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Protective Efficacy of a DNA Influenza Virus Vaccine Is Markedly Increased by the Coadministration of a Schiff Base-Forming Drug

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Effective vaccination against heterologous influenza virus infection remains elusive. Immunization with plasmid DNA (pDNA) expressing conserved genes from influenza virus is a promising approach to achieve cross-variant protection. However, despite having been described for more than a decade, pDNA vaccination still requires further optimization to be applied clinically as a standard vaccination approach. We have recently described a simple and efficient approach to enhance pDNA immunization, based on the use of tucaresol, a Schiff base-forming drug. In this report we have tested the ability of this drug to increase the protection conferred by pDNA vaccination against influenza virus infection. Our results demonstrate that a significant protection was achieved in two strains of mice by using the combination of pDNA and tucaresol. This protection was associated with an elevated humoral and cellular response and a switch in the type of the T helper cell (Th) immune response from type 2 to type 1. This vaccine combination represents a promising strategy for designing a clinical study for the protection from influenza and similar infections.

Influenza virus infection remains a major health problem worldwide with annual epidemics, which are often complicated by significant morbidity and mortality despite the availability of rationalized vaccination protocols based on the use of inactivated virus (8, 22, 26). There are, however, two major drawbacks with this vaccine: firstly, the protection induced by the vaccine is short-lived and therefore requires annual administration, and secondly, the vaccine is only capable of eliciting a strain-specific antibody response (16, 22, 26). The latter drawback is of paramount importance because influenza strains are highly and continuously variable because of antigenic shift, which occurs as a result of RNA segments exchanged between virus strains and antigenic drift due to point mutations (26). This highlights the need for a vaccine with higher efficacy. Optimally, such a vaccine should be capable of inducing protection against heterologous virus strains and should induce long-lived responses and be highly efficient. Several studies in recent years have reported on the induction of heterologous protection against influenza using plasmid DNA (pDNA) immunization through various routes of immunization (9, 16, 17, 31). Heterologous protection was obtained through targeting the conserved internal viral proteins for recognition by Th and cytotoxic T lymphocytes (CTL) following pDNA immunization (31).

Plasmid DNA immunization is a promising approach for the management of infectious diseases, based on its ability to induce long-term protective memory responses which are not usually seen with other types of noninfectious vaccines (3, 17). Yet, pDNA immunization suffers from a limited efficacy due to the induction of a weaker immune response than that induced by some traditional vaccines such as live-virus vaccines (6, 10). This has contributed to the premise that pDNA immunization is probably not an appropriate vaccination strategy against influenza virus infection (22). In an attempt to overcome this limitation we have tested the ability of tucaresol, an immunopotentiating drug, to enhance the efficacy of an influenza pDNA vaccine. Tucaresol (Tuc) is a Schiff base-forming molecule that is capable of interacting with molecules expressed on the surface of T cells in a process that leads to costimulation (24, 25). We have recently identified tucaresol as a potent enhancer for humoral and cellular immune responses induced by pDNA immunization (6). Tucaresol markedly enhanced the antigen specific immune response as measured in vitro by its ability to elevate the immune response against viral and bacterial antigens (6).

In the present study, we have tested whether these adjuvant effects of tucaresol for pDNA vaccination are also achievable in vivo against influenza virus challenge, as measured by immunizing mice with either influenza hemagglutinin (HA)- or nucleoprotein (NP)-encoding plasmids independently or in combination with tucaresol. Tucaresol administration following HA or NP pDNA immunization enhanced the protective efficacy of the vaccine and resulted in both increased survival and decreased morbidity, as manifested by reduced weight loss in mice from two different strains. These in vivo effects were associated with an augmented humoral response, as assessed by the increase in specific immunoglobulin (Ig) response, and with potent cytotoxic T-cell responses.

