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Humoral and Cellular Immune Response to RNA Immunization with Flavivirus Replicons Derived from Tick-Borne Encephalitis Virus

Judith H. Aberle, Stephan W. Aberle, Regina M. Kofler, and Christian W. Mandl*

Institute of Virology, Medical University of Vienna, Vienna, Austria

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A new vaccination principle against flaviviruses, based on a tick-borne encephalitis virus (TBEV) self-replicating noninfectious RNA vaccine that produces subviral particles, has recently been introduced (R. M. Kofler, J. H. Aberle, S. W. Aberle, S. L. Allison, F. X. Heinz, and C. W. Mandl, Proc. Natl. Acad. Sci. USA 7:1951–1956, 2004). In this study, we evaluated the potential of the self-replicating RNA vaccine in mice in comparison to those of live, attenuated vaccines and a formalin-inactivated whole-virus vaccine (ImmunInject). For this purpose, mice were immunized using gene gun-mediated application of the RNA vaccine and tested for $CD8^+$ T-cell responses, long-term duration, neutralizing capacity, and isotype profile of specific antibodies and protection against lethal virus challenge. We demonstrate that the self-replicating RNA vaccine induced a broad-based, humoral and cellular (Th1 and $CD8^+$ T-cell response) immune response comparable to that induced by live vaccines and that it protected mice from challenge. Even a single immunization with 1 μg of the replicon induced a long-lasting antibody response, characterized by high neutralizing antibody titers, which were sustained for at least 1 year. Nevertheless, it was possible to boost this response further by a second injection with the RNA vaccine, even in the presence of a concomitant $CD8^+$ T-cell response. In this way it was possible to induce a balanced humoral and cellular immune response, similar to infection-induced immunity but without the safety hazards of infectious agents. The results also demonstrate the value of TBEV replicon RNA for inducing protective long-lasting antiviral responses.

Flaviviruses are small enveloped viruses with a single positive-sense RNA genome that include a number of important arthropod-borne human pathogens, such as West Nile virus, the dengue viruses, yellow fever virus, Japanese encephalitis virus, and tick-borne encephalitis virus (TBEV) (4). The development of new vaccines against flaviviruses represents an impressive challenge because these pathogens are considered to be emerging diseases due to their geographic spread and intensified transmission over the past few years (43).

The administration of self-replicating RNA from a noninfectious flavivirus mutant has recently been introduced as a new vaccine approach (18). This strategy is based on the induction of immunity upon expression of almost the entire viral genome from a self-replicating RNA within the vaccinated host. The RNA was derived from tick-borne encephalitis virus and carries a specifically engineered deletion that prevents assembly of infectious virus particles as well as other modifications that promote secretion of immunogenic subviral particles (18).

The flavivirus genome encodes three structural proteins (capsid protein C; protein prM, which is the precursor to the small membrane protein M; and the large envelope protein E) and several nonstructural proteins in a single open reading frame (24). By specifically introducing a large deletion of approximately two-thirds of the capsid protein, replicons can be generated which replicate in the cell but are incapable of producing infectious virus particles. The introduction of additional specifically engineered mutations in the prM signal sequence causes an efficient export of solely capsidless subviral particles (18) which are not infectious but retain structural and antigenic properties similar to those of infectious virions (7, 38) and are thus highly immunogenic (13). Such an RNA vaccine should be as safe as conventional inactivated vaccines because of its complete lack of infectivity (28). Nevertheless, it exhibits important characteristics of a live-virus vaccine, such as in vivo particle formation and release, RNA replication, and authentic nonstructural protein expression, which should induce an immune response resembling that elicited by a natural infection, as characterized by induction of a robust cellular immune response and long-term duration of immunity. In addition to the antigen-specific adaptive response, RNA vaccines offer the prospect of natural adjuvanticity and stimulation of the innate immune response by double-stranded RNA intermediates arising during the replication process (22, 23).

The feasibility of immunization with the self-replicating noninfectious RNA introduced by gene gun microcarrier bombardment was recently demonstrated in adult mice (18).

