bacteria, though up to 20% of the total RNA in bacterial cells is tRNAs (9) and tRNAs are relatively more stable than mRNA (10), it has been found that tRNA separated from *Salmonella enterica* serovar Typhimurium LT2 provided mice protection against challenge with *Salmonella* (19). Bacterial tRNA, but not tRNA preparations of eukaryotic origin, can give dose-dependent protection of mice against *Encephalomyocarditis* virus infection (33).

However, the functional involvement of tRNAs in the immune system and the recognition of tRNAs by TLR largely remain unknown. Mammalian TLRs play a key role in host defense during pathogen infection by regulating and linking innate and adaptive immune responses (26). About 11 or 12 TLRs in humans have been described, and for most of them, specific ligands have been identified (15). TLR3 was determined to be the factor that recognizes synthetic dsRNAs (1, 2, 25) or mRNA (18), TLR4 was determined to recognize lipopolysaccharide (28), and TLR9 was determined to recognize unmethylated CpG DNA (21). Moreover, TLRs can respond to host-derived molecules that are released from injured tissues or cells, for example, surfactant protein A (12), fibrinogen (32), heparan sulfate proteoglycan (16), β-defensins (5), and chromatin-immunoglobulin (IgG) complexes (22).

These results brought us to determine whether bacterium-derived tRNAs have important adjuvant function in mammalian immune response and which motif of tRNAs in bacteria is important for immune response, as well as whether mammalian TLRs can recognize bacterium-derived tRNAs as a pathogen-associated molecular pattern.

With the development of in vitro RNA transcription technology, it becomes possible to prepare tRNA fragments by T7 RNA polymerase. In this study, we evaluated the functional involvement of different tRNA fragments as hepatitis B surface antigen (HBsAg) adjuvant in the BALB/c mice and the recognition of tRNA fragments by TLRs. Results show that the 3′ CC ACCA sequence of tRNA^{Ala}(UGC) is an important motif for inducing a Th1 immune response and that the 3′ CCACCA sequence of tRNA^{Ala}(UGC) can be significantly recognized by TLR3.

MATERIALS AND METHODS

Preparation of tRNA^{Ala}(UGC) fragments by runoff transcription. Genomic DNA was prepared from *Escherichia coli* K-12 by a genomic DNA preparation kit (QIAGEN). Primers were designed according to an *E. coli* K-12 genomic sequence (GenBank accession no. NC_000913), and all primers are listed in

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TABLE 1. Designed primers for the PCR[a]

Primer	Sequence
F	GGGGCTATAGCTCAGCTGGGAGAG
R	TGGTGGAGCTATGCGGGATCGAAC
1-76F	TAATACGACTCACTATA**G**GGAGAGGGGCTATAGCTCAGCTGGGAGAG
1-76R	TGGTGGAGCTATGCGGGATCGAAC
26-76F	TAATACGACTCACTATA**G**GGAGAGCCTGCTTTGCACGCAGGAGGTCT
26-76R	TGGTGGAGCTATGCGGGATCGAAC
40-76F	TAATACGACTCACTATA**G**GGAGACAGGTCTGCGGTTCGATCCCGCAT
40-76R	TGGTGGAGCTATGCGGGATCGAAC
62-76F	TAATACGACTCACTATA**G**GGAGACGCATAGCTCCACCA
62-76R	TGGTGGAGCTATGCGGGATCGAAC
1-70F	TAATACGACTCACTATA**G**GGAGAGGGGCTATAGCTCAGCTGGGAGAG
1-70R	AGCTATGCGGGATCGAAC
26-70F	TAATACGACTCACTATA**G**GGAGAGCCTGCTTTGCACGCAGGAGGTCT
26-70R	AGCTATGCGGGATCGAAC
40-70F	TAATACGACTCACTATA**G**GGAGACAGGTCTGCGGTTCGATCCCGCAT
40-70R	AGCTATGCGGGATCGAAC
62-70F	TAATACGACTCACTATA**G**GGAGACGCATAGCT
62-70R	AGCTATGCGTCTCCCTAT

[a] The forward primers contain T7 promoter canonical sequence TAA TAC GAC TCA CTA TA**G** GGA GA (underlined), where the bold G corresponds to the start of the RNA and which represents the cap structure in the final tRNA fragment products.

Table 1. Primers F and R were used for the amplification of DNA fragment encoding $tRNA^{Ala}$(UGC) from *E. coli* genomic DNA. PCR products were purified with a gel purification kit (QIAGEN). This PCR product was cloned into pCR2.1 with the TOPO TA cloning kit (Invitrogen, CA), and the new plasmid $pCRtRNA^{Ala}$ was constructed. The positive $pCRtRNA^{Ala}$ clone was sequenced. DNA sequence transcribing $tRNA^{Ala}$(UGC) was cut from the plasmid $pCRtRNA^{Ala}$ with BamHI and EcoRI. The DNA fragment transcribing $tRNA^{Ala}$(UGC) was purified with the QIAGEN gel purification kit. And this DNA fragment was used for the next PCR so that T7 promoter could be added onto it. In order to add the 23-bp T7 promoter sequence upstream of the PCR product, the second PCR was performed. The primer pairs 1-76F and 1-76R, 26-76F and 26-76R, 40-76F and 40-76R, 62-76F and 62-76R, 1-70F and 1-70R, 26-70F and 26-70R, 40-70F and 40-760, and 62-70F and 62-70R were used for PCR to produce T7 fusion DNA fragments transcribing $tRNA^{Ala}$1-76 [corresponding to positions 1 through 76], $tRNA^{Ala}$26-76, $tRNA^{Ala}$40-76, $tRNA^{Ala}$62-76, $tRNA^{Ala}$1-70, $tRNA^{Ala}$26-70, $tRNA^{Ala}$40-70, and $tRNA^{Ala}$62-70, respectively.