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MATERIALS AND METHODS

Plasmid construction and testing. All genes were inserted in the plasmid PUA CMV SMI2 (P). The resulting plasmids with the influenza antigens HA and NP were designated P-HA and P-NP. Details about the subcloning and the testing of these plasmids have been published elsewhere (20).

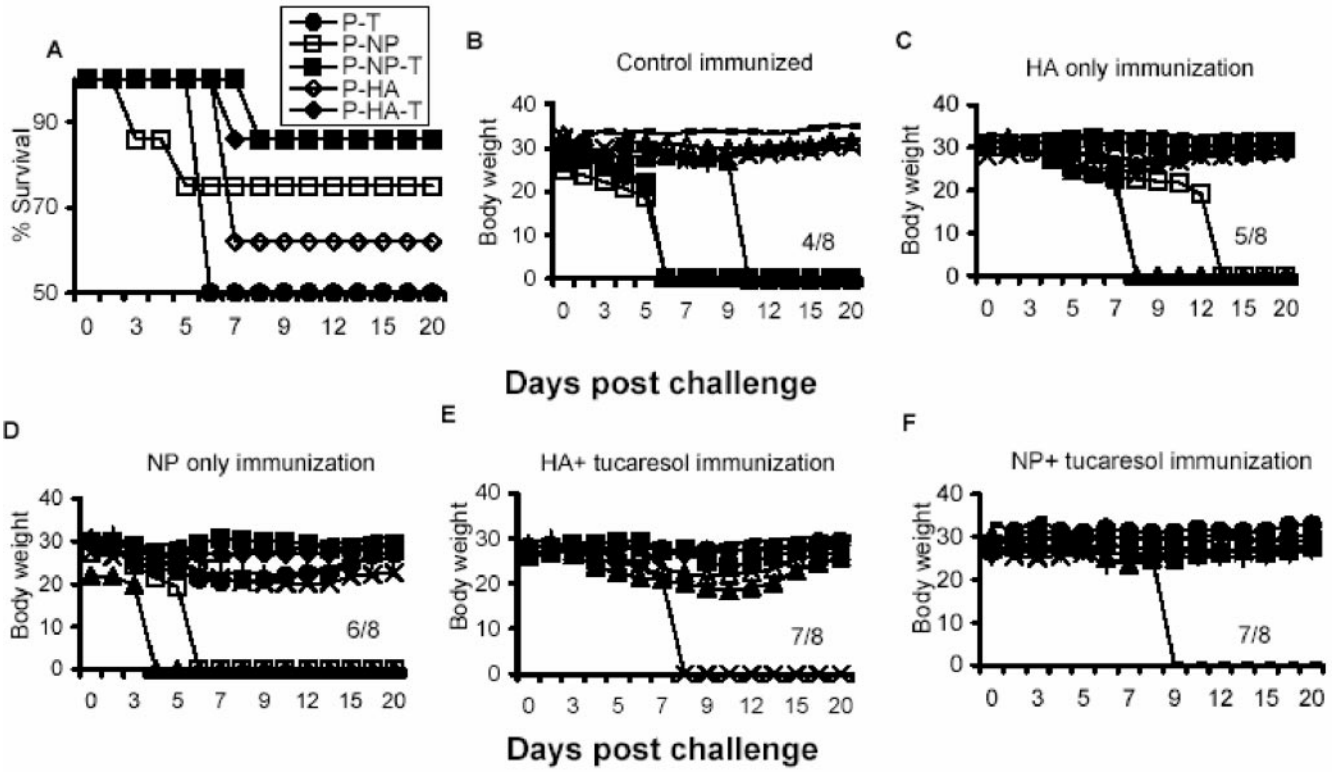

FIG. 1. Tucaresol enhances the protective efficacy of HA- and P-NP-based pDNA vaccines against influenza virus infection. C57BL/6 mice were immunized twice with a gene gun and then challenged intranasally with a LD_{50}. Mice from groups treated with tucaresol received 1 mg of tucaresol subcutaneously 24 h after pDNA immunization. (A) Percent survival among mice after virus challenge. (B to F) Body weight (grams) after virus challenge. Dead mice are indicated by 0-g weight. Numbers above the abscissa represent the number of live mice out of the total number of mice per group. *P* equals 0.039 for P+Tuc compared to either P-NP+Tuc or P-HA+Tuc vaccination, and *P* is >0.05 for all other comparisons.

