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A DNA Aptamer Prevents Influenza Infection by Blocking the Receptor Binding Region of the Viral Hemagglutinin*

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Influenza A virus infection is a major source of morbidity and mortality worldwide. Current means of control for influenza are based on prophylaxis by vaccines and on treatment by the available specific influenza neuraminidase inhibitor drugs. The approach taken in the present study is to prevent and/or ameliorate influenza infection by site-specific blocking of the viral binding to host cell receptors. We describe a novel oligonucleotide, known also as an aptamer, which has been designed to complement the receptor-binding region of the influenza hemagglutinin molecule. It was constructed by screening a DNA library and processing by the selective evolution of ligands by exponential enrichment (SELEX) procedure. We show that this DNA aptamer is indeed capable of inhibiting the hemagglutinin capacity of the virus, as well as in the prevention of viral infectivity *in vitro*, in tissue culture. Furthermore, it inhibits viral infection by different influenza strains in an animal model, as manifested by 90–99% reduction of virus burden in the lungs of treated mice. The mode of action of this aptamer is by blocking the binding of influenza virus to target cell receptors and consequently prevention of the virus invasion into the host cells.

Influenza infections remain an important cause of morbidity and mortality, particularly in the elderly population, and carry the risk of pandemics; they also impose a considerable economic burden worldwide. Present means of prevention and therapy are not entirely satisfactory, as the current vaccines do not provide a complete solution because of their limited efficacy and the frequent genetic variations of the virus, and anti-viral drugs are only marginally effective (1–3). The more recently developed specific anti-influenza drugs consisting of neuraminidase inhibitors, comprising analogues of *N*-acetylneuraminic acid (*e.g.* oseltamivir and zanamivir), provide a limited beneficial effect, reducing the duration of infection by 1 day, from 7 to 6 days (2, 4).

The influenza virus envelope carries two major immunogenic surface glycoproteins: hemagglutinin (HA)[1] and neuraminidase. The HA plays a key role in initiating viral infection by binding to sialic acid-containing receptors on host cells and thus mediating the subsequent viral entry and membrane fusion (5–7). It exists in the viral membrane as homotrimeric spikes, each monomer containing a globular domain with a conserved sialic acid-binding pocket surrounded by antigenically variable antibody-binding sites (8). The receptor-binding site within the HA is probably not exposed to the immune system because of the conformational restriction of the trimeric form of HA in native conditions. Nonetheless, as we recently demonstrated (9), the recombinant globular region HA-(91–261) is capable of inducing both humoral and cellular immune responses against the intact virus, leading to a partial protection of the immunized mice against infection. This region includes one of the major conserved antigenic epitopes (HA-(91–108)) of all H3 strains (10, 11), as well as the region of HA1 (HA-(116–261)) encompassing the receptor-binding pocket (8). Because infection by the influenza virus is initiated by the binding of the virus to host cell receptors, the approach taken in the present study was to try and prevent infection by the blocking of this binding. Using the above mentioned conserved region of the HA molecule as a template, we designed a novel molecule aptamer, which would bind directly to the virus HA receptor-binding region and eventually prevent its interaction with the host cells.

An aptamer is a DNA or RNA oligonucleotide that has been selected *in vitro* for specific binding to a target molecule. The process through which these molecules are isolated is called selective evolution of ligands by exponential enrichment (SELEX) (12). It starts with a pool of DNA oligonucleotides containing a region of randomized nucleotides (usually 30–100 nucleotides) flanked by conserved sequences that contain primer-binding sites for use in the PCR (polymerase chain reaction). An iterative process involving the binding of the DNA oligonucleotides to a target molecule, isolation of the tightest binders, followed by their PCR amplification is used to isolate those molecules that conform best to the selection criteria (13). In the past few years, aptamers have been evolved to bind proteins that are associated with a number of disease states (14–16), thus yielding powerful antagonists of such proteins. In the present study, using this SELEX procedure, an aptamer was selected for its binding to the globular region of influenza HA, HA-(91–261). We demonstrated that this aptamer was capable of blocking the binding of the virus to host cells and consequently preventing viral infection both *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

SELEX—Preparation and selection of the aptamer were performed by a modification of the method used by Morris *et al.* (15). The aptamer library containing a central, randomized sequence of 30 nucleotides flanked by common primers 5′-AAT TAACCCTCACTAAAGGG-$(N)_{30}$-TATGGTCGAATAAGTTAA-3′ was synthesized in a 380B DNA synthe-

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[1] The abbreviations used are: HA, hemagglutinin; SELEX, selective evolution of ligands by exponential enrichment; ssDNA, single-stranded DNA; Ni-NTA, nickel-nitrilotriacetic acid; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; HAU, hemagglutination units; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole.

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sizer (Applied Biosystems). The ssDNA aptamer library was denatured at 80 °C for 10 min and then cooled on ice for 10 min. For the selection process, 30 nmol of this product were mixed with 25 μg of the recombinant His-tagged HA-(91–261) peptide (9) in 500 μl of selection buffer (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$, 100 μg tRNA, 0.2% bovine serum albumin) at 37 °C for 30 min. The bound aptamer·HA-(91–261) complex was purified by adding 25 μl of Ni-NTA superflow (Qiagen). After washing three times with 1 ml of selection buffer, the complex was resuspended in 40 μl of 10 mM Tris buffer and amplified by PCR using the above 5′ primer (denoted T3 primer) 5′-AATTAACCCTCACTAAAGGG-3′ and the 3′ primer 5′-TTAACTTAT-TCGACCATA-3′ under the conditions of 70 pmol/primer, 2 μl of 10 mM deoxy-NTP, in a final volume 50 μl at 46 °C, for 30 cycles. After three repeats of this SELEX procedure, amplified nucleotides were cloned by inserting the PCR product into pGEM-T vector and transformed into *Escherichia coli*.

Oligonucleotides—The three oligonucleotides used in this study, synthesized in the 380B DNA synthesizer (Applied Biosystems) had the following sequences: A22, 5′AATTAACCCTCACTAAAGGGCTGAGTC-TCAAAACCGCAATACACTGGTTGTATGGTCGAATAAGTTAA-3′; A21, 5′-AATTAACCCTCACTAAAGGGCGCTTATTTGTTCAGGTTGG-GTCTTCCTATTATGGTCGAATAAGTTAA-3′; and NP-(147–158), 5′-ACTTATCAGCGGACCCGTGCCTTTAGTTCGTACTGGTGAT-3′.

Mice—BALB/c mice at the age of 10–12 weeks old were purchased from Harlan Laboratories (Rehovot, Israel). In each experiment 5–10 mice were employed/group, and 2–3 repetitive experiments were conducted.

Viruses—Influenza strains A/Port Chalmers/1/73 (H3N2), A/Texas/1/77 (H3N2), A/PR/8/34 (H1N1), and A/Japan/57 (H2N2) were grown in the allantoic cavity of 11-day-old embryonated hen eggs (Bar On Hatchery, Hod Hasharon, Israel). Virus growth and purification were performed according to standard methods as described by Barret and Inglis (30). The titer of virus used for infection was evaluated by the infection of Madin-Darby canine kidney (MDCK) cells (31), and hence, virus titer was expressed as the tissue culture infective doses leading to 50% infected cells (TCID50).

Cells—MDCK cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with heat-inactivated 10% fetal calf serum (Biological Industries). Chicken blood for red blood cell preparation was obtained from the Faculty of Agriculture, Hebrew University (Rehovot, Israel).