T7 RNA polymerase (Invitrogen, CA) was used for in vitro runoff transcription of T7-fusion DNA fragments transcribing different $tRNA^{Ala}$(UGC) fragments at 37°C for 2 to 3 h to prepare $tRNA^{Ala}$1-76, $tRNA^{Ala}$26-76, $tRNA^{Ala}$40-76, $tRNA^{Ala}$62-76, $tRNA^{Ala}$1-70, $tRNA^{Ala}$26-70, $tRNA^{Ala}$40-70, and $tRNA^{Ala}$62-70. Then $tRNA^{Ala}$1-76, $tRNA^{Ala}$26-76, $tRNA^{Ala}$40-76, $tRNA^{Ala}$62-76, $tRNA^{Ala}$1-70, $tRNA^{Ala}$26-70, $tRNA^{Ala}$40-70, and $tRNA^{Ala}$62-70 products were purified by a combination of hydrophobic chromatography on phenyl-Sepharose and reverse-phase high-performance liquid chromatography as described in the literature (6). The RNA sequences of different tRNA fragments are shown in Fig. 1.

Animals. Twelve groups of 7- to 8-week-old BALB/c mice were used in the experiments. In each group, there were five mice. Recombinant HBsAg (Aldevron, Fargo, N.Dak.) was used. Group A mice were injected with phosphate-buffered saline (PBS) solution as a control; group B mice were injected with 50 μg HBsAg; group C mice were injected with 50 μg HBsAg and 50 μg phosphorothioate-modified CpG oligodeoxynucleotides (ODN), respectively. The sequence of phosphorothioate-modified ODN was 5′ TCC ATG ACG TTC CTG ACG TT 3′. Group D mice were injected with 50 μg HBsAg and 50 μg phosphorothioate-modified non-CpG ODN as control, respectively. The sequence of phosphorothioate modified non-CpG ODN was 5′ TCC AAT GAG CTT CCT GAG TCT 3′. Group E mice were injected with 50 μg HBsAg and 50 μg $tRNA^{Ala}$1-76; group F mice were injected with 50 μg HBsAg and 50 μg $tRNA^{Ala}$26-76; group G mice were injected with 50 μg HBsAg and 50 μg $tRNA^{Ala}$40-76; group H mice were injected with 50 μg HBsAg and 50 μg $tRNA^{Ala}$62-76, group I mice were injected with 50 μg HBsAg and 50 μg $tRNA^{Ala}$1-70; group J mice were injected with 50 μg HBsAg and 50 μg $tRNA^{Ala}$26-70; group K mice were injected with 50 μg HBsAg with 50 μg $tRNA^{Ala}$40-70; and group L mice were injected with 50 μg HBsAg with 50 μg $tRNA^{Ala}$62-70. All mice were immunized by intramuscular injection in the hind leg muscles at 0, 2, and 4 weeks and bled at 0, 2, 4, 6, and 8 weeks by retroorbital puncture. Mice were killed at week 8. Spleens were removed and passed through a sieve. Cells were centrifuged at 1,200 rpm for 5 min, the supernatant was discarded, and red blood cells were lysed with lysing buffer (0.14 M ammonium chloride, 20 mM Tris, pH 7.5). The red-blood-cell-lysed splenocytes were considered antigen-presenting cells (APCs) for T-lymphocyte proliferation assays and cytokine assays. To prepare T-lymphocyte-enriched splenocytes, B lymphocytes were removed by nylon wool fiber columns (Polysciences, Inc., Warrington, PA). Eluted cells are referred to as T-lymphocyte-enriched splenocytes. After three washes in RPMI 1640, T lymphocytes were resuspended in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, 8 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 100 μM 2-mercaptoethanol. T-lymphocyte-enriched splenocytes were used for cytokine assays, T-lymphocyte proliferation assays, and cytotoxic-T-lymphocyte (CTL) assays.

Evaluation of antibodies responses. Antibodies specific to HBsAg (total IgG, IgG1, and IgG2a) were quantified by an enzyme-linked immunosorbent assay (ELISA) method. Briefly, 96-well plates (Costar) were coated with 100 μl HBsAg (10 μg/ml) in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Plates were blocked with 1% milk in PBS for 1 h at 37°C. Subsequently, the plates were incubated with the serum samples diluted with 1% milk and 1% Tween 20 in PBS for 2 h at 37°C. The goat anti-mouse IgG, IgG1, and IgG2a horseradish peroxidase conjugate (Amersham) was incubated 1 h at 37°C. Subsequently the plates were incubated with the substrate solution (200 mM Na_2HPO_4, 100 mM citrate, 1 mg/ml *O*-phenylenediamine dihydrochloride, and 0.1% H_2O_2) for 15 min at room temperature. One hundred microliters of 1 M phosphoric acid was added to stop the reaction. The plates were read at 492 nm in a microplate reader (Bio-Rad). End-point titers were determined according to the method of Reed and Muench (29). At least three separate ELISAs were performed for each experiment.