In this study, we have evaluated the vaccine potential of the self-replicating but noninfectious RNA in comparison to live-virus infection and a formalin-inactivated whole-virus vaccine (ImmunInject). Comparative analysis included the testing of $CD8^+$ T-cell responses, long-term duration, neutralizing capacity, and isotype profile of the TBEV-specific antibody response and protection against lethal virus challenge. We demonstrate that immunization with the noninfectious self-replicating RNA vaccine induces a protective humoral and cellular ($CD8^+$ and Th1 cell responses) immune response in mice. The antibody response to the RNA vaccine did not drop off with time and was characterized by high neutralizing (NT) antibody titers, which were sustained for at least 1 year. Nev-

* Corresponding author. Mailing address: Institute of Virology, Kinderspitalgasse 15, A-1095 Vienna, Austria. Phone: 43-1-404-90, ext. 79502. Fax: 43-1-404-90-9795. E-mail: christian.mandl@meduniwien.ac.at.

ertheless, it was possible to further boost this response by a second injection with the RNA vaccine, even in the presence of a concomitant $CD8^+$ T-cell response. In this way it was possible to induce a balanced, long-lasting and protective immune response, similar to infection-induced immunity.

MATERIALS AND METHODS

Mutant viruses and replicons. All mutants were derived from the wild-type infectious cDNA clone of TBEV strain Neudoerfl (25). Two previously described attenuated mutants were used as live vaccine strains. (i) Mutant C(Δ28–43) carries an in-frame deletion within the region coding for capsid protein C, removing 16 amino acid residues (no. 28 to 43) of this protein (17). (ii) Mutant 3′Δ10847 carries a deletion within the 3′-noncoding region that removes nucleotides 10378 to 10847 of the TBEV genome (26).

The replicon mutants C(Δ28–89) and C(Δ28–89)-S both carry an in-frame deletion removing 62 amino acid residues (no. 28 to 89) of protein C. In addition, replicon C(Δ28–89)-S has specific point mutations within the carboxy-terminal anchor region of protein C (the signal sequence for protein prM) which promote the secretion of subviral particles (18).

Immunization and virus challenge. Pathogen-free female BALB/c mice aged 6 to 8 weeks were purchased from Charles River Laboratories (Sulzfeld, Germany) and were maintained under pathogen-free conditions.

Groups of 12 BALB/c mice were immunized as follows. (i) One immunization was performed with self-replicating viral RNA C(Δ28–89)-S using the GeneGun, delivering ≈1 μg of RNA per mouse: RNA was synthesized in vitro from the corresponding cDNA clones by T7-mediated transcription, as described previously (25). Afterward, the DNA template was removed enzymatically, and RNA was coated onto gold microcarrier particles essentially as described previously (27). (ii) One immunization was performed (10^4 PFU subcutaneously) with live, attenuated viruses [3′Δ10847 and C(Δ28–43)]. (iii) The third immunization was with commercial TBE vaccine, containing 1 μg formalin-inactivated virus (FSME ImmunInject; Baxter-Immuno, Vienna, Austria). For booster immunizations with the mutant C(Δ28–89)-S and ImmunInject, this procedure was repeated 4 weeks later.

Eight weeks after the initial (or 4 weeks after booster) immunization, all mice were bled and tested for the presence of TBEV-specific antibody. For further analysis, from each group of 12 mice, 4 were monitored for maintenance of antibody responses for 52 weeks, 4 were sacrificed and their spleens were isolated for testing $CD8^+$-T-cell responses, and 4 were challenged by intraperitoneal inoculation with 1,000 50% lethal doses of the highly mouse-pathogenic TBEV strain Hypr (46), and after a 4-week observation period, sera were collected from mice that had survived challenge.