Hemagglutination Assay—Chicken red blood cells were suspended in Alsever solution, and the concentration was brought to 0.5% in PBS. Assay was performed in micro-titer plates with 50 μl of sample containing the influenza virus and 50 μl of 0.5% chicken red blood cells. Wells were inspected for agglutination, and the results were evaluated as hemagglutination units (HAU). For hemagglutination inhibition assay, a serial dilution of either the aptamer or lung homogenate extracts were added to the virus prior to the addition of chicken red blood cells.

Immunization of Mice with HA-(91–261) Peptide—The recombinant fragment HA-(91–261) was prepared as described previously (9). Briefly, cDNA encoding the globular region of HA protein (91–261 amino acid residues) was expressed in *E. coli* and purified in Ni-NTA column (Qiagen, Beer-Sheva, Israel) using their N-terminal histidine tag. For the intranasal immunization, each mouse was administered with a droplet of 50 μl of recombinant peptide solution containing 1 μg/μl HA-(91–261) in PBS. The mice were boosted twice at 3-week intervals, using the same amount of antigen used for the initial immunization. The mice were bled 1 week after the last immunization.

Enzyme-linked Immunosorbent Assay (ELISA)—High binding capacity ELISA plates (Immunoplate, Nunc, Denmark) were coated with 100 μl of purified virus containing 100 HAU/ml of various influenza viral strains diluted in PBS by incubating at 4 °C overnight. After several washings, the wells were blocked with PBS containing 1% bovine serum albumin, 100 μl of serial-diluted serum samples were added to each well, and the plates were incubated at 37 °C for 2 h. After washing five times with PBS-T (PBS containing 0.05% (v/v) Tween 20), the bound antibodies were detected by peroxidase-labeled goat anti-mouse IgG conjugates (Jackson Immunoresearch Laboratories). Reaction was revealed by incubating with 3,3′,5,5′-tetramethyl benzidine (Sigma) solution for 30 min at room temperature. After stopping the reaction with 50 μl of 2 M H_2SO_4, the plates were read with a multichannel ELISA reader (Titertek, Multiskan MCC/340 MK II; Helsinki, Finland) at 450 nm.

Reverse Screening of Aptamer—Biotinylated ssDNA of each plasmid from individual clones was synthesized using the B-T3 20-mer, which has the same sequence as the 5′ primer (T3 primer) in the aptamer. The biotinylated 5′ primer, B-T3 20-mer, was purchased from Stratagene (La Jolla, CA). The solid phase ELISA plate was prepared by coating with 100 μl/well of 100 μg/ml streptavidin diluted in 0.1 M $NaHCO_3$ and incubated overnight at 37 °C. After washing four times with PBS, the wells were blocked with 200 μl of PBS containing 1% bovine serum albumin by incubating at room temperature for 2 h followed by washing three times with PBS-T. After washing, 100 μl of 2.5 pmol/100 μl of the individual biotinylated ssDNA (expectant oligonucleotides) and, as negative control, B-T3 primer were added to the wells and incubated at 37 °C for 2 h followed by washing four times with PBS-T. After this step, 100 μl/well of 10 HAU/well virus or 2 μg/well histidine-labeled HA-(91–261) peptide were added and incubated at 37 °C for 2 h. After washing the wells four times with PBS-T, anti-His antibody or anti-virus serum were added to the corresponding wells. Reverse screening assay was completed by following the procedures of ELISA after this step.

Viability of MDCK Cells (MTT Assay)—MDCK cells were laid on 96-well plates (7×10^4/well) 1 day before the experiments, as such or infected with influenza virus, in the presence or absence of aptamer. They were incubated in maintaining medium (Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum) at 37 °C for 72 h. The viability assay was performed according to Levi *et al.* (31) by adding 4 mg/ml MTT (Sigma) dissolved in PBS to the cell cultures and incubating at 37 °C for 3 h. The plates were then centrifuged at 800 $\times$ *g* for 10 min. The supernatants were aspirated, and the formazan dye was dissolved in 150 μl/well isopropyl alcohol (Merck). The optical density values were quantitated using an ELISA reader at 540 nm.

Immunostaining—For immunostaining, 5×10^5 MDCK cells were laid on glass coverslips. After 24 h, influenza virus (Port Chalmers/1/73, H3N2) was added with or without 1 h of preincubation with A22. After 48 h, the cells were permeabilized with 3% paraformaldehyde containing 0.5% Triton X-100, and subsequently, the cultures were fixed with freshly prepared 3% paraformaldehyde. Influenza surface antigen HA was detected by incubating the cultures with a mouse monoclonal antibody specific for influenza HA (diluted 1:100) (Santa Cruz Biotechnology). All antibody incubations were carried out for 1 h at room temperature in a humidified chamber, followed by three washes in PBS. Primary antibodies were detected with Cy3-conjugated goat anti-mouse immunoglobulin (Jackson Immunoresearch Laboratories) secondary antibodies. Nuclei were visualized by staining with 2 μg/ml DAPI (Sigma). Immunofluorescence microscopy was performed using a Nikon Eclipse E600 microscope. Photographs were taken using the Spot software program. Images were processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Infection of Mice with Influenza Virus—The mice were inoculated intranasally with a sublethal dose of infectious allantoic fluid containing 2.5×10^3 TCID50. Preliminary experiments for the evaluation of this viral dose on BALB/c mice revealed that it is equivalent to 0.3 lethal doses 50 (LD_{50}). The mice were sacrificed 6 days after the inoculation (the peak of virus titer), and the lungs were removed and homogenized in PBS containing 0.1% bovine serum albumin (10% w/v). After homogenization, the samples were centrifuged to remove debris and stored at −70 °C. In the day of the assay, after thawing the lung homogenates (a process which releases the virus from the cells), they were injected (100 μl of 10-fold serial dilution) into the allantoic cavity of 9–11-day-old embryonated eggs. Following incubation for 48 h at 37 °C and overnight at 4 °C, allantoic fluid was removed, and virus presence was determined by hemagglutination assay as described above. The results of these assays were presented as log egg infection doses 50% (30).

Histology—For histological analysis of lungs, mice were sacrificed 6 days after infection, and pieces of the lungs were put into 10% neutral buffered formalin (pH 7.0), sectioned, and stained with hematoxylin and eosin. All slides were analyzed and evaluated by a uninformed observer.

Statistical Analysis—The *p* values were calculated by Student's *t* test, and $p < 0.05$ was considered significant according to the Statistical Package for the Social Sciences version 10.0 software program.