Cytokine assays. Totals of 5×10^6 T-lymphocyte-enriched splenocytes and 2.5×10^6 APCs (irradiated; 2,000 rad) were plated for the interleukin-4 (IL-4), and gamma interferon (IFN-γ) cytokine assays in triplicate in 96-well round-bottom polystyrene plates. For IL-12 cytokine assays, 5×10^6 T-lymphocyte-enriched splenocytes and 2.5×10^6 nonirradiated APCs were plated in 96-well plates and incubated for 42 h at 37°C in 5% CO_2 with HBsAg at a concentration of 3 μg/ml. The culture supernatants were used for cytokine assays. A mouse IL-4 ELISA kit (Pharmingen), a mouse IL-12 ELISA kit (Pharmingen), and a mouse IFN-γ ELISA kit (Pharmingen) were used to assay the concentrations of different cytokines.

T-lymphocyte proliferation assays. Totals of 5×10^6 T-lymphocyte-enriched splenocytes and 2.5×10^6 APCs (irradiated; 2,000 rad) were incubated for 4 days at 37°C in the presence of HBsAg (3 μg/ml). All proliferation assays were performed in triplicate in 96-well plates. [^{3}H]thymidine (0.5 μCi/well; Amersham) was added 12 h before harvesting. Cells were harvested on glass microfiber papers. Thymidine incorporation was assessed by liquid scintillation spectrometry. Results are expressed as stimulation indices of the mean cpm from triplicate

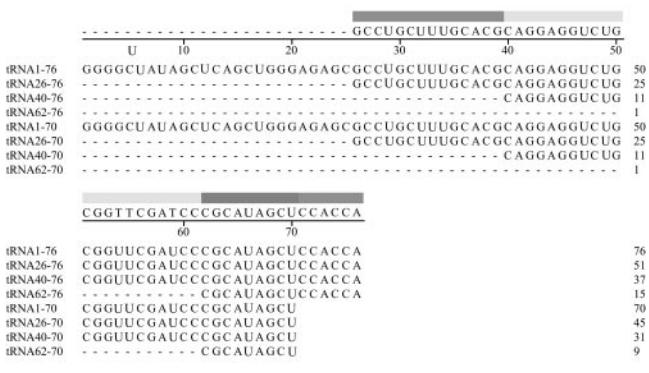

FIG. 1. RNA sequences of different $tRNA^{Ala}$(UGC) fragments. The 5′ cap structure sequence (GGGAGA) is not listed in the figure.

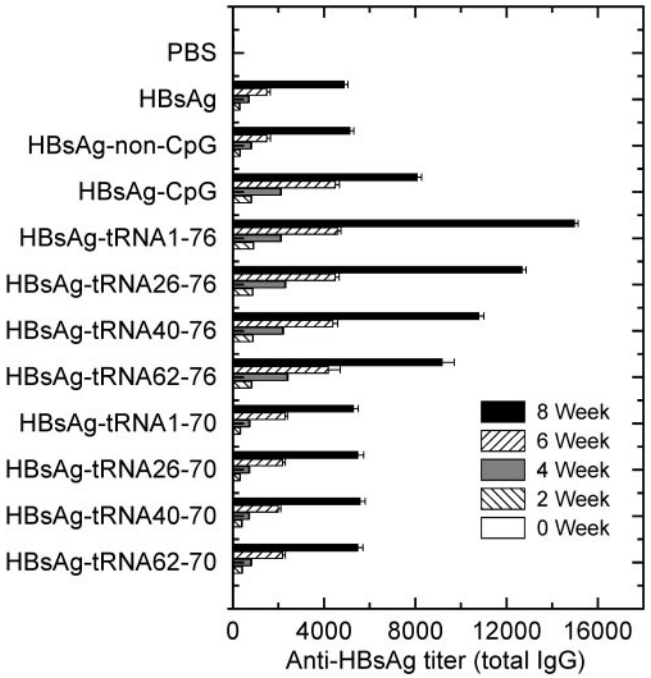

FIG. 2. The anti-HBsAg antibodies (total IgG) after immunization with HBsAg and different adjuvants in BALB/c mice. The sera were collected at weeks 0, 2, 4, 6, and 8, and the antibody titers were determined. Data shown are representative of three independent experiments and are expressed as mean ± SD.