Mice immunization and challenge. C57BL/6 and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine). These mice were propagated and maintained in our specific pathogen-free environment in the Microbiology and Tumor Biology Center animal house at the Karolinska Institute. DNA immunization was accomplished by gene gun immunization as previously described (3, 16). Mice, immunized with P, P-HA, or P-NP plasmids, were injected with tucaresol (referred to hereafter as P+Tuc, P-HA+Tuc, or P-NP+Tuc). Tucaresol [4-(2-formyl-3-hydroxy-phenoxymethyl) benzoic acid], kindly provided by John Rhodes, GlaxoSmithKline, Stevenage, United Kingdom, was injected subcutaneously in the flank at a site separate from the DNA immunization site. Tucaresol injection was applied as a single injection of 1 mg of tucaresol/100 μl of phosphate-buffered saline/mouse, 24 h after the pDNA immunization. Mice received a booster vaccination 4 weeks after priming with the same schedule of plasmid and tucaresol. On day 49, the mice were challenged with 50,000 50% tissue culture infective doses of the influenza virus A/PR8/34(H1N1) intranasally at 50 μl/mouse (20). This dose was titrated to be the 50% lethal dose (LD_{50}) in inoculated C57BL/6 mice. Mice were then monitored for survival and weight loss.

Generation of antigen-specific CTL and cytotoxicity assays. Peptide epitope-specific CTL lines were prepared using the NP 8.147 H-$2K^d$ binding peptide (TYQRTRAI) (12, 30) as follows: 4 weeks after the last immunization, pooled splenocytes from five mice of each immunization group were cultured at 3×10^6 cell/ml in a 25-cm^3 flask (Costar, Life Technologies, Paisley, Scotland) pulsed with 1-μg/ml peptide in a total volume of 10 ml of Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, 100 mg of streptomycin/ml, 50 μM β-mercaptoethanol, and 2 mM L-glutamine (all from Life Technologies). On day 4, interleukin-2 was added to a final concentration of 5 IU/ml (Peprotech, Rocky Hill, N.J.). After 6 days, the cultures were assayed for cytotoxicity as follows: D2F2, an H-$2K^d$-expressing mammary adenocarcinoma (kindly provided by Wei Zen Wei, Karmanos Cancer Institute, Wayne State University, Detroit, Mich.), or the H-2^d-expressing mastocytoma P815 (American Type Culture Collection, Manassas, Va.) were used as target cell lines. Cell-mediated cytotoxicity was measured by ^{51}Cr release assays. One million target cells were incubated at 37°C in the presence of 200 μCi of sodium ^{51}Cr chromate (Amersham, Little Chalfont, United Kingdom) for 1 h, washed three times, and resuspended in complete medium at 10^5 cells/ml in the presence or absence of 10-μg peptide. The test was performed by incubating T cells together with 4×10^3 to 5×10^3 target cells at different effector-to-target ratios in triplicate wells at a final volume of 200 μl in V-bottomed 96-well plates. Cultures were incubated for 4.5 h at 37°C, after which supernatants were harvested and used to determine specific lysis using the following equation: percent specific release = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Cytotoxicity was quantified by calculating the number of lytic units in 10^7 effector cells that could lyse 12% of target cells (4×10^3 cells), referred to as LU_{12}. A calculation was made using the following formula: $LU_{12}/10^7 = 10^7/[(E{:}T_{12})\ (4 \times 10^3)]$, where $E{:}T_{12}$ is the effector-to-target ratio at which 12% of the target cells are killed, as described by Friberg et al. (14). $E{:}T_{12}$ was used instead of the more commonly employed $E{:}T_{20}$, since the percent lysis in the P-NP-only group did not reach 20%.