$CD8^+$ T-cell assay. For the analysis of $CD8^+$ T-cell responses, splenocytes from immunized mice (responder cells) were isolated and cultured with p815 cells (ATCC TIB-64), a murine major histocompatibility complex (MHC) class I^+ mastocytoma cell line, which lacks MHC class II expression (51), that were either used as uninfected controls or infected with TBEV (1,000 50% tissue culture infective doses) for 48 h (stimulator cells), resulting in approximately 90% TBEV-infected cells, as judged by immunofluorescence. Approximately 5×10^5 stimulator cells and 5×10^6 responder cells were plated together with costimulatory antibodies CD28/49d (0.5 and 1 mg/ml, respectively) in 24-well, flat-bottom plates (Nunclon, Nuns, Denmark) in RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 nM glutamine, and 50 μM 2-mercaptoethanol in a humified chamber at 37°C. Cells were stained 6 h later for phenotypic and intracytoplasmic gamma interferon (IFN-γ) analysis. To enhance intracellular accumulation of IFN-γ, 5 μg/ml brefeldin A was added for the final 4 h of culture. Cells were incubated at room temperature with 20 mM EDTA for 15 min, vortexed, and subsequently incubated with fluorescence-activated cell sorting (FACS) lysing solution for 10 min. Cells were washed with FACS buffer and resuspended in 100 μl FACS buffer. Cell suspensions were added to 500 μl of FACS Permeabilizing Solution 2 and incubated for 10 min at room temperature. Fixed and permeabilized cells were stained for 30 min with R-phycoerythrin-conjugated rat anti-mouse IFN-γ (clone XMG1.2), Cy-chrome-conjugated rat anti-mouse CD8a (Ly-2) (clone 53–6.7), fluorescein isothiocyanate-conjugated rat anti-mouse CD3 (clone 17A2), and the corresponding immunoglobulin G1 (IgG1) isotype control (clone R3-34) obtained from BD Pharmingen, following the manufacturer's instructions. After two washes with FACS buffer, cells were resuspended in FACS buffer and analyzed by flow cytometry. All buffers were purchased from BD. Cells were analyzed on a FACSCalibur, and at least 15,000 events were acquired for each preparation. All samples were tested in duplicate. Results were given as percentage of $CD8^+$ T lymphocytes expressing IFN-γ upon restimulation with TBEV-infected cells, calculated by using the formula $(a - b) \times 100$, where a = percentage $CD8^+$ IFN-γ^+ cells in the presence of TBEV-infected p815 cells, and b = percentage $CD8^+$ IFN-γ^+ cells in the presence of mock-infected p815 cells. The statistical significance of differences in $CD8^+$ T-cell frequencies among groups was analyzed by analysis of variance (ANOVA); post hoc tests were computed to assess differences between pairs of groups of immunized or control mice.

ELISA for virus-specific antibody and IgG isotype determination. Eight weeks after the first immunization, serum samples from individual mice were tested for the presence of TBEV-specific antibody by enzyme-linked immunosorbent assay (ELISA) using purified live TBEV as a coating antigen and a goat anti-mouse IgG-horseradish peroxidase conjugate (Nordic, Lausanne, Switzerland) for the detection of bound antibody, as described previously (11). The longevity of the antibody responses was tested in pools of equal serum aliquots from four mice of each group at weeks 12, 24, and 52 after the first immunization. All samples were tested in duplicate. Student's t test (using log-transformed data) was used to determine differences between groups with or without booster immunizations.

The IgG isotype distribution was determined by ELISA 8 weeks after the initial immunization using pooled sera from 12 mice and 1 μg/ml purified TBEV as antigen and a Mouse Typer SubIsotyping kit (Bio-Rad) for detection, according to the manufacturer's instructions.

Determination of neutralizing antibody. NT antibodies in immunized mice were determined using a modified protocol of the previously described TBEV neutralization assay (14). Briefly, aliquots of twofold dilution series of serum (starting with a 1:10 dilution) were mixed with TBEV to give a final virus concentration of 5 PFU and incubated for 1 h at 37°C. A baby hamster kidney 21 (BHK-21) cell suspension (1×10^5 cells in a volume of 100 μl) was then added, and the cells were incubated at 37°C for 4 days. On day 4 postinfection, 50-μl aliquots from the supernatants were tested at a 1:3 dilution for the presence or absence of viral antigen in a four-layer ELISA as described previously (12). The NT antibody titers were expressed as the reciprocal of the serum dilution that was able to suppress virus infection to such an extent that detection of viral antigen in the supernatant by ELISA remained below the cutoff value (optical density at 450 nm, >0.2). The test was repeated twice, and the results were averaged. In each test, titrations of virus controls in the absence of antibodies and one negative and three positive serum controls were included.