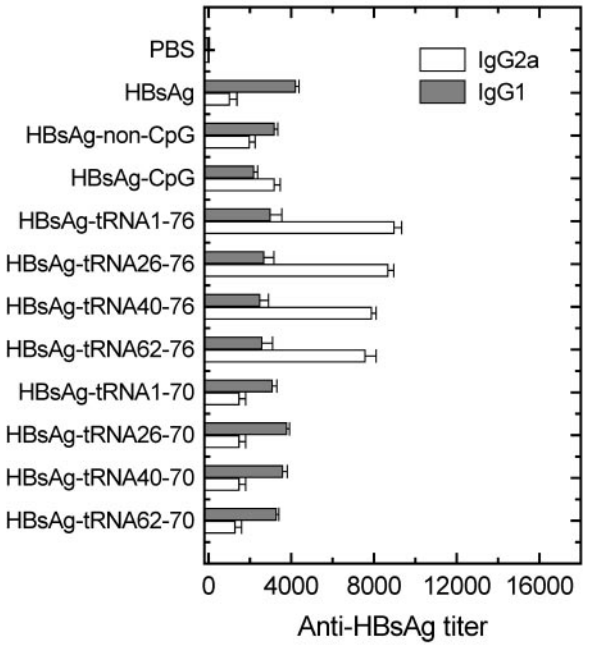

FIG. 3. The anti-HBsAg subtype IgG1 and IgG2a responses after immunization with HBsAg and different adjuvants at week 8. Data shown are representative of three independent experiments and are expressed as means ± SD.

cultures in the presence of antigen divided by the mean cpm of triplicate cultures obtained with medium only.

CTL response assays. A total of 5×10^7 T-lymphocyte-enriched splenocytes was restimulated in vitro with the addition of HBsAg (10 μg/ml) in 12-well plates, and cultured for 6 days, supplemented with 25 IU IL-2 in each well in the last two days. P815-S cells were used as target cells. P815-S cells were a mastocytoma cell line derived from BALB/c mice and transfected with hepatitis B virus S protein genes. P815 cells were used as a control. The target cells were labeled for 1 h with ^{51}Cr (100 μCi/10^6 cells), and then they were added to effector cells at effector cell-to-target cell ratios of 12.5:1, 25:1, and 50:1, respectively. The cell mixtures were incubated in 96-well round-bottom tissue culture plates in 0.2 ml of complete RPMI medium for 4 h at 37°C in a 5% CO_2 humidified atmosphere. At the end of the 4 h culture, the supernatants were processed for the determination of ^{51}Cr release. The percentage of specific release was calculated as follows: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Total release was measured by resuspending target cells in lysis buffer. Spontaneous release was obtained from targets incubated with medium alone.

Transfection of cells and luciferase reporter assays. Human embryonic kidney 293 (HEK293) cells were seeded in 24-well plates (10^5 cells/well). Cells were transiently transfected with a NF-κB reporter construct pNF-κB-luc (Stratagene) when the cells reached 30 to 50% of confluence along with constructs expressing various TLRs using the 293fectin transfection reagent (Invitrogen, CA). All plasmids were prepared with the Endofree Maxiprep plasmid kit (QIAGEN). Briefly, 100 ng of reporter construct was cotransfected with 100 ng of human pUNO-hTLR3, pUNO-hTLR7, pUNO-hTLR8, or pUNO-hTLR9 construct (Invivogen, CA). After 20 h of transfection, cells were stimulated with different tRNA fragments for 6 h in triplicate, and transfection efficiency was compared with TLR agonists by a TLR agonist kit (Invivogen, CA) with human TLR3 agonist poly(I:C), human TLR7 agonist imiquimod, human TLR8 agonist ssRNA40, and human TLR9 agonist ODN2006 were used. After the stimulation, supernatants were discarded and cells were lysed in 100 μl of lysis buffer (8 mM $MgCl_2$, 1% Triton X-100, 15% glycerol, 1 mM dithiothreitol, 25 mM Tris, pH 8.0) for 5 min at room temperature. Lysed cells were analyzed for luciferase activity in lysis buffer complemented with 2 mM luciferin and 1 mM ATP by using a luminometer (Microlumat Plus, Berthold, Germany). The experimental data were expressed as means ± standard deviations (SD) from at least three independent experiments, each performed in duplicate.

Statistical analysis. All values were the averages of triplicate assays. The significance of differences between the experiment group and control group was determined by Student's *t* test, with *P* values of >0.05 being considered not significant.

RESULTS

Antibody response. After BALB/c mice were vaccinated with HBsAg and different adjuvants, the antibody responses (total IgG) of HBsAg in BALB/c mice were determined from 0 week to 8 week. As shown in Fig. 2, anti-HBsAg titers varied with the vaccine formulations. The highest titer was attained with HBsAg-tRNA^{Ala}1-76 vaccine formulations at week 8 ($P < 0.05$). The total IgG responses of HBsAg-tRNA^{Ala}1-70, HBsAg-tRNA^{Ala}26-70, HBsAg-tRNA^{Ala}40-70 and HBsAg-tRNA^{Ala}62-70 were significantly lower than that of HBsAg-tRNA^{Ala}1-76 at week 8 ($P < 0.05$). The mice immunized with HBsAg-CpG also significantly improved the anti-HBsAg IgG from week 4 to week 8 ($P < 0.05$), however the mice immunized with HBsAg–non-CpG ODN did not induce antibody titers similar to those induced by HBsAg-CpG. In our experiment, CpG adjuvant experiment results were comparable to many other research groups (24), vaccination with HBsAg alone did not induce strong anti-HBsAg antibody titer; however, a combination of HBsAg and tRNA^{Ala}1-76 can induce the strongest anti-HBsAg titer.