ELISA procedures. Sera from mice were collected and used in direct enzyme-linked immunosorbent assays (ELISAs) as described previously (20). Recombinant NP was used at a concentration of 5 μg/ml in carbonate buffer to coat the wells of 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) overnight at 4°C. Sera were then added in duplicate at a 1:100 dilution and incubated overnight at 4°C. Binding antibodies were detected using IgG- (preabsorbed against mouse IgM), IgG1-, and IgG2a-specific, alkaline phosphatase-conjugated goat anti-mouse sera (Southern Biotech, Birmingham, Ala.).

RESULTS AND DISCUSSION

It has recently been shown that tucaresol efficiently enhances the immune response to pDNA immunization (6). This was manifested as an increased humoral and cellular response to

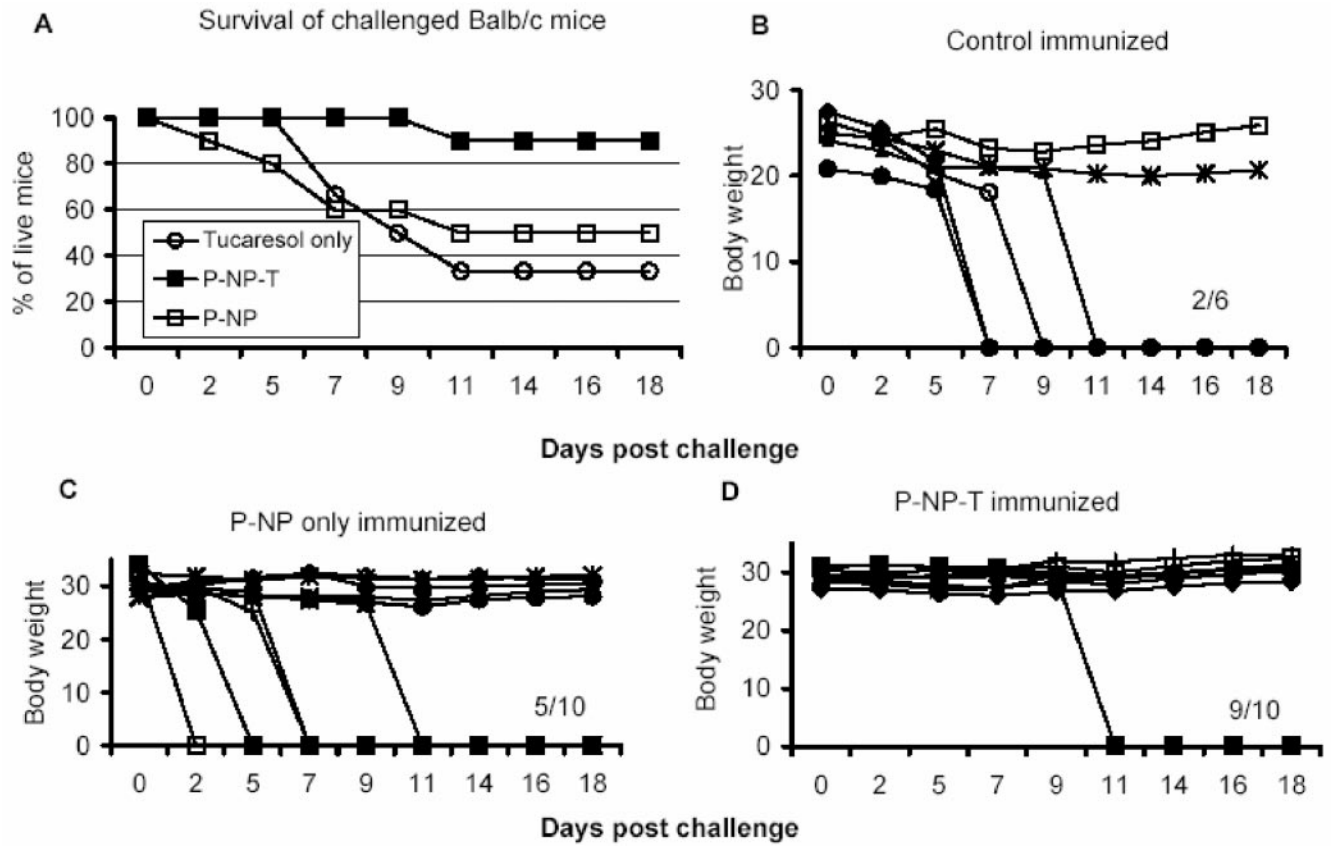

FIG. 2. Tucaresol enhances the protective efficacy of P-NP-based pDNA vaccines against influenza virus infection in BALB/c mice. Mice were immunized twice with a gene gun and then challenged intranasally with a LD_{50}. Mice from groups treated with tucaresol received 1 mg of tucaresol subcutaneously 24 h after pDNA immunization. (A) Percent survival after virus challenge. (B to D) Body weight (grams) after virus challenge. Mouse death is indicated by 0-g weight. Numbers above the abscissa represent the number of live mice out of the total number of mice per group. P is <0.001 for control compared to P-NP+Tuc, P equals 0.017 for P-NP+Tuc compared to P-NP vaccination, and P equals 0.23 for control compared to P-NP vaccination.

the mycobacterial heat shock protein 65 (Mhsp65) and Epstein-Barr virus nuclear antigen 4 (EBNA-4) genes determined using in vitro assays for antibody production, cytokine release, T-cell proliferation, and cytotoxicity. As the ability to enhance the in vivo protective efficacy of a pDNA vaccine by tucaresol has not been tested, we designed this study to investigate whether an influenza virus-targeted pDNA vaccine could be improved by including tucaresol in the immunization protocol. We utilized plasmids coding for NP and HA, which are the two antigens that