RESULTS

$CD8^+$ T-cell responses. A $CD8^+$ T-cell response is induced most efficiently in live virus infection, in which processed peptides derived from endogenously synthesized proteins are presented to the immune system in association with MHC class I proteins. To investigate $CD8^+$ T-cell responses induced by the self-replicating noninfectious RNA, mice were either immunized with 1 μg of the replicating RNA mutant C(Δ28–89)-S using a gene gun or injected with live, attenuated TBEVs (3′Δ10847) and C(Δ28–43) or a commercial TBEV vaccine, consisting of formalin-inactivated whole virus adsorbed onto $Al(OH)_3$ as an adjuvant (ImmunInject). One group of mice immunized either with the replicon C(Δ28–89)-S or with ImmunInject was given a second injection 4 weeks after initial immunization. $CD8^+$ T cells were visualized by IFN-γ staining. Intracellular IFN-γ staining was done after culturing the spleen cells from immunized animals with *H-2*^{d}-compatible, TBEV-infected MHC class I^+ BALB/c-p815 cells or mock-infected control cells. The results are shown in Fig. 1. Every mouse that had been inoculated with the self-replicating RNA vaccine had a significant $CD8^+$ T-cell response after a single immunization (mean ± standard error, 0.4% ± 0.1%), with frequencies comparable to those observed when mice were inoculated with live viruses: 0.5% ± 0.1% for 3′Δ10847 and 0.5% ± 0.2% for C(Δ28–43). A second immunization with the RNA, given to mice 4 weeks after the initial injection, resulted in a significant increase in $CD8^+$ T-cell frequencies (1.7% ±

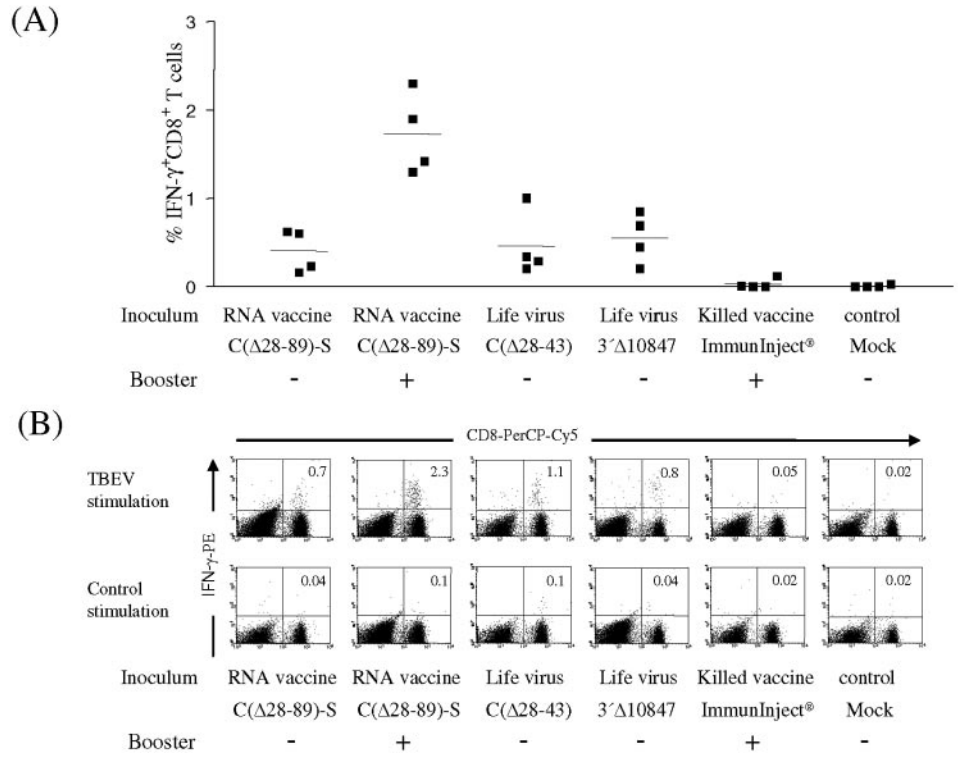

FIG. 1. IFN-γ expression-based detection of $CD8^+$ T-cell responses. Groups of four BALB/c mice were inoculated with the replicon mutant C(Δ28–89)-S or live, attenuated viruses [3′Δ10847 and C(Δ28–43)] or with a formalin-inactivated TBEV vaccine (ImmunInject). One group of mice immunized with the replicon C(Δ28–89)-S or with ImmunInject was given a second injection 4 weeks after initial immunization. $CD8^+$ T cells specifically recognizing TBEV-infected cells were visualized by IFN-γ staining. Intracellular IFN-γ staining was done after culturing the spleen cells from immunized mice with MHC class I^+ BALB/c-p815 cells or mock-infected control cells. (A) Percentages of $CD8^+$ T cells specifically recognizing TBEV-infected cells in individual mice. The squares represent the mean of duplicate samples obtained from individual mice; lines represent overall mean of all samples. (B) Representative set of flow cytometry data. Dot plots were generated by gating on $CD3^+$ T lymphocytes. Percentages of IFN-γ^+ cells within $CD3^+$ $CD8^+$ cells are indicated in the upper right quadrant.