RESULTS

Cross Reactivity of HA-(91–261) Fragment with Different Viral Strains—The globular region of HA has been chosen for the aptamer selection, because it encompasses the conserved receptor-binding site, which is not exposed in the intact HA molecule or in its trimeric spikes on the virus. Hence, antibodies induced by this globular region are expected to react with different strains that share this structure. To induce such antibodies, we have cloned the peptide comprising the HA-(91–261) region that includes the binding zone to the oligosaccharide receptor of target

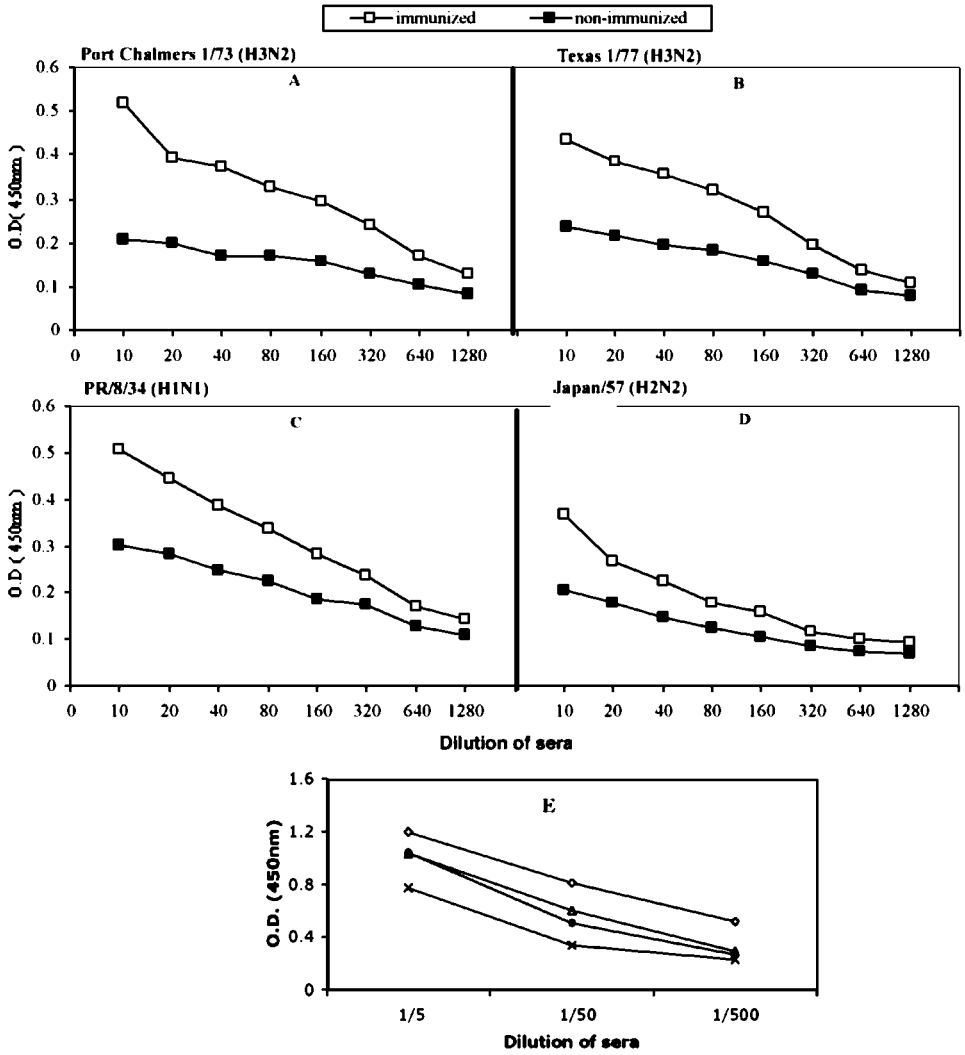

FIG. 1. **Reactivity of anti-sera from mice immunized with the recombinant HA-(91–261) peptide with several strains of influenza virus.** High binding capacity ELISA plates were coated with various virus strains: *A*, A/Port Chalmers/1/73 (H3N2); *B*, A/Texas/1/77 (H3N2); *C*, A/PR/8/34 (H1N1); *D*, A/Japan/57 (H2N2). The IgG levels were measured by ELISA in serum samples of immunized (□) and nonimmunized (■) mice. *E*, strain specificity-reactivity of anti-serum induced by A/Texas/1/77 with A/Texas/1/77 (◇), A/Port Chalmers/1/73 (△), A/PR/8/34 (●), and A/Japan/57 (×). *O.D.*, optical density.

cells. We have shown previously (9) that this protein fragment (denoted HA-(91–261) peptide) induced significant levels of IgG antibodies that recognized and neutralized the intact influenza virus A/Texas/1/77 (H3N2). As shown in Fig. 1, *A–D*, these anti-HA-(91–261) antibodies react with different influenza viral strains, including H1N1 (A/PR/8/34) and H2N2 (A/Japan/57), at a comparable level to their reactivity with the H3N2 (A/Texas/1/77 and A/Port Chalmers/1/73) viruses. This is in contrast to the strain-specific immune response induced by the intact A/Texas/1/77 virus (Fig. 1*E*). These results indicate that the HA-(91–261) globular region of the HA molecule may lead to broad spectrum neutralizing activity.

Selection and Construction of the Aptamer—Using this HA-(91–261) peptide, we attempted to design novel molecules that can bind directly to the virus HA and eventually prevent the interaction of the virus with the host cells. To this end, recourse was taken to the development of aptamers, namely oligonucleotides derived from an *in vitro* evolution process called SELEX, in which the peptide is used as a template. According to the described procedure (12), a nucleotide library was synthesized containing a random segment of 30 nucleotides flanked by 5′ and 3′ common primers as conserved linkers to amplify the selection process. This random library was hybridized with the HA-(91–261) peptide in a selection buffer and purified by Ni-NTA resin. After purification, the ssDNAs were amplified by PCR using the linker sequences. The SELEX process was repeated three times followed by the cloning of the final PCR product and transformation into *E. coli*. For the screening of oligonucleotides, biotinylated ssDNA from individual clones were screened by reverse ELISA for their binding to the HA-(91–261) fragment and the intact influenza virus, respectively. The two oligonucleotides with the highest binding activity were selected from the library and denoted "A21" and "A22," respectively. As illustrated in Fig. 2*A*, in their binding levels to the HA-(91–261) fragment, they were similar and showed highly significant differences from the control ssDNA ($p = 0.042$ and $p = 0.0008$, respectively). On the other hand, there was a significant difference between A21 and A22 in binding to the intact virus, where A22 showed higher binding than A21 ($p = 0.017$), and both showed significant binding compared with the control (Fig. 2*B*). The two oligonucleotides A21 and A22 were isolated and sequenced. Their sequences and the proposed secondary structures, as determined by using the DNA draw program (18), are illustrated in Fig. 2, *C* and *D*. The secondary structure of an additional oligonucleotide, NP-(147–158), which served as a negative control in the *in vivo*