were reported to be effective in inducing protection from influenza virus infection when used as pDNA vaccines in mice (17, 20, 31, 32). Mice were immunized with P+Tuc, P-NP, P-HA, P-HA+Tuc, or P-NP+Tuc and then challenged with a predetermined LD_{50} of the virus. In C57BL/6 mice, 50% of the control P+Tuc-immunized mice lost weight and died (Fig. 1A and B). Immunization with P-NP or P-HA protected 69% of the mice (Fig. 1A, C, and D). Including tucaresol in the immunization protocol protected most of the mice that were immunized with P-HA+Tuc or P-NP+Tuc from death, with 88% of the immunized mice surviving the challenge (Fig. 1A, E, and F). Therefore, including tucaresol in the immunization protocol leads to about a threefold decrease in the death caused by the infection (from 31% in the groups immunized with P-NP or P-HA to 12% in those immunized with P-HA+Tuc or P-NP+Tuc). Significant protection was only obtained when tucaresol was included in the vaccination ($P = 0.039$), while mice vaccinated with P-NP or P-HA alone were not protected compared to mice immunized with control plasmid only ($P > 0.05$).

In BALB/c mice immunized with the control Tuc, most of the mice (67%) suffered from weight loss and died (Fig. 2A and B). In the P-NP-immunized group, 50% of the mice lost weight and died, which suggests some protection due to this vaccine (Fig. 2A and C). This level of protection is similar to what was achieved in the original report on NP-based pDNA vaccine for influenza virus, despite using a different challenge protocol (31). Remarkably, in the P-NP+Tuc-immunized group only one mouse died, indicating 90% efficacy of this vaccine combination (Fig. 2A and C). This adjuvant effect of tucaresol on DNA vaccination is statistically significant compared to either control vaccination ($P < 0.001$) or to P-NP vaccination ($P = 0.017$). For both B6 and BALB/c mice taken together, 50 to 67% of the mice in the control-immunized groups succumbed to the infection. Weight loss was also apparent in most of these mice. In mice immunized with P-NP or P-HA some protection was afforded, with 62% protected from