Antibody isotype profiles. Different IgG antibody isotypes were used to evaluate the type of Th response, with a predominance of IgG2a and IgG1 antibodies indicating Th1- and Th2-like responses, respectively. Antibody isotype profiles were determined at week 8. HBsAg recombinant-protein-based immunization gave clear Th2 responses with low IgG2a (Fig. 3). In contrast,

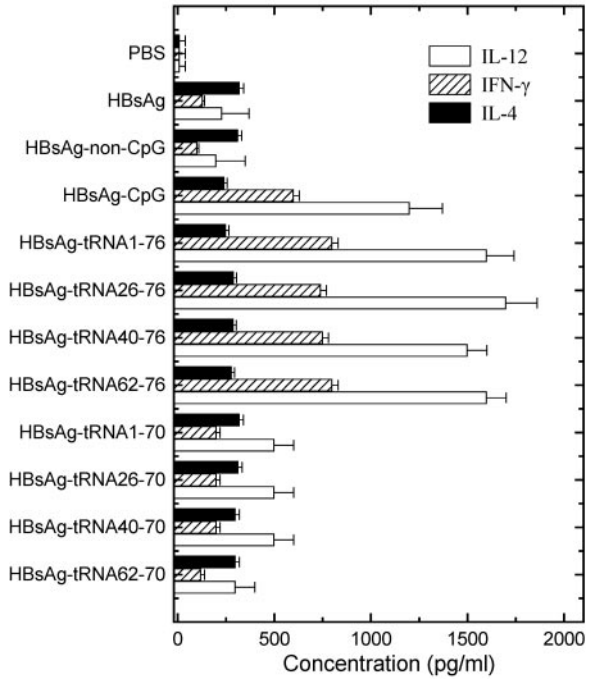

FIG. 4. Cytokine responses of T-lymphocyte-enriched splenocytes separated from BALB/c mice after immunization with HBsAg and different adjuvants. Data shown are representative of three independent experiments and are expressed as means ± SD.

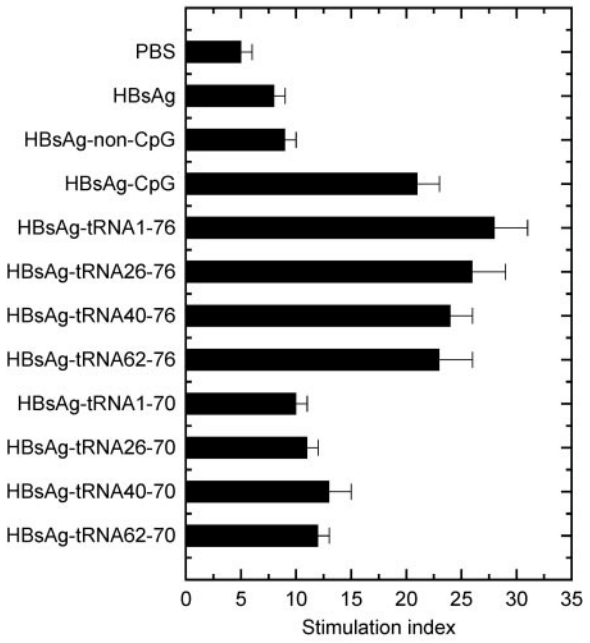

FIG. 5. Lymphoproliferative response of T-lymphocyte-enriched splenocytes separated from BALB/c mice after immunization with HBsAg and different adjuvants. Data shown are representative of three independent experiments and are expressed as means ± SD.

HBsAg-tRNA^{Ala}1-76, HBsAg-tRNA^{Ala}26-76, HBsAg-tRNA^{Ala}40-76, HBsAg-tRNA^{Ala}62-76, and HBsAg-CpG induced higher levels of IgG2a antibodies, which indicates Th1 responses ($P < 0.05$), but HBsAg-tRNA^{Ala}1-70, HBsAg-tRNA^{Ala}26-70, HBsAg-tRNA^{Ala}40-70, HBsAg-tRNA^{Ala}62-70 lacked the ability to induce Th1 response (Fig. 3).

Cytokines analysis. After spleen T lymphocytes were separated from the vaccinated BALB/c mice, spleen T lymphocytes were stimulated with HBsAg in vitro; the IL-12, IL-4, and IFN-γ cytokine levels are shown in Fig. 4. In our experiments, HBsAg-tRNA^{Ala}1-76, HBsAg-tRNA^{Ala}26-76, HBsAg-tRNA^{Ala}40-76, HBsAg-tRNA^{Ala}62-76 induced the high levels of IL-12 and IFN-γ responses relative to control group mice ($P < 0.05$) (Fig. 4), but they did not significantly induce IL-4 response (Fig. 4). The results show that HBsAg-tRNA^{Ala}1-76, HBsAg-tRNA^{Ala}26-76, HBsAg-tRNA^{Ala}40-76, HBsAg-tRNA^{Ala}62-76 can induce Th1-like cytokines IL-12 and IFN-γ. Moreover, HBsAg-tRNA^{Ala}1-70, HBsAg-tRNA^{Ala}26-70, HBsAg-tRNA^{Ala}40-70, and HBsAg-tRNA^{Ala}62-70 did not significantly induce IL-12 and IFN-γ responses. T lymphocytes from HBsAg-CpG-immunized mice secreted significantly higher levels of IL-12 and IFN-γ relative to HBsAg–non-CpG control group ($P < 0.05$) (Fig. 4). These results show that the 3′ CCACCA sequence of tRNA^{Ala} (UGC) is important motif for Th1 immune response.