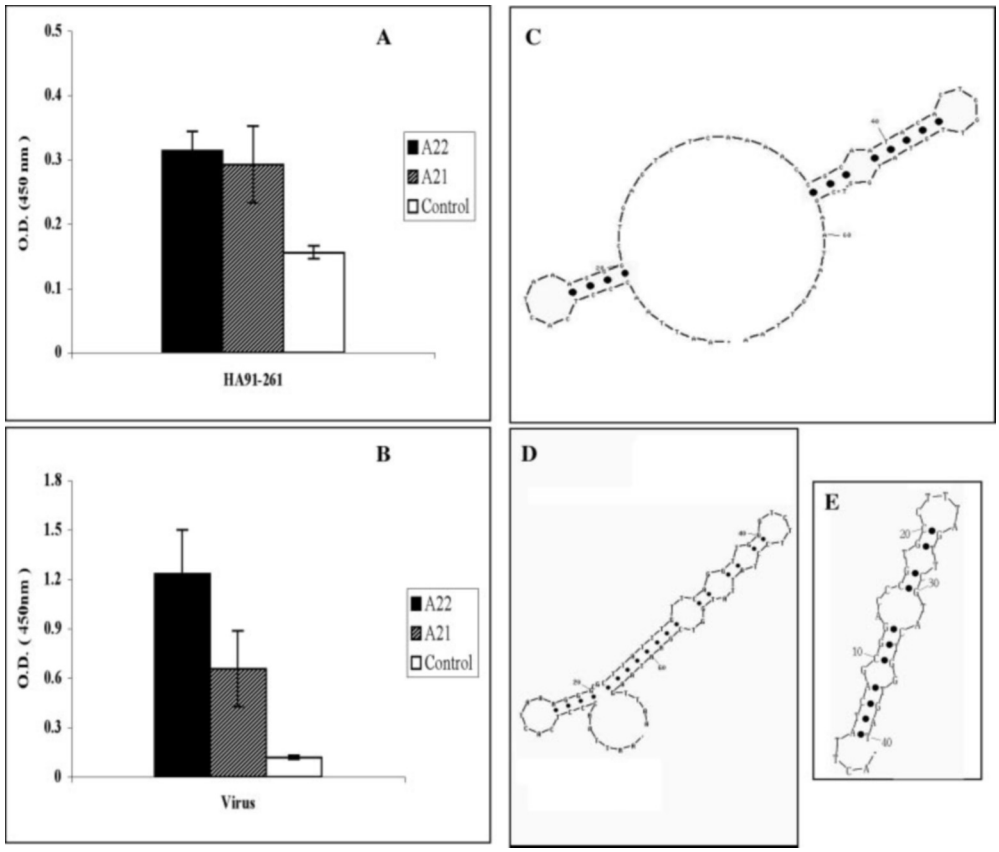

FIG. 2. **Properties of aptamers.** Binding levels of aptamers A21 and A22, as well as control ssDNA (unselected random nucleotides) to the HA-(91–261) peptide (*A*) and to the virus (*B*) as measured by ELISA. *C*, proposed secondary structure of the aptamer A22 comprising the oligonucleotide sequence 5′-AATTAACCCTCACTAAAGGGCTGAGTCTCAAAACCGCAATACACTGGTTGTATGGTCGAATAAGTTAA-3′, as drawn by the DNAdraw software program. *D*, proposed structure of A21. *E*, proposed structure of the control oligonucleotide, coding for the NP-(147–158) region of influenza nucleoprotein. The sequences are listed under "Experimental Procedures." *O.D.*, optical density.

experiments, is shown in Fig. 2*E*. The aptamers were then synthesized and evaluated as described in the following.

Inhibitory Effect of A22 Aptamer on the in Vitro Influenza Infectivity—The first evaluated parameter is the capacity to inhibit the hemagglutination activity of the virus, namely the capacity to agglutinate chicken red blood cells. The aptamer A22, which evinced the higher binding capacity to the virus, indeed demonstrated hemagglutination inhibition. Thus, at a 12.5-pmol concentration, it inhibited completely the hemagglutination by 4 HAU of the A/Texas/1/77 (H3N2) virus, and at a concentration of 20 pmol, it inhibited the hemagglutination by 10 HAU of influenza A/Japan/57 (H2N2) and 15 HAU of A/Port Chalmers/1/73 (H3N2), respectively. The capacity of A22 to inhibit viral infectivity was then investigated *in vitro* by measuring the viability of MDCK cells exposed to influenza virus (A/Port Chalmers/1/73, H3N2). Fig. 3*A* demonstrates the effect of A22 in preventing cell death and illustrates its dose-dependent nature. As shown, exposure of the MDCK cells to 5×10^2 TCID50 of the virus led to almost total cell death. The cell viability increased as a function of the concentration of the A22 added to the cell culture and reached its peak between 50 and 100 pmol of A22 ($r = 0.972$, $p = 0.028$). The slight decline in effectivity at the highest concentration is possibly because of some detrimental effect to the uninfected cells caused by high concentration of A22. Accordingly, a concentration of 50 pmol was selected to determine the effect of A22 on the infection of MDCK cells by another strain of influenza, A/Japan/57 (H2N2), in comparison to the inhibition of infection by the A/Port Chalmers/1/73 (H3N2) strain. The results (Fig. 3*B*) demonstrate that A22 had a comparable inhibitory effect on the MDCK death caused by the two viral strains. It led to a reduction of 80% in the cell mortality caused by A/Port Chalmers and 70% reduction of mortality caused by the A/Japan, two influenza strains that differ in their HA. Interestingly, as also shown in Fig. 3*B*, the less reactive aptamer A21 was also capable of reducing the *in vitro* infectivity of the viruses, although less effectively than A22. The inhibition of viral infectivity of the MDCK is specific, because a nonrelevant DNA (coding for the region NP-(147–158) of influenza nucleoprotein) did not lead to any change in cell mortality caused by the viral infection.

The effect of A22 in preventing viral binding and entry to cells was demonstrated also by microscopic analysis. As seen in the upper part of Fig. 4, using light microscopy, the whole structure of the MDCK cells was damaged by the viral infection (Fig. 4*A*). In comparison, in the presence of A22, destruction was inhibited, and the cell structure was largely conserved (Fig. 4*B*). Furthermore, the mere treatment with A22 did not affect the structure of the cells (Fig. 4*C*), indicating that the damage was caused only by the viral infection. These findings are corroborated in the fluorescence microscopy images in the lower part of Fig. 4. Here the presence of the virus was detected with Cy3-labeled specific anti-HA monoclonal antibodies. As shown, although viral presence is clearly manifested in the infected cells (Fig. 4*D*), it is almost entirely prevented by the addition of A22 (Fig. 4*E*). Untreated cells appeared identical to the cells treated with A22 (Fig. 4*F*).

Mode of Action of A22 Evidence for Aptamer-Virus Interaction—To eliminate the possibility that the prevention of infection is due to binding of the aptamer to the target cells rather