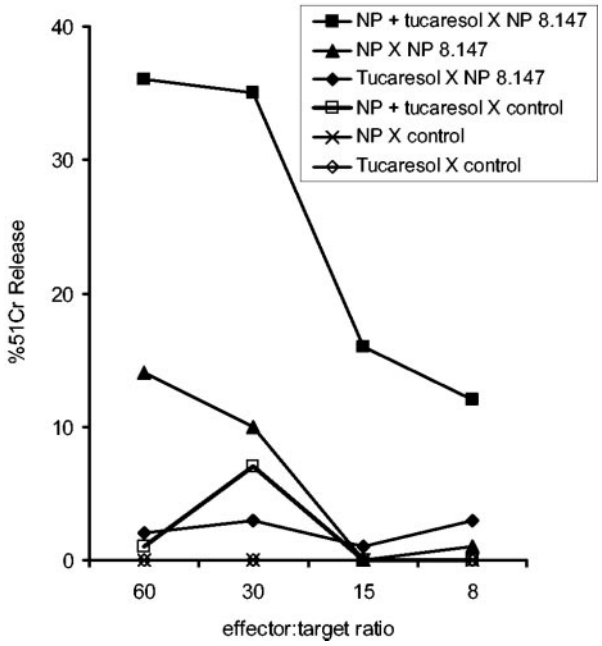

FIG. 3. Marked enhancement of influenza virus-NP-specific cytotoxicity is induced by administrating tucaresol and P-NP pDNA vaccine. Effector cells are derived from splenocytes of mice immunized with tucaresol only, P-NP, or P-NP+Tuc. Four weeks after the last immunization, pooled splenocytes from five mice of each immunization group were pulsed with the NP 8.147 peptide or the control peptide and cultured as described in Materials and Methods for 6 days. Thereafter, their cytotoxic activity was measured in a standard ^{51}Cr release assay at the indicated effector-to-target ratios using P815 cells as targets.

weight loss and death while 38% succumbed. Remarkably as well, in the P-HA+Tuc- or P-NP+Tuc-immunized mice only 12% died, and the majority of the mice (88%) did not suffer from weight loss and survived the challenge. This is an overall more than threefold decrease in mortality, which suggests that tucaresol represents an effective adjuvant for protective DNA vaccination against influenza. It is noteworthy that the protection afforded as a result of P-NP+Tuc administration to BALB/c mice was more prominent than that afforded to B6 mice by the same immunization, particularly since P-NP immunization alone could only protect one more mouse than control immunization did (Fig. 2). Other approaches to enhance DNA vaccination include the use of cytokine-encoding plasmids such as granulocyte-macrophage colony-stimulating factor, which has been shown to enhance the protective efficacy of an influenza DNA vaccine (21). Tucaresol was previously compared to gamma interferon and granulocyte-macrophage colony-stimulating factor for their efficiency as DNA vaccine adjuvants, and tucaresol was found to be at least as strong an adjuvant as either of these two cytokines (6).

It has been shown that NP-based DNA immunization results in heterologous protection that is T-cell mediated (15, 32). To dissect the protective immune response induced by DNA vaccinations, Fu et al. and Ulmer et al. have performed antibody depletion of the CD4 or CD8 subsets of T cells (15, 32). Their data suggested that while both T-cell subsets are important for the protection, CD8 T cells are more efficient in protecting the mice from weight loss and death. We have therefore investigated whether tucaresol enhances the CD8-mediated T-cell response induced by NP pDNA immunization. For this purpose, target cells pulsed with the NP epitope NP 8.147 restricted by H-2K^d (12, 30) were used in a cytotoxicity assay. Effector cells were derived from mice immunized with Tuc, P-NP, or P-NP+Tuc. No significant cytolytic activity was detected using splenocytes from Tuc or P-NP against targets that were either unpulsed or pulsed with the NP epitope (Fig. 3). Of particular importance, we were able to detect significant cytotoxicity against target cells pulsed with the NP epitope with splenocytes from P-NP+Tuc-immunized mice (Fig. 3). To quantify the effect of tucaresol on CTL activity, we calculated