0.2%) ($P < 0.0001$, ANOVA; $P < 0.01$, post hoc test). No $CD8^+$ T-cell responses were observed in mice immunized with the inactivated whole-virus vaccine or in mock-infected control mice. These results indicate that the RNA vaccine, like live-virus infection, can efficiently induce $CD8^+$ T cells, whereas the formalin-inactivated whole-virus vaccine failed to induce a detectable $CD8^+$ T-cell response.

Seroconversion and longevity of the antibody response. In order to assess the feasibility of using RNA immunization to elicit antibodies in mice, specific ELISA titers were determined in groups of 12 mice 8 weeks after the initial immunization and compared to those induced after immunization with live, attenuated viruses and with the inactivated virus vaccine. A single immunization with 1 μg of the RNA vaccine was sufficient to induce 100% (12/12) seroconversion. As can be seen in Fig. 2, the titers obtained for individual RNA-immunized mice showed minimal variation, indicating that this method is highly reproducible and reliable. One hundred percent seroconversion was also achieved by immunization with either live, attenuated viruses or the inactivated whole-virus vaccine, but with higher interindividual variation in antibody titers. A single immunization with 1 μg RNA elicited specific IgG titers comparable with those induced with live attenuated viruses. A second immunization with the self-replicating RNA vaccine, given to mice 4 weeks after the initial injection, resulted in a significant increase in TBEV-specific IgG antibody ($P < 0.0001$, Student's t test), with titers approximately twofold lower than those elicited with the inactivated-virus vaccine (Fig. 2).

To determine whether the self-replicating noninfectious RNA vaccine could induce long-lasting immunity, antibody

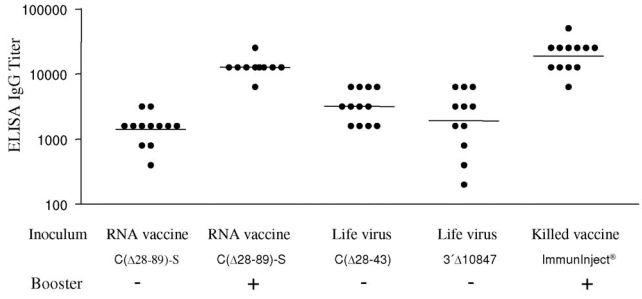

FIG. 2. Geometric mean (lines) and individual values (black dots) of TBEV-specific IgG antibody titers 8 weeks after initial immunization with replicon RNA C(Δ28–89)-S, live, attenuated viruses [3′Δ10847 and C(Δ28–43)], or the formalin-inactivated TBEV vaccine (ImmunInject), as determined by ELISA; one group of mice immunized with the replicon C(Δ28–89)-S or with ImmunInject was given a second injection 4 weeks after the initial immunization.

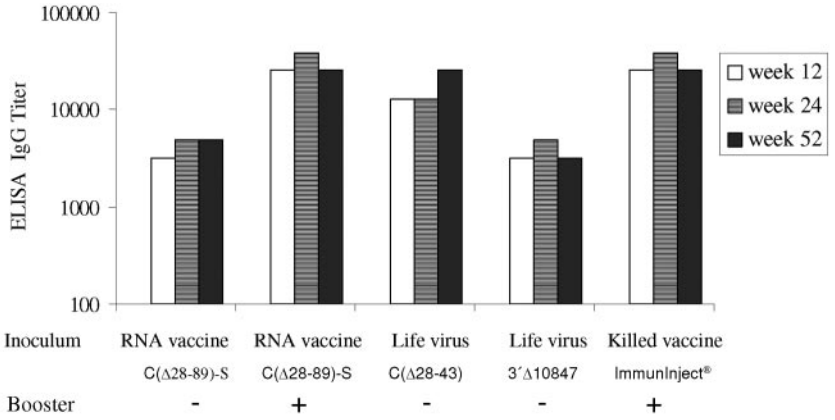

FIG. 3. Long-term IgG antibody response at weeks 12, 24, and 52 after initial immunization with the mutant C(Δ28–89)-S, live, attenuated viruses [3′Δ10847 and C(Δ28–43)], or the formalin-inactivated TBEV vaccine (ImmunInject), as determined by ELISA; bars represent the mean of duplicate assays using pooled sera ($n = 4$). One group of mice immunized with the replicon C(Δ28–89)-S or with ImmunInject was given a second injection 4 weeks after the initial immunization.

titers in pooled sera from four mice of each group were analyzed sequentially for 52 weeks. Figure 3 shows that the antibody titers at 52 weeks were not significantly different from those seen at 8 weeks after initial immunization, indicating the induction of long-lasting antibody responses. A similar long-term maintenance of specific antibodies was observed using live, attenuated viruses and inactivated-whole-virus vaccine as well.