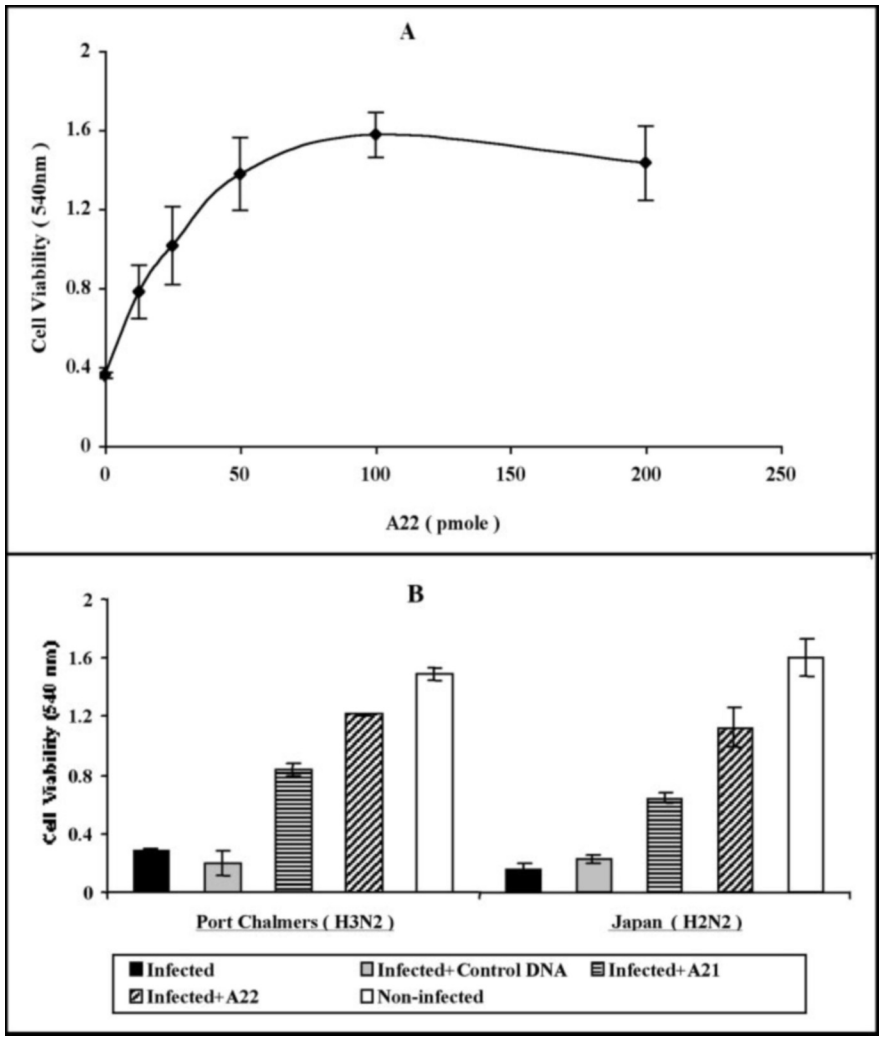

FIG. 3. **The effect of A22 on the viability of MDCK cells infected by 5×10^2 TCID50 of influenza virus in tissue culture.** The viability was measured by the MTT method in the presence of increasing concentrations of A22. *A*, dose-dependent inhibition of cell death caused by infection with the Port Chalmers strain of influenza. The highest protective effect was achieved by A22 at the concentration range of 50–100 pmol. *B*, comparison of the protective effect of A22 (50 pmol), A21 (50 pmol), and control DNA (NP-(147–158)) (50 pmol) in the prevention of MDCK cell death infected with two strains of influenza, Port Chalmers (H3N2) and Japan/57 (H2N2).

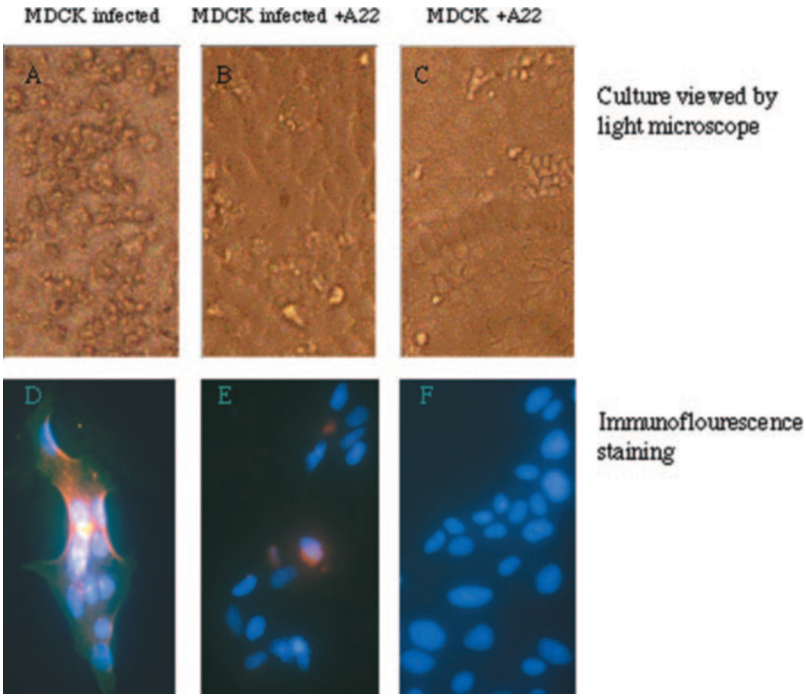

FIG. 4. **Microscopic analysis of the effect of A22 on influenza infection of cells.** *Upper panels*, light microscope views of MDCK cells after infection with 5×10^2 TCID50 influenza virus (*A*) and after pre-treatment with 50 pmol A22, with (*B*) or without (*C*) infection. *Lower panels*, immunofluorescence images of MDCK cells after 2 days of incubation with influenza virus (*D*), with influenza virus and A22 (*E*), or treatment with A22 alone (*F*). The cells were fixed with 3% paraformaldehyde and permeabilized in paraformaldehyde containing 0.5% Triton X-100. The cells were immunostained for the presence of virus using mouse anti-HA antibodies and goat anti-mouse IgG conjugated to Cy3 as a secondary antibody. Nuclei were visualized by staining with DAPI. HA and nuclei are shown in *red* and *blue*, respectively.

than to the virus, a pre-incubation experiment was performed. Before infection with 2.5×10^2 TCID50 of A/Port Chalmers/1/73 (H3N2) virus, the MDCK cells were incubated with 50 pmol of A22 for 30 or 60 min followed by repeated washing (twice) to remove unbound aptamer. Assessment of their viability (Fig. 5*A*) revealed no significant difference between the

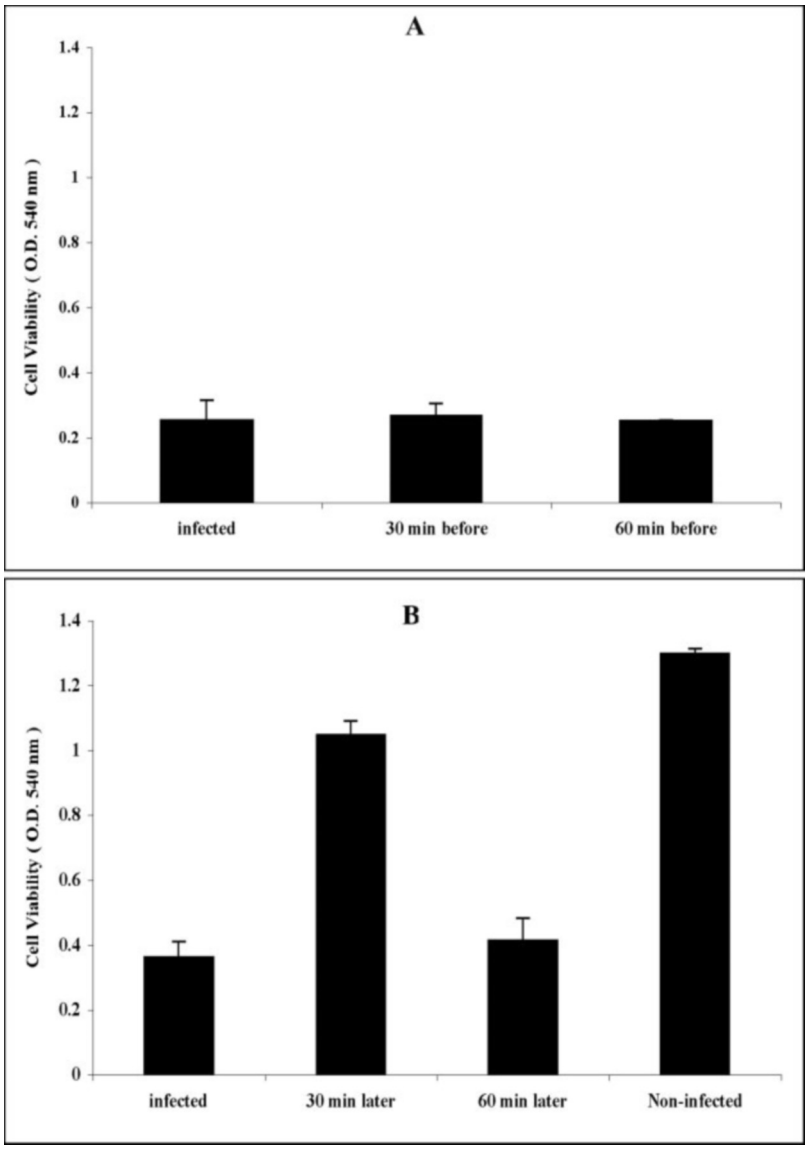

FIG. 5. **Mode of action of the aptamer in inhibiting viral infection of cells.** *A*, effect of pre-incubation of A22 with the MDCK cells prior to the viral infection. MDCK cells were infected with the virus after their prior incubation with 50 pmol A22 for 30 and 60 min, respectively. There is no significant difference between the viability of the cells in the A22-treated or untreated groups ($p = 0.34$ for 30 min and $p = 0.484$ for 60 min), indicating that A22 does not bind to the cells. *B*, effect of prior exposure of MDCK cells to the virus with the protective effect of A22. MDCK cells were incubated with influenza virus for 30 or 60 min before treatment with 50 pmol A22 for an additional 60 min. *O.D.*, optical density.