T-lymphoproliferative response. After HBsAg with different adjuvants were used to immune BALB/c mice, T lymphocytes were culture in vitro, and stimulated with HBsAg. Figure 5 shows the lymphoproliferative response, HBsAg-tRNA^{Ala}1-76, HBsAg-tRNA^{Ala}26-76, HBsAg-tRNA^{Ala}40-76, and HBsAg-tRNA^{Ala}62-76 induced significantly lymphoproliferative response ($P < 0.05$). However HBsAg-tRNA^{Ala}1-70, HBsAg-tRNA^{Ala}26-70, HBsAg-tRNA^{Ala}40-70, and HBsAg-tRNA^{Ala}62-70 did not significantly induce lymphoproliferative response. Moreover HBsAg-CpG induced the lymphoproliferative response ($P < 0.05$); however, HBsAg–non-CpG ODN did not induce significantly lymphoproliferative response. Results show that tRNA^{Ala}(UGC) with the 3′-end CCACCA sequence induced greater lymphoproliferative responses than tRNA^{Ala}(UGC) without the 3′ CC ACCA motif in HBsAg immunization.

CTL response. Figure 6 shows the CTL responses with P815-S as target cells, the specific cytotoxic-T-lymphocyte responses by HBsAg-tRNA^{Ala}1-76, HBsAg-tRNA^{Ala}26-76, HBsAg-tRNA^{Ala}40-76, and HBsAg-tRNA^{Ala}62-76 were significantly higher than those of HBsAg-tRNA^{Ala}1-70, HBsAg-tRNA^{Ala}26-70, HBsAg-tRNA^{Ala}40-70, and HBsAg-tRNA^{Ala}62-70 at different effector cell-to-target cell ratios (50:1, 25:1, and 12.5:1). The specific cytotoxic-T-lymphocyte responses from HBsAg-CpG-treated mice were significantly higher than that from HBsAg–non-CpG ODN-treated mice ($P < 0.05$) (Fig. 6). Moreover, there was no significant CTL response with P815 cells as target cells (data not shown). Our results show that the 3′ CCACCA sequence of tRNA^{Ala}(UGC) has an important function to induce HBsAg-specific CTL responses in BALB/c mice.

The TLR3 recognizes different tRNA^{Ala}(UGC) fragments. HEK293 cells were transiently transfected with plasmid pUNO-hTLR3 or pUNO-hTLR7, pUNO-hTLR8, and pUNO-hTLR9; we then stimulated the HEK293 cells with different tRNA^{Ala}(UGC) fragments and measured the luciferase activity driven by a cotransfected NF-κB reporter construct, pNF-κB-luc. The results are shown in Fig. 7. tRNA^{Ala}1-76, tRNA^{Ala}26-76, tRNA^{Ala}40-76, and tRNA^{Ala}62-76 strongly activated cells transfected with human TLR3 but not human

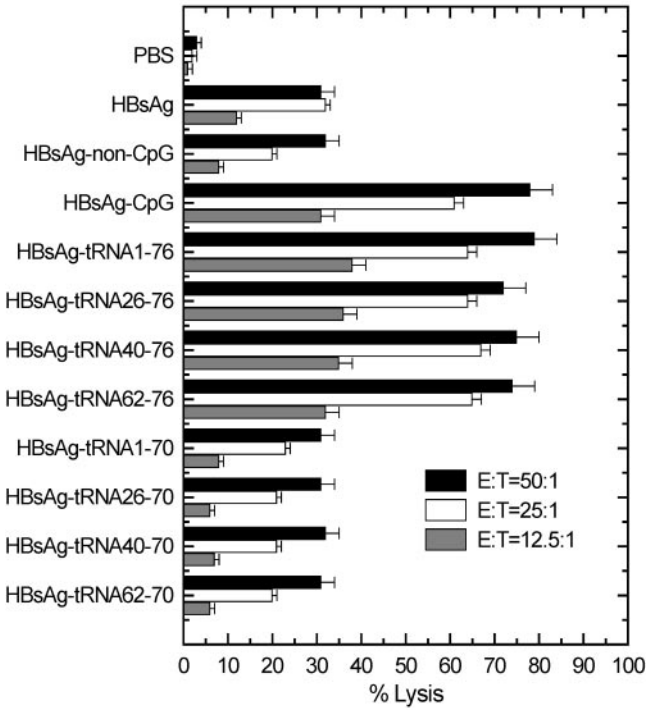

FIG. 6. The CTL response after immunization with HBsAg and different adjuvants. Lytic activities of effector cells against target cells were assessed. The effector cell-to-target cell ratios (E:T) were set at 12.5:1, 25:1, and 50:1. Data shown are representative of three independent experiments and are expressed as means ± SD.