Neutralizing antibody response and protection. Neutralization tests revealed that even a single immunization with 1 μg of the self-replicating noninfectious RNA induced neutralizing antibodies whose titers increased further by a second immunization with the RNA vaccine. Neutralizing antibodies were maintained for at least 52 weeks (Table 1).

To test if the induced immune response correlated with protection, groups of four mice were challenged by injecting them intraperitoneally with 1,000 times the 50% lethal dose of the highly mouse-pathogenic TBEV strain Hypr. We have previously shown that 100% protection was achieved after booster immunization with the self-replicating RNA vaccine (18). Here, we show that even a single immunization of 1 μg of the RNA vaccine was sufficient to confer 100% protection. Complete protection was also achieved in mice immunized with live, attenuated viruses or with the formalin-inactivated whole-virus vaccine, whereas the challenge was lethal for the mock-infected controls (Table 1). The data presented in this section indicate that the self-replicating RNA vaccine elicits long-lasting neutralizing antibody responses in mice and confers protection similar to attenuated-live-virus infection.

TABLE 1. Neutralizing antibody response and protection against virus challenge

Inoculum	Booster[a]	NT antibody titer at wk:		Protection (no. surviving/ no. tested)	Fold increase of IgG titer in surviving mice after challenge
		8	52		
RNA vaccine					
C(Δ28–89)-S	−	10	15	4/4	32
C(Δ28–89)-S	+	80	30	4/4	1.5
Live virus					
C(Δ28–43)	−	60	20	4/4	3
3′Δ10847	−	15	10	4/4	2
Killed vaccine					
ImmunInject	+	20	20	4/4	0
Control					
Mock	−	<10	<10	0/4	ND[b]

[a] +, mice were given a second injection 4 weeks after the first immunization; −, no booster immunization.
[b] ND, not determined because no survivors after challenge.

To assess whether the observed immune response against challenge could inhibit replication of the challenge virus, postchallenge sera were tested for increases in antibody titer. As is shown in Table 1, postchallenge antibody titers from mice that had received a booster immunization with the replicon or with the inactivated whole-virus vaccine were not significantly different from the corresponding prechallenge titers. In contrast, postchallenge sera from mice that had received only a single immunization with the RNA vaccine exhibited a significant increase in the antibody titers. This indicates that the immunity, though protective, allowed replication of the challenge virus to occur at least to a certain extent.

Th1/Th2 response. The isotype distribution of the serum antibody depends upon the cytokine profile secreted by different Th cell subsets. In mice, IgG2a is associated with the Th1 subset (IFN-γ), whereas IgG1 is associated with the Th2 subset (49), and this correlation of IgG isotypes and Th cytokine pattern has previously been confirmed for TBEV-specific Th cells (1). IgG2a antibody has been shown to be the predominant isotype raised after experimental natural infection with other flaviviruses (35, 42, 44), while the IgG1 response predominated after immunization with the inactivated virus vaccine (1, 35). To investigate the T-helper cell response induced by self-replicating RNA vaccination, the IgG1/IgG2a isotype profile of the TBEV-specific antibody response was determined. As can be seen in Table 2, immunization with the self-replicating RNA vaccine induced a Th1-dominated response, as indicated by higher titers of IgG2a and low or undetectable IgG1. A Th1 response was also induced in mice

TABLE 2. Isotypes of TBEV-specific antibodies

Inoculum	Booster[a]	TBEV-specific antibody titer			Bias of T-helper response[b]
		IgG1	IgG2a	IgG1/IgG2a	
RNA vaccine					
C(Δ28–89)-S	−	<100	800	1:>8	Th1
C(Δ28–89)-S	+	800	6,400	1:8	Th1
C(Δ28–89)	−	400	1,600	1:4	Th1
Live virus					
C(Δ28–43)	−	100	3,200	1:32	Th1
3′Δ10847	−	<100	3,200	1:>32	Th1
Killed vaccine					
ImmunInject	+	12,800	800	16:1	Th2

[a] +, mice were given a second injection 4 weeks after the first immunization; −, no booster immunization.
[b] The type of Th response was assigned based on the IgG subclasses of TBEV-specific antibodies.

inoculated with the live, attenuated viruses [3′Δ10847 and C(Δ28–43)] (Table 2). In contrast, the antibody response to the inactivated-whole-virus vaccine showed IgG1 subclass dominance, consistent with a Th2-dominated response.