survival level of the nontreated and the A22-treated cells, nor any difference between the two times of exposure of the cells to A22. In all cases, cell survival is very low, because infection is not hindered. These results indicate that the effect of A22 in preventing the cell infection and mortality, as demonstrated in Fig. 3, is not by direct blocking of the sialic acid-containing receptors on host cells.

On the other hand, as demonstrated in Fig. 5*B*, the aptamer A22 can exert its protective effect even after initial interaction of the virus with the host cell receptors took place. The MDCK cell lines were infected with 2.5×10^2 TCID50 of A/Port Chalmers/1/73 virus for either 30 or 60 min prior to the addition of 50 pmol of A22. The results (Fig. 5*B*) show that, if added after 60 min of incubation of the cells with the virus, the effect of A22 was not significant any longer; cell mortality was high, whereas the difference between this group and the noninfected cells was significant. On the other hand, the cells incubated with the virus for only 30 min prior to the treatment with A22, showed high cell viability, not much different from that of the noninfected cells ($p = 0.022$). According to these results, it may be concluded that A22 is effective in preventing infection, even after a short (up to 30 min) exposure of the cells to the virus.

Capacity of A22 to Prevent Influenza Infection in Vivo—The efficacy of A22 to prevent influenza infection *in vivo* was evaluated by its administration to mice either concomitantly, before, or even after viral infection. The mice were divided into four groups according to the timing of treatment with A22. Each mouse was infected intranasally with a sublethal dose of influenza A/Texas/1/77, consisting of 2.5×10^3 TCID50 of the virus diluted in 50 μl of PBS. The mice in the "0-day" group were inoculated intranasally with a mixture of the virus and 50 μl of 2.5 nmol/ml A22. The mice in the "−1-day" and "+2-day" groups were administered 50 μl of 2.5 nmol/ml A22 intranasally either 1 day before or 2 days after the viral exposure, respectively.

In a preliminary experiment, the mice were not sacrificed, and their body weight loss was followed during 16 days after viral infection. The results demonstrate that in the control group consisting of infected animals, the mice lost about 20% of their body weight at the peak of infection. In contrast, in all the treatment groups (+2-day, 0-day, and −1-day), only marginal weight loss was observed after the infection, with faster recovery of their original weight (Fig. 6*A*).

In a parallel experiment, the mice were sacrificed 6 days after the inoculation of virus, and their lungs were subjected to virus titer determination as well as to histological examination. The viral load in the lungs of the mice in the four different groups, as determined by the whole egg titration method (Fig. 6*B*), is indicative of the protective capacity of A22. As shown, in

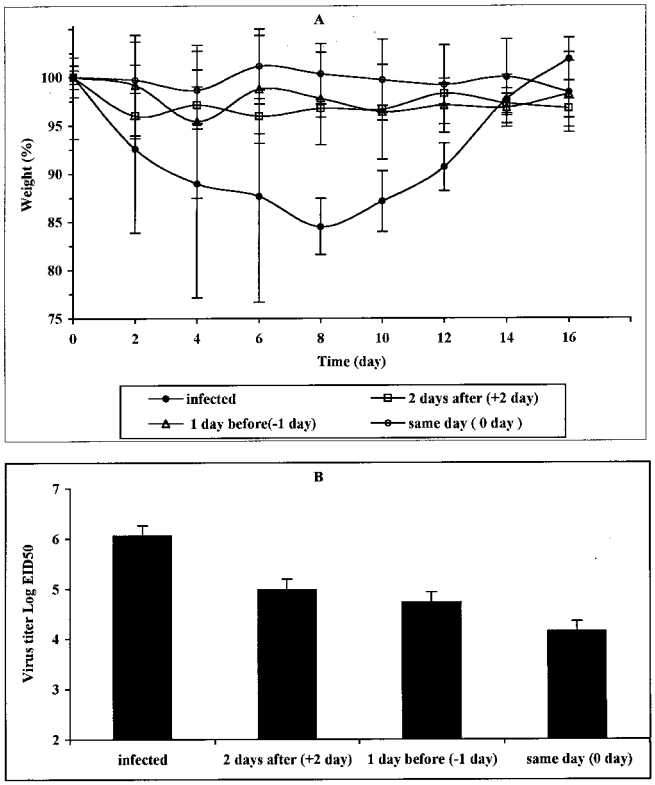

FIG. 6. **Inhibition of *in vivo* influenza infection by A22.** *A*, the effect of A22 treatment on the body weight loss of the mice infected with influenza. In contrast to the control-infected group, A22-treated mice showed significantly lower weight loss and recovered weight much more quickly ($p < 0.05$). *B*, the effect of A22 on the viral burden in the lungs of influenza-infected mice. All mice were infected with a sublethal dose, namely 2.5×10^3 TCID50 of A/Texas/1/77 influenza virus. The mice were divided into four groups according to the time of their treatment with A22 (1 day before, concomitantly, or 2 days after infection). Six days following the infection, the mice were sacrificed, their lung homogenized and assayed for virus level by the hemagglutination inhibition test. The results are presented as log egg infection doses 50% (*EID50*). The level of reduction of viral load in all three groups of the A22-treated mice is statistically significant when compared with the mice in infected groups ($p < 0.05$).

the lungs of mice treated intranasally with 50 μl of 2.5 nmol/ml A22 (125 pmol/mouse), either 1 day before or 2 days after the inoculation, the viral load was about 1.2 log lower than in the infected control group (>90% decrease). This difference in viral load is statistically significant ($p < 0.05$). The mice in the 0-day group manifested even higher protective effect when compared with the +2-day and −1-day groups. This mode of treatment led to 2.4 log (over a hundred-fold) reduction in lung viral titer ($p < 0.01$), thus indicating that A22 is most effective when administered concomitantly with the virus.

This inhibition of infection is corroborated by the histological analysis of the lungs (Fig. 7), which demonstrates that, in contrast to the noninfected lungs from normal mice (Fig. 7*A*), infected mice showed typical pathology of the lungs, including bulk expansion of mononuclear cells and collapsed areas (Fig. 7*B*). In comparison, in the lungs of mice treated with A22 (125 pmol/mouse), especially in the 0-day and −1-day groups (Fig. 7, *C* and *D*, respectively), there was far less mononuclear cellular infiltration, and most of the alveoli were open. In the lungs of the +2-day group, both damaged and undamaged sites could be observed (Fig. 7, *E* and *F*), with about 60% of the lung showing no damage. These findings demonstrate that administration of A22 reduces the inflamed areas in the lungs in a highly significant way.

