Microbiol. Immunol., **49**(4), 349–353, 2005

Effect of Cytokine-Encoding Plasmid Delivery on Immune Response to Japanese Encephalitis Virus DNA Vaccine in Mice

Kaushik Bharati, Mohan Babu Appaiahgari, and Sudhanshu Vrati*

National Institute of Immunology, New Delhi 110 067, India

Received October 28, 2004; in revised form, January 19, 2005. Accepted January 25, 2005

***Abstract*: We have previously shown that immunization of mice with plasmid pMEa synthesizing Japanese encephalitis virus (JEV) envelope protein induced anti-JEV humoral and cellular immune responses. We now show that intra-muscular co-administration of mice with pMEa and pGM-CSF, encoding murine granulocyte-macrophage colony-stimulating factor or pIL-2, encoding murine interleukin-2 given 4 days after pMEa, augmented anti-JEV antibody titers. This did not enhance the level of protection in immunized mice against JEV. However, intra-dermal co-administration of pMEa and pGM-CSF in mice using the gene gun, enhanced anti-JEV antibody titers resulting in an increased level of protection in mice against lethal JEV challenge.**

***Key words*: Flavivirus, Adjuvant, Neutralization, Gene gun**

Japanese encephalitis virus (JEV) is a member of the *Flaviviridae* family of animal viruses, which includes several viruses of immense medical importance such as those causing dengue and yellow fever. The virus is distributed in vast geographic areas, including India, China, Japan, and virtually all of South-East Asia. JEV, transmitted by mosquitoes, is responsible for frequent epidemics of encephalitis affecting mostly young adults. Approximately 3 billion people live in JEV-endemic areas and up to 50,000 Japanese encephalitis cases are reported annually, of which ~10,000 prove to be fatal. Moreover, a high proportion of the survivors exhibit serious neurological and psychiatric sequelae (22). A mouse brain-grown, formalin-inactivated vaccine is available internationally, but is inherent with certain drawbacks; it is expensive to manufacture, provides immunity of uncertain duration, and may cause allergic reactions due to the inclusion of murine encephalogenic basic protein or gelatin stabilizer (15, 17). Thus, there is an urgent need to produce an alternate vaccine that may be safer and cheaper, and efforts are underway in this direction in various laboratories (9).

In recent years, plasmid DNA-based vaccination has emerged as a novel vaccination strategy having several advantages over diverse traditional vaccines. Thus, similar to infectious vaccines such as attenuated pathogens, plasmid vectors lead to activation of $CD8^+$ cytotoxic T-lymphocytes (CTLs) that play a major role in limiting the spread of the intracellular pathogens. In addition, DNA vaccines are non-infectious, are incapable of replication, do not express unwanted pathogenic proteins, and thus have enhanced safety compared to live attenuated vaccines. A number of candidate DNA vaccines against JEV have been developed using plasmids that express various structural or non-structural JEV proteins, and in a mouse model, these plasmids were found to provide protection of varying degree against lethal JEV challenge (16). Of these, plasmids expressing JEV envelope (E) protein were found to be most promising as they induced JEV neutralizing antibodies that are important indicators of protection (10).

Previously we described plasmid pMEa synthesizing JEV prM and E proteins and its immunogenicity in mice (8). These studies showed that intra-muscular (IM) or intra-dermal (ID) immunization of mice with pMEa generated protective immunity that was significantly lower than the near complete protection induced by the mouse brain-derived, formalin-inactivated commercial JEV vaccine. Thus, in order to improve the

**Address correspondence to* Dr. Sudhanshu Vrati, Virology Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, JNU Complex, New Delhi 110 067, India. Fax: +91–26162125. E-mail: vrati@nii.res.in

Abbreviations: APC, antigen presenting cell; CTL, cytotoxic T-lymphocyte; E, envelope; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; ID, intra-dermal; IL-2, interleukin-2; IM, intra-muscular; JEV, Japanese encephalitis virus.

efficacy of JEV DNA vaccines, methods need to be explored that could enhance anti-JEV antibody titers and in particular the virus-neutralizing antibodies.

In recent years immunological adjuvants such as the cytokine-encoding genes have increasingly been used to enhance the efficacy of DNA vaccines (2, 4, 6, 7, 12, 13, 18, 19, 23, 24). Two intensely investigated cytokines with proven adjuvant effects in the context of DNA vaccines are granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2). In the present study we investigated whether the immune responses elicited by the plasmid pMEa in mice could be augmented by administration of plasmids encoding murine GM-CSF and IL-2.

To study the effect of the cytokine-encoding plasmids on anti-JEV antibody titers, groups of 4-week-old BALB/c mice were immunized with plasmid pMEa with or without the murine GM-CSF-encoding plasmid pGM-CSF (kindly provided by Dr. O. Burrone, ICGEB, Trieste) and murine IL-2-encoding plasmid pIL-2 (kindly provided by Dr. R. Tuteja, ICGEB, New Delhi) by direct IM injection of the DNA using a needle. Figure 1 shows that anti-JEV antibodies were detectable in all immunization groups 2 weeks after the primary immunization. The antibody titers increased following the booster doses. Administration of pGM-CSF along with pMEa led to an enhancement of anti-JEV antibody titer. Thus after the second booster dose, ~2-fold higher antibody titers ($P=0.06$) were recorded in mice immunized with pMEa+pGM-CSF when compared with titers in those mice immunized with pMEa alone. Co-administration of pIL-2 with pMEa did not affect anti-JEV antibody titers. However, when pIL-2 was given 4 days after the delivery of pMEa, the titers increased ~2.6-fold compared to those obtained from pMEa injection alone ($P=0.07$).