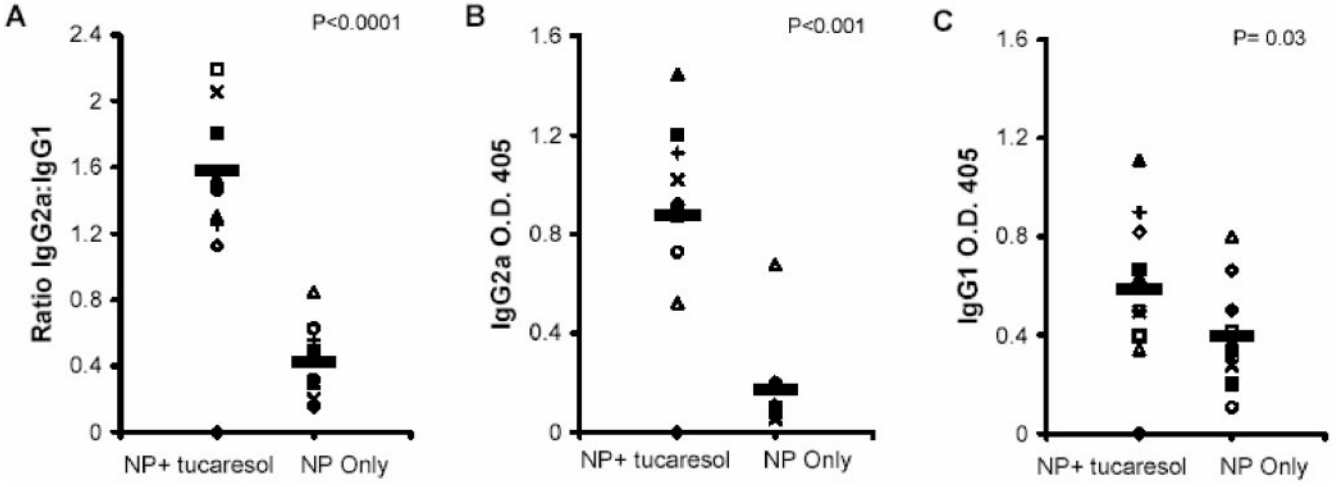

FIG. 4. Tucaresol enhances the production of Th1-associated antibody response and inverts the ratio of Th1 to Th2 induced by pDNA gene gun immunization. (A) The ratio of IgG2a to IgG1 was determined by specific ELISA and measured for each mouse. The concentration of anti-NP-specific antibodies was measured by ELISA at 405-nm optical density. Isotype-specific secondary antibodies were used to detect IgG2a (B) and IgG1 (C) concentrations in sera. Each symbol represents one mouse. Sera from 10 mice were tested per each group. *P* values are indicated in the upper right quadrant of each panel.