Protection against Infection by Various Influenza Strains—Because the receptor-binding region of the HA is a highly conserved region, it was of interest to test whether the protective effect of A22 is manifested also toward infection with other influenza strains. The results are shown in Fig. 8*A*, which demonstrate the reduction in the lung virus titer in mice infected with three strains of influenza as a result of treatment with A22 on the day of infection. In this particular experiment, the efficacy of the aptamer was lower than in the experiment depicted in Fig. 6. However, it demonstrates that A22 reduced the level of infection by all the tested strains, namely A/PR/8/34 (H1N1), A/Japan/37 (H2N2), as well as A/Texas/1/77 (H3N2). These findings corroborate the results of the *in vitro* assay presented in Fig. 3*B*. In contrast to A22, a control-irrelevant nucleotide, coding for influenza nucleoprotein region NP-(147–158) led to an insignificant change in the viral titer. It is of interest that the aptamer A21, although less effective than A22, was still capable of reducing the lung virus titer of A/Texas/1/77 (H3N2) (Fig. 8*B*).

DISCUSSION

Several anti-influenza compounds have been described in the literature (32). Of those, amantadine and rimantadine act by blocking an ion channel formed by the M2 protein that spans the viral membrane, whereas zanamivir and oseltamivir are specific neuraminidase inhibitors that act by preventing the progeny virus to penetrate the surfaces of infected cells. The efficacy of all these drugs is rather limited (2–4). None of the currently available drugs is directed at the hemagglutinin, which is one of the major membranal surface glycoproteins of the virus. Several compounds were reported to achieve anti-influenza activity by targeting the HA (mostly sialic acid analogues), but their effectiveness has not been described in definite terms (19, 20).

Because the effective protection associated with the HA-specific humoral immunity, resulting from vaccination, is due mainly to the neutralization of virus activity, the use of HA-specific monoclonal antibodies has been proposed as an effective therapeutic treatment for preventing the spreading of the virus (21, 22). However, their preventive effect on infection is apparently of limited applicability, because it depends on the isotype, specificity and structure of the antibody, and the treatment dose (23). During the first presentation of the SELEX procedure and DNA aptamers, a large number of nucleic acid molecules were developed that display activities not usually found in nature. These ssDNA or RNA aptamers were accepted as substitutes for monoclonal antibodies and are considered uniquely useful in diagnosis and treatment, and if they bind tightly to a protein, they inhibit a specified function of that protein and have no harmful side effects (24, 25). To date, several studies have reported on the use of aptamers in diagnosis and therapy (25) of a few viral, bacterial, and parasitic infections (26–28). In the present study, we propose a novel DNA aptamer, denoted A22, that is specific toward the influenza HA globular region and consequently prevents the binding of intact virus to the receptors on the target cells. This feature correlates strongly with its capacity to reduce the level of viral infection, both *in vitro* and *in vivo*.

The A22 aptamer was developed based on the HA-(91–261) peptide as a template and using the SELEX procedure. In this novel procedure, a construct library containing a segment of 30 nucleotides was used. Such a library will generate more than 10^{18} types of different ssDNAs, but only those that possess a fitting structure will hybridize with the peptide. Iteration of the selection procedure eventually leads to a small number of fitting aptamers (15). In the present study, it yielded just two novel oligonucleotides, A21 and A22, binding equally to the

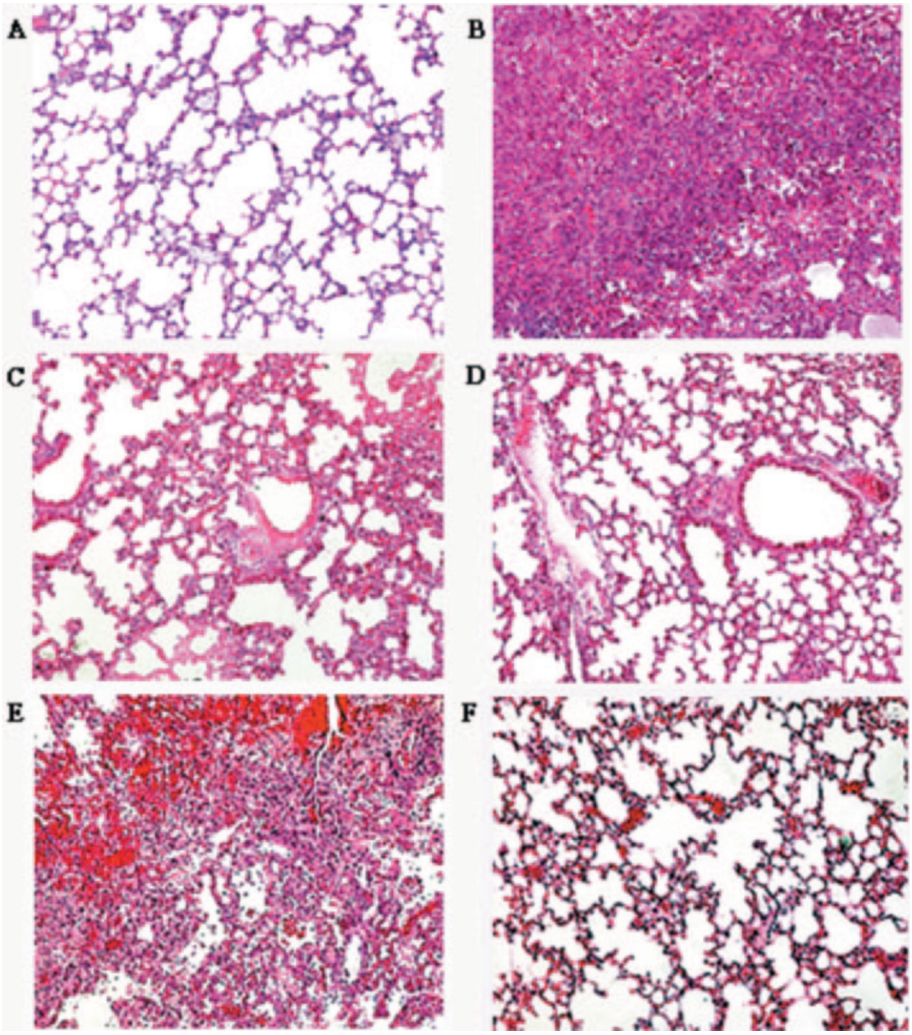

FIG. 7. **Histological analysis of the lungs from infected mice after staining with hematoxylin and eosin.** The mice in various groups, as described under "Experimental Procedures" and in Fig. 6, were sacrificed 6 days after their intranasal inoculation with influenza virus, and small portions of their lungs were removed and put into 10% neutralized buffered formalin for histological examinations. *A*, lung from a normal, noninfected control group. *B*, lung from infected mouse. *C*, lung from a mouse of the −1-day group treated with A22 aptamer 1 day prior to the infection. *D*, lung of the 0-day group treated with A22 concomitantly with the infection. *E* and *F*, two different sections from a lung of the +2-day group treated with A22 2 days following the infection. It is estimated that about 60% of the lung of that mouse corresponded to the pattern in *F*, which is similar to the histology of the noninfected control, whereas 40% of the lung contained bulk expansion of mononuclear cells (*E*).