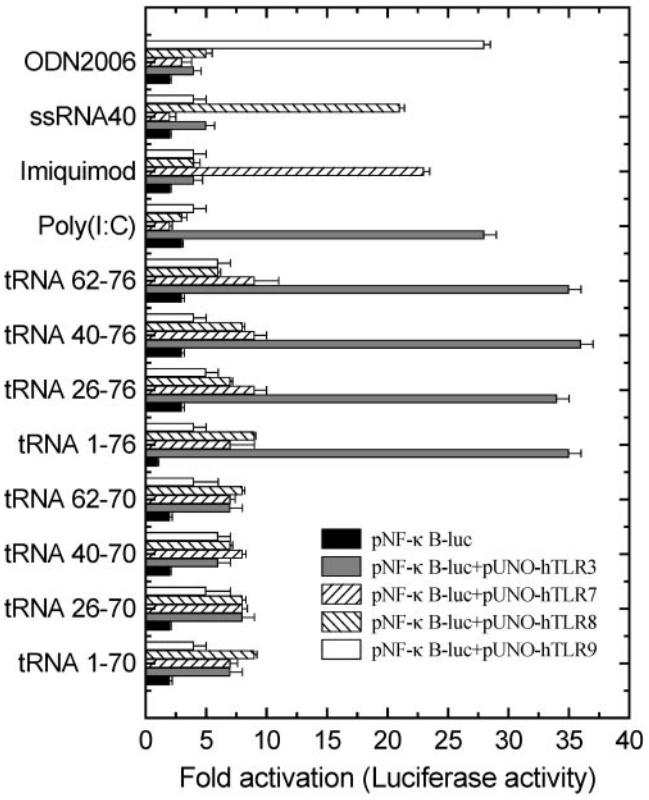

FIG. 7. In vitro NF-κB-driven luciferase reporter assays of human TLR3, TLR7, TLR8, and TLR9 activation by different fragments of $tRNA^{Ala}$(UGC). These are expressed as activation (*n*-fold) (luciferase activity upon stimulation divided by luciferase activity of the nonstimulation). Data shown are representative of three independent experiments and are expressed as means ± SD.

TLR7, TLR8, and TLR9 to express luciferase ($P < 0.05$) (Fig. 7). However $tRNA^{Ala}$1-70, $tRNA^{Ala}$26-70, $tRNA^{Ala}$40-70, and $tRNA^{Ala}$62-70 failed to activate cells transfected by TLR3, TLR7, TLR8, and TLR9 constructs from the human origin to express luciferase (Fig. 7). The lower responses of TLR3-transfected human cells upon $tRNA^{Ala}$1-70, $tRNA^{Ala}$26-70, $tRNA^{Ala}$40-70, and $tRNA^{Ala}$62-70 stimulation were due to the lack of the 3′ CCACCA motif. In the control experiments, TLR9 agonist ODN2006, TLR8 agonist ssRNA40, TLR7 agonist imiquimod, and TLR3 agonist poly(I:C) strongly stimulated HEK293 cells transfected by TLR9, TLR8, TLR7, and TLR3 to express luciferase ($P < 0.05$), respectively. From our experiments, we deduced that the 3′ CCACCA sequence of $tRNA^{Ala}$(UGC) is the necessary motif for the binding of $tRNA^{Ala}$(UGC) and TLR3. It is important that this sequence is recognized by TLR3.

DISCUSSION

In eukaryotes, in which the 3′ CCA sequence is rarely carried by the tRNA gene, the tRNA nucleotidyltransferase is essential and 3′ CCA must be posttranscriptionally added. In contrast, in bacteria, the 3′ CCA is generally carried by the tRNA gene; for example, bacteriophages (27) and *E. coli* bacteria (11) studied to date contained the 3′ CCA sequence. Mitochondrial tRNA 3′-end metabolism is related to many human diseases (23). A mutation or interference of mitochondrial tRNA 3′-end metabolism possibly induces a danger signal in humans. In our experiments, the bacterium-derived 3′ CCACCA sequence of $tRNA^{Ala}$(UGC) could be considered an interference factor of mature mammalian tRNA, and this sequence is crucial for the immune response to bacterial invasion.

The immunogenicity of sequence-dependent RNA has not been well studied for vaccine adjuvant development. Only recently, chemically modified synthetic CpG RNA has been described as an sequence-dependent RNA adjuvant (34). However, the chemical synthetic and modified RNA might not really reveal the immune response characteristic of RNA. We used the T7 RNA polymerase to transcribe different tRNA fragments. The final tRNA fragments were purified with a high-performance liquid chromatography method. In our experiments, HBsAg-$tRNA^{Ala}$ with the 3′ CCACCA sequence can induce an IgG2a response significantly higher than the IgG1 response in BALB/c mice (Fig. 3). However, HBsAg-$tRNA^{Ala}$ without the 3′ CCACCA sequence did not induce a significant IgG2a response. These results show that the 3′-end CCACCA sequence of $tRNA^{Ala}$(UGC) is an important motif for the induction of the Th1 response in BALB/c mice.