These findings are in agreement with our previous study using DNA-based immunogens that had shown that the presentation of protein E in particulate form is essential for induction of a Th1 immune response, even when a gene gun was used (1), which typically leads to a Th2-dominated response (48). To investigate whether particle generation would also influence the nature of the Th response to self-replicating RNA vaccination, the IgG isotype distribution induced by the self-replicating RNA construct C(Δ28–89)-S, which has been shown to export subviral particles, was compared to that of another RNA construct, C(Δ28–89), which fails to efficiently secrete subviral particles (18). Generally, higher IgG titers were induced in mice immunized with the mutant C(Δ28–89)-S, but Th1 dominance was elicited with both RNA constructs (Table 2). These results indicate that self-replicating RNA vaccination can induce a strong Th1 response and is thus able to overcome the Th2 bias imposed by the gene gun, even without the formation of subviral particles.

DISCUSSION

The delivery of noninfectious self-replicating RNA by gene gun bombardment as described here is very efficient and represents a promising new type of vaccine against flaviviruses. Our study was performed with TBEV, but this approach will probably be applicable to other flaviviruses as well, such as the dengue viruses or West Nile virus, for which no vaccines are currently available (34).

The safety profile of this replicon RNA vaccine should be similar to those of inactivated or subunit vaccines, because it is not infectious and thus cannot spread from the inoculation site. Nevertheless this kind of vaccine exhibits important properties characteristic of live vaccines such as in vivo production of particulate antigen in native form (subviral particles), RNA replication, and authentic nonstructural protein expression. Here we demonstrate that the replicon RNA indeed effectively induced the full complement of immune responses, including both humoral and cellular responses ($CD8^+$ and Th1 cell responses) similar in magnitude to those achieved by live, attenuated viruses. Since $CD8^+$ T cells are induced most efficiently when the processed peptides are derived from endogenously synthesized viral proteins and are thus presented to the immune system in association with MHC class I proteins, our findings indicate that, following RNA vaccination, viral antigens are presented efficiently in the context of class I MHC antigens and are able to induce robust $CD8^+$ T-cell responses in mice. The precise role of the T-cell immunity in flavivirus infection is not yet clearly defined, but previous studies suggest that in many primary virus infections it is essential for limiting virus growth by eliminating virus-infected cells or terminating virus replication by the production of antiviral cytokines (10, 30). Self-replicating RNA constructs for heterologous gene expression have previously been shown to successfully generate humoral and $CD8^+$ T-cell responses to the encoded immunogens when delivered intramuscularly or intradermally into adult mice (2, 45, 47, 52). In addition, naked RNA immunization has been used to demonstrate the potential of replicon-based vectors to induce protective antiviral and antitumor immunity in animal models (2, 9, 21, 23, 45, 50).

Immunization with the self-replicating RNA strongly induced a $CD8^+$ T-cell response. In the case of intracellular infections, $CD8^+$ cytotoxic T-lymphocyte (CTL) responses are related to Th1 cytokine predominance (IFN-γ and IgG2a). However, T-cell responses have been more difficult to categorize in the context of gene gun-mediated DNA vaccination because it was shown that immunized mice or rhesus monkeys developed significant IFN-γ and CTL activity, but nevertheless presented mostly IgG1 antibodies (Th2-like) (29, 33). Interestingly, our data show that self-replicating RNA vaccination induced a Th1-dominated immune response (IgG2a), similar to live virus infection, and was thus able to overcome the Th2 bias imposed by the gene gun (48). This characteristic feature of the RNA vaccine was demonstrated in the BALB/c mouse model, while different mouse strains, especially outbred mice, may produce heterologous Th responses (16, 35, 41). The immune responses induced by the RNA vaccine may even be more variable in genetically diverse populations of nonhuman primates, but this issue clearly requires further testing.