HA-(91–261) construct, of which A22 showed a higher binding capacity to the intact influenza virus than A21 (Fig. 2*A*). The reactivity of A22 with influenza virus was demonstrated by its capacity to inhibit the viral-induced hemagglutination of chicken red blood cells, indicating that this aptamer indeed binds to the viral HA.

A more important issue is whether this binding can lead to inhibition of viral infection, and this was assessed both *in vitro* and *in vivo*; *in vitro*, by its capacity to inhibit the virus-induced cell mortality in tissue culture (Fig. 3*A*). An important finding is the efficacy of A22 in preventing the infection of cells by more than one viral strain (Fig. 3*B*). This is probably because of the conserved nature of the structure of the receptor-binding region in the HA molecule, rendering it a common target across strain variations, as shown also by immunological procedures. Thus, in contrast to the intact HA, which elicits antibodies specific to a particular viral strain and does not recognize the highly conserved receptor-binding site on HA because of conformational restrictions (17), antibodies induced by the globular region are not expected to have this limitation and, consequently, interact with several viral strains (Fig. 1).

The finding that aptamer A21, which is equivalent to A22 in its binding to the HA-(91–261) region but is of lower binding to the intact virus and was also capable of inhibiting viral infection, although less effectively than A22, points to a plausible mode of action of the aptamers, namely via direct interaction with the receptor-binding region of the viral HA, which is encompassed in the globular region. This prevention of viral attachment to the cell receptors and its subsequent penetration into the MDCK cells is corroborated by microscopic analysis of the cells (Fig. 4).

The interference with viral entry into the cells could stem either from the binding of A22 to the virus or from its binding to the target cells. In both cases, the results would be the blocking of the virus-cell interaction. However, the finding that a prior incubation of the MDCK cells with A22 for up to 60 min does not diminish, at all, their infection by the virus and the ensuing cell mortality (Fig. 5*A*) indicates that the effect of A22 is not by direct binding to the cells, but rather by its binding to the viral surface. These results are in accord with the capacity of A22 to inhibit the HA activity of influenza. It is of interest that A22 can exert its preventive effect on viral infection even *after* the initial infection process has been initiated, probably by dissociating the virus-cell interaction before complete fusion has occurred. Consequently, if the MDCK cells are exposed to the virus for 30 min or less prior to the introduction of A22, the latter can still prevent cell mortality (Fig. 5*B*). These findings lend further support to the proposed mode of action of the A22 aptamer and are also relevant to its prospects of being a potential anti-influenza agent. Accordingly, in a situation of *in vivo* infection, such an agent might exert its effect even after exposure to the infective virus, as corroborated by the results *in vivo*.

The most relevant parameter of influenza is its infectivity *in vivo* leading to disease. Hence, the capacity of the aptamer A22 to reduce the level of the disease in an *in vivo* model is of high

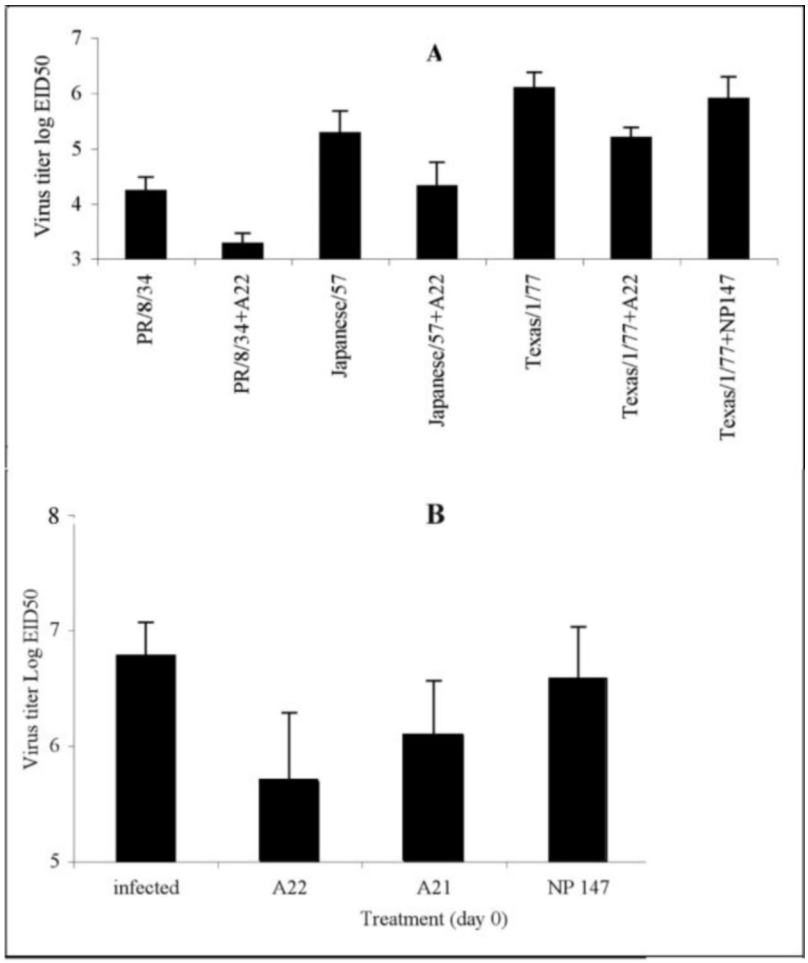

FIG. 8. *A*, inhibition by A22 (125 pmol) of influenza infection by different strains of influenza. Mice were infected with 2.5×10^3 TCID50 of the respective virus. *B*, inhibition of infection with A/Texas/1/77 by the two aptamers A22 and A21 in comparison with a control of unrelated nucleotide.

significance. This inhibition of viral infection was established using three criteria: 1) prevention of weight loss (Fig. 6*A*), 2) decrease of viral load in the lungs (Fig. 6*B*), and 3) restriction of the level of inflammation and cellular infiltration in the lungs of infected mice (Fig. 7) as a result of intranasal treatment with the aptamer. According to all three criteria, A22 was most effective when administered concomitantly with the viral infection leading to ~95% reduction in viral burden. An attempt to apply "preventive treatment" by administration of A22 1 day prior to infection resulted in a less effective inhibitory effect on infection, probably because the DNA is partially degraded, and hence less of it is available for binding the virus administered 24 h later. Even more significant is the finding that a single treatment with A22 *in vivo* 2 days following the infection, namely a "therapeutic treatment," still leads to almost 95% reduction in viral titer in the lungs of the mice.

It is of some interest to examine the level of inhibition of infection by A22 in comparison with the degree of inhibition of influenza observed in mice after administration of the currently available neuraminidase inhibitors. In the case of zanamivir, it was reported that in mice, daily intranasal administration of 0.1 mg/kg body weight led to 90% survival, and 1 mg/kg body weight led to 100% survival, whereas oral treatment was only effective at higher doses (29). This efficacy is comparable with the ~95% inhibition of infection achieved with 0.13 mg/kg body weight of A22 as observed in this study. It is therefore apparent that in the animal model, A22 is as effective as the neuraminidase inhibitors. It should be borne in mind, however, that in patients, the neuraminidase inhibitors are applied by the oral route and used daily after infection.

In conclusion, we report the design of a novel molecule, comprising a ssDNA aptamer, which inhibits influenza infection by interfering with the virus-host cell interaction, via blocking of the receptor-binding region of the surface glycoprotein HA. The effect achieved *in vivo* with a single dose of the aptamer is highly significant. This novel aptamer is therefore of interest, as it might be considered a suitable candidate for development as an alternative, selective inhibitory anti-influenza agent.

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