the LU_{12} contained within each of the effector cell groups from Fig. 3. This value was found to be 1,250 for effector cells of P-NP+Tuc-immunized mice, 147 for cells from P-NP-immunized mice, and 6 LU_{12} for effector cells of mice that were only treated with tucaresol. This shows that tucaresol enhances the CTL activity of P-NP immunization and results in an almost 10-fold increase in the cytotoxic activity. These data are similar to those obtained in previous studies with tucaresol (4, 6). In line with our present results, we did not detect cytolytic response using splenocytes from mice immunized only with a plasmid coding for a mycobacterial heat shock protein (Mhsp65) while we were able to detect a strong cytolytic response using splenocytes from the Mhsp65-immunized mice that have also received tucaresol (4–6). It appears therefore that tucaresol is a very potent enhancer of the cytolytic response with the ability to increase the precursor frequency or the efficacy of CD8 T cells in pDNA-immunized mice about 10 times compared to mice that did not receive tucaresol. This effect of tucaresol is particularly important for providing resistance to infections caused by viruses and intracellular bacteria (1). The role of $CD8^+$ CTL in the protection from heterologous influenza virus infection is well established by the use of $CD8^+$ CTL clones, CTL lines, or T-cell receptor (TCR) transgenic mice and believed to be dependent on the cytotoxic potential of the cell (13, 18, 19). It appears that CTL prevent the disease by first slowing the spread of the infection and then eliminating the pathogen reservoir (11, 13). Plasticity in the recognition of influenza virus-specific CTL (induced by natural infection) is not restricted to cross-strain protection but extends to other viral infections (29). A vaccine based on inducing CTL by utilizing full-length gene DNA vaccination is also superior to that utilizing single epitope peptide vaccination because such a DNA vaccine produces a broad type of response, thus decreasing the potential development of epitope-loss variants induced by point mutations that were reported to occur in mice transgenic for influenza virus-specific TCR (23). The induction of a strong CTL response by a combination of pDNA vaccination and tucaresol may therefore further reduce the risk of development of epitope loss variants.

High-titer IgG production is the result of B-cell differentiation into plasma cells that have received help by cytokine-producing T cells (1). Tucaresol has the ability to enhance the Th1-mediated immune response, as measured by both increased Th1 cytokine production and by inducing Th1-associated antibody-isotype switching and production (6, 25). It has recently been reported that the Th1-associated antibody response to Mhsp65 pDNA immunization was significantly enhanced by tucaresol administration, as detected by an increased level of IgG2a in the sera of the immunized mice (6). In the present study, we collected sera from Tuc-, P-NP-, or P-NP+Tuc-immunized mice to determine the type and level of the NP-specific antibody response induced by the immunization. Tucaresol has clearly favored a Th1-type antibody response, as measured by the IgG2a/IgG1 ratio seen in sera from P-NP+Tuc-immunized mice compared to sera from P-NP-immunized mice (Fig. 4A; $P < 0.0001$). This was accounted for by a significant increase in the levels of IgG2a in sera of mice immunized with P-NP+Tuc (Fig. 4B; $P < 0.001$). A predominantly Th1-favored antibody response is beneficial for protection from a viral infection, while a Th2 response is helpful for clearing the infection (1). Therefore, an ideal vaccine should enhance Th1 responses while concomitantly maintaining Th2 responses. Tucaresol-pDNA vaccination seems to be able to fulfill these requirements, since tucaresol also increased the Th2 response to NP, though to a much lesser extent (Fig. 4C; $P = 0.03$). These data are similar to those obtained in an earlier study in which tucaresol was used to enhance the antibody response to Mhsp65 (6).

Taken together, our data suggest a simple and direct modification to enhance gene gun-based pDNA immunization against influenza. Although limited information exists on the efficacy of pDNA immunization in humans, effective pDNA immunization has been achieved in both humanized animal models and in clinical studies in patients as well as volunteers (2, 5, 17, 33). The molecular mechanism by which tucaresol exerts its immunopotentiating effects remains to be defined. It has been shown that tucaresol binds to cell surface molecules on the T cells and provides a costimulatory signal which is different from that induced by TCR signaling (7). Nonetheless, tucaresol has been clinically used and proven to be efficiently taken up and to be safe even in doses substantially higher than those used here (27, 28). This could provide a significant incentive for clinical testing of a vaccine combination that contains a P-NP-based pDNA and tucaresol. Such a vaccination strategy is predicted to be safe and, according to the data presented here, efficient and would result in long-lasting protection (3) against heterologous strains of the virus (31).

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