The type of the Th response is also influenced by the form of the expressed antigen. In a previous DNA immunization study, we showed that a DNA construct that gives rise to secreted subviral particles can induce a balanced Th1/Th2 response, even when the construct is applied by the gene gun (1). Assembly of subviral particles was not required for the generation of a Th1 response upon RNA vaccination, as indicated by the observation that a strong Th1 response was induced even with an RNA construct that does not lead to efficient subviral particle secretion. This indicates that self-replicating RNA vaccination may employ different mechanisms for immune activation than DNA vaccines (36). A basic difference between self-replicating RNA and DNA vaccines is the virus-like RNA replication occurring inside transfected host cells, which can trigger a series of "danger signals" and results in the formation of double-stranded RNA intermediates (22). Double-stranded RNAs are potent inducers of interferons and function as strong adjuvant for cellular and humoral immune responses by the induction of apoptosis in transfected host cells (22, 36).

Although it is clear that T cells do play an important role in the resolution of viral infections, the major mediators of protection against flaviviruses are neutralizing antibodies, which are mainly directed against the envelope protein E (19, 34). The ability to induce significant levels of neutralizing antibodies is therefore an important characteristic of the RNA vaccine. Our previous studies provided strong evidence that the presentation of protein E in particulate form is essential for inducing neutralizing antibodies (1, 13, 18). The self-replicating noninfectious RNA mutant used in the present study has specifically engineered mutations in the prM signal sequence that cause an increased export of highly immunogenic subviral particles containing the viral surface antigens. Therefore, the mechanism of this RNA vaccine to induce high levels of neutralizing antibody in mice seems to be similar to that of subviral particle vaccines (1, 13). Gene gun inoculation of plasmid DNA expressing the prM and E genes of TBEV has also been shown to induce high levels of neutralizing antibodies in rhesus macaques (40). A side-by-side comparison of our previously studied TBEV prM/E-expressing DNA vaccine (1) and the self-replicating RNA vaccine indicated that 1 μg of RNA or DNA injected into BALB/c mice by gene gun resulted in identical TBEV-specific IgG titers (unpublished observation). We would therefore expect it to be possible to generate adequate immune responses with the RNA vaccine in primates as well.

Although neutralizing antibody appears to be the major protective component in mice, protection against flavivirus infection in the absence of detectable neutralizing antibody has been reported (3, 5), and the involvement of antibodies and CTL populations directed against nonstructural proteins is well documented (8, 15, 19, 20, 31, 39). In agreement with these findings, we have previously shown that protection can be achieved by RNA immunization using constructs that do not induce detectable neutralizing antibody (18). Ongoing investigations indicate that immunization with a replicon lacking almost all of the structural protein genes can provide partial protection (R. Gehrke and C. W. Mandl, unpublished observation). This suggests that both cellular and humoral immune responses against a variety of proteins may be induced by this kind of vaccine, and several of these may contribute to protection. Therefore, an optimal flavivirus vaccine might require presentation of both structural and homologous nonstructural proteins.

Despite the sustained high levels of antibody and $CD8^+$ T cells following self-replicating RNA vaccination, the humoral and $CD8^+$ T-cell response could be boosted by a second injection of the RNA vaccine. This is consistent with the idea that, upon a subsequent injection, newly transfected cells can express sufficient antigen to boost the immune response before being destroyed by $CD8^+$ T cells (6). In addition, the efficacy of RNA vaccination should not be affected by a specific immune response against the RNA itself, making multiple immunizations possible. This is particularly important because the efficacy of vaccines can be compromised by preexisting immunity to the vaccine vehicle (37). The ability to transfect cells without interference by antibodies may be important for immunization of infants still carrying neutralizing antibodies from the mother. Whereas live vaccines, such as the measles virus vaccine, are readily neutralized by maternal antibodies (32), RNA immunization should not be affected in this manner.

Finally, the self-replicating RNA vaccine induced a memory response. A long-lasting antibody response was generated even after a single immunization with 1 μg of the self-replicating RNA and was maintained at peak levels for at least 1 year. More importantly, the neutralizing antibody response was sustained for at least 1 year, which is quite remarkable, considering the short intracellular half life of RNA, its degradation by ubiquitous RNases, and the rapid death of transfected host cells. The exact mechanism accounting for long-lasting immunity is not clear, but it is a characteristic of immunity induced by virus infection and has been successfully exploited by some of the most efficient antiviral vaccines available.

In conclusion, self-replicating noninfectious RNA immunization produced long-term humoral immunity and cellular immunity quantitatively and qualitatively similar to those of live attenuated viruses but without the safety hazards of infectious agents. These findings indicate the potential for developing new safe and powerful vaccines.

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