About 20 years ago, the effects of tRNAs on IFN production in the cultured Lpa cells were been reported. Zahorska et al. determined that IFN production was obtained at concentrations of 50 μg/ml poly(I:C) in Lpa cells (40). Recently, Alvarado-Vásquez et al. (3) analyzed the capacity of a fungal tRNA from *Aspergillus niger* to protect HEp-2 cells against a

viral infection. When HEp-2 cells were incubated with tRNA or poly(I:C), Alvarado-Vásquez et al. found that HEp-2 cells treated with tRNA were less susceptible to adenovirus 6 infection than those incubated with poly(I:C), and tRNA protected HEp-2 cells against adenovirus 6 infection due to IFN-β synthesis induced by tRNA. Alvarado-Vásquez et al. indicated that tRNAs are more efficient at protecting against virus infection than the synthetic dsRNA poly(I:C).

Several research groups have reported the induction of Th1-like cytokine IL-12 by RNA. Riedl et al. (30) found that RNAs from eukaryotic cells can improve the Th1 immune response and that codelivery of eukaryotic RNAs with HBcAg nucleocapsids facilitates priming of antiviral Th1 cytokines. Bacci et al. (4) reported that dendritic cells (DCs) transfected with yeast RNAs can produce IL-12 but not IL-4, and IL-12 can induce Th1-dependent antifungal resistance when delivered in mice. Koski et al. (20) showed that RNAs derived from bacterial sources, when transfected into DCs, induce high-level IL-12 secretion towards the Th1 phenotype. Cella et al. (7) showed that human DCs can be activated by dsRNAs; this activation results in increased production of IFN. In our experiments, $tRNA^{Ala}$(UGC) with the 3′ CCACCA sequence not only induce IFN-γ production in T-lymphocyte-enriched splenocytes but also induce IL-12 in the splenocytes. High levels of IFN-γ and IL-12 are important for increasing the cellular capacity to resist infection.

It has been found that RNA can improve DC, NK, or CD8 T-cell activity. Vidalain et al. reported that DCs acquire the ability to kill tumoral cells when treated with dsRNA (37). Sivori et al. (31) determined that freshly isolated NK cells acquired the ability to lyse immature DCs after stimulation with dsRNA and IL-12. In our experiments, mice treated with HBsAg-$tRNA^{Ala}$ containing the 3′ CCACCA sequence have significantly improved CTL responses.

Moreover, the recognition of tRNA by TLRs in HEK293 cells was analyzed. Unlike the CpG DNA, which has been known to be recognized by TLR9, RNAs were reported to be recognized by different receptors; for example, dsRNA can be recognized by TLR3 (2), ssRNA can be recognized by TLR7 and TLR8 (8, 14), and mRNA can be recognized by TLR3 (18). The different TLRs in charge of different RNA for signaling were believed to be due to the sequence or structure difference of RNAs used in different research groups. TLR3 was demonstrated to signal in response to poly(I:C) dsRNA (2). Phosphothioate-protected synthetic ssRNA was demonstrated to be recognized by TLR7 or TLR8 (8, 14). Imiquimod can be recognized by TLR7 (38).

tRNA differs from synthetic ssRNA homopolymer [poly(A), poly(G), poly(C), or poly(U)], in which it contains considerable secondary structure, including a double-stranded region and a single-stranded region. This led us to test whether tRNA can be recognized by TLR3, TLR7, TLR8, or TLR9. In our experiments, we found that the recognition of $tRNA^{Ala}$(UGC) by TLR3 depended on the 3′ CCACCA sequence of $tRNA^{Ala}$(UGC).

Accumulating evidence suggests that it is possible for RNA, including mRNA, tRNA, rRNA, or a small RNA species, such as small nuclear RNA derived from pathogens or damaged hosts, to stimulate the innate immune system. In the present work, we demonstrated that the 3′ CCACCA end of $tRNA^{Ala}$(UGC) is important for antigen-specific humoral and cell-mediated immune responses in BALB/c mice characterized by enhanced secretion of IFN-γ and IL-12 cytokines, increasing IgG2a isotype, and strong cytotoxic-T-lymphocyte induction. The representative effect of the 3′ CCACCA $tRNA^{Ala}$(UGC) was the enhanced induction of Th1 differentiation. Among all the adjuvants used in our experiments, very successful immunization occurred only with the formulation containing the 3′ CCACCA motif of $tRNA^{Ala}$(UGC) or the formulation containing CpG oligonucleotides. Moreover, the 3′ CCACCA sequence of tRNA can be recognized by TLR3, allowing TLR3 signaling. Although the relationship between TLR3 signaling and Th1-like immune response in TLR3 knockout mice (as the animal model) still needs further investigation, the findings of the present study suggest that it is practical to develop a effective and cheap vaccine adjuvant, because bacterial tRNAs are relatively cheaper than CpG ODN and more stable than other kind of RNA. Our earlier success with $tRNA^{Ala}$(UGC) indicates the importance of immunogenicity of tRNAs.

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