In parallel, groups of 4-week-old BALB/c mice were immunized with plasmid pMEa with or without cytokine-encoding plasmids pGM-CSF by ID delivery of the DNA using a gene gun. Figure 1 shows that anti-JEV antibodies were detectable in all immunization groups 2 weeks after the primary immunization and these titers increased following the booster doses. For different immunization groups, anti-JEV antibody titers were generally higher in ID-immunized mice than in those immunized IM. Mice immunized ID with pMEa and pGM-CSF developed ~2-fold higher anti-JEV titers than those immunized with pMEa alone ($P=0.44$). Timing of pGM-CSF delivery did not make any appreciable difference. Thus mice that received pGM-CSF together with pMEa had similar anti-JEV titers, as did those receiving pGM-CSF 4 days before the inoculation of pMEa.

Analysis of the isotypes of anti-JEV antibodies indicated that the IM inoculation of the plasmids generated higher levels of IgG2a compared to IgG1 and the reverse was true in the case of ID inoculation of the plasmids by the gene gun (data not shown). Thus IM

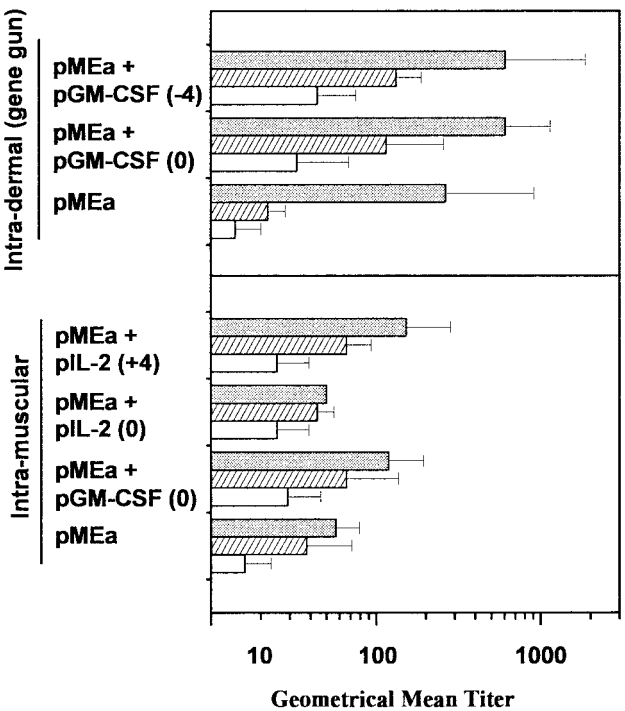

Fig. 1. Groups of 4-week-old BALB/c mice were immunized with pMEa, with or without the plasmid encoding murine GM-CSF or IL-2, by the IM needle injection or ID using the gene gun. For the IM immunizations, mice were injected with 50 μg pMEa with or without 50 μg of plasmids pGM-CSF or pIL-2 into the quadricep muscle mass of the hind leg. Numbers in parenthesis next to the cytokine-encoding plasmids indicate the time of injection. Thus, 0 indicates that the cytokine-encoding plasmid was given at the same time as pMEa; +4 indicates that the plasmid was given 4 days after pMEa and −4 indicates that the plasmid was given 4 days ahead of pMEa delivery. All mice received two booster doses (containing the same amount of the plasmid as in the primary dose) 3 and 5 weeks after the primary injection. For the ID immunizations, plasmid DNA was delivered into the epidermal layer of the skin using a hand-held helium-driven Helios gene gun (Bio-Rad, U.S.A.) as described before (8). Each animal received two shots (0.5 μg) of pMEa together with or without the cytokine-encoding plasmid for priming and one shot each (0.25 μg) of pMEa together with or without the cytokine-encoding plasmid for subsequent booster doses. Mice were bled 1 week before the first booster dose and 1 week after each booster dose. ELISA was used to assay the 2-fold serial dilutions of sera (starting at 1:12.5) for the end-point anti-JEV antibody titers (8). Shown above are geometrical mean titers (as bars) and standard deviation. The hollow bars represent the pre-booster titers, the hashed bars represent the titers after the first booster dose, and the gray-colored bars represent the titers after the second booster dose.

inoculation of plasmid DNA generated Th1-type immune responses whereas these were of Th2-type for the ID delivery of DNA using the gene gun. Co-administration of the cytokine-encoding plasmids did not alter the quality of the immune responses.

To study the effect of the cytokine-encoding plasmid delivery on JEV neutralizing antibody titers, serum samples from mice obtained 1 week after the second booster dose were studied by the plaque reduction neutralization assay (21). Figure 2 shows that IM administration of pMEa along with pGM-CSF resulted in a small enhancement (~1.7-fold) of JEV neutralizing antibody titers when compared with those obtained after pMEa administration ($P=0.18$). Co-administration of pIL-2 had no effect on JEV neutralizing antibody titers induced by pMEa. In general, compared to the IM inoculation of the plasmids, the ID delivery of plasmids using the gene gun induced higher JEV neutralizing antibody titers. Thus, pMEa given ID induced 3-fold higher JEV neutralizing titers than those induced by the IM inoculation of pMEa ($P=0.07$). Administration of pGM-CSF along with pMEa did not alter JEV-neutralizing antibody titers in comparison with those obtained after pMEa delivery. However, administration of pGM-CSF 4 days ahead of pMEa delivery, led to ~1.5-fold increase in JEV neutralization titer when compared with titers obtained in mice immunized with pMEa alone ($P=0.31$).

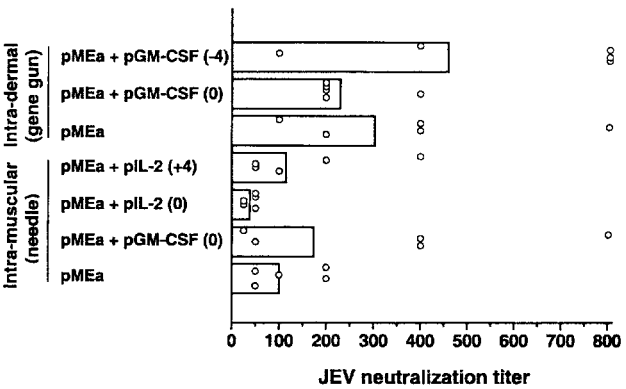

Fig. 2. Mice were immunized with pMEa, with or without the plasmid encoding murine GM-CSF or IL-2, by the IM needle injection or ID using the gene gun. Numbers in parenthesis next to the cytokine-encoding plasmids indicate the time of injection as described before. Mice were bled 1 week after the second booster dose. Two-fold serial dilutions of sera (starting at 1:25) were assayed for the end-point anti-JEV neutralizing antibody titers (21). The bars above show the geometrical mean titers. The open circles show JEV neutralizing antibody titers in individual mice.

To study the effect of the cytokine-encoding plasmids on the efficacy of the immunization, the immunized mice were challenged by intra-cerebral inoculation of a highly lethal dose (100 LD_{50}) of JEV. Table 1 shows that the mice immunized IM with pMEa demonstrated 80% protection against lethal JEV challenge and the level of protection did not increase further with the co-administration of the cytokine-encoding plasmids. The ID immunization of mice using the gene gun gave 40% protection and this figure increased to 80% when cytokine-encoding plasmids were co-administered ($P=0.26$). None of the unimmunized control mice was protected against the virus challenge.

Marrow-derived antigen-presenting cells (APCs) play a central role in priming naïve T cells after DNA vaccination (1). A substantial body of evidence indicates that the growth, proliferation, differentiation, as well as recruitment of APCs, including the dermal

Table 1. Protection of mice challenged with the lethal dose of JEV

Immunogen	Route and mode of immunization	No. of mice surviving/ challenged	Protection (%)
pMEa	IM, Needle	4/5	80
pMEa+pGM-CSF (0)	IM, Needle	4/5	80
pMEa+pIL-2 (0)	IM, Needle	4/5	80
pMEa+pIL-2 (+4)	IM, Needle	4/5	80
pMEa	ID, Gene gun	2/5	40
pMEa+pGM-CSF (0)	ID, Gene gun	4/5	80
pMEa+pGM-CSF (−4)	ID, Gene gun	4/5	80
None	—	0/5	0

Mice were immunized with pMEa, with or without the plasmid encoding murine GM-CSF or IL-2, by the IM needle injection or ID using the gene gun. Numbers in parenthesis next to the cytokine-encoding plasmids indicate the time of injection. Mice were challenged with 100-fold 50% lethal dose (LD_{50}) of JEV given intra-cerebrally. Mice were observed for mortality for the next 4 weeks. The table above shows the numbers of mice surviving the virus challenge and the percent protection.

Langerhans cells, is strongly influenced by GM-CSF, a secreted glycoprotein of the haematopoietic colony-stimulating factor family. Therefore, the prominent role of GM-CSF, when used as an adjuvant appears to be its capability to function as an attractant, and as a growth and differentiation factor for the local APCs. Thus, co-inoculation of a plasmid expressing murine GM-CSF with a rabies DNA vaccine enhanced anti-rabies virus antibody titers in mice (23). In addition, inoculation of pGM-CSF 5 days prior to the exposure to plasmid encoding β-gal antigen brought about an enhanced IgG response to β-gal (5). Similarly, administration of pGM-CSF 3 days prior to immunization with the cottontail rabbit papillomavirus E6 DNA vaccine resulted in greatly augmented effects of the vaccination (11). It can thus be seen that different workers have used different protocols for pGM-CSF administration with respect to the DNA vaccine. In our experiments, we have compared co-administration of pGM-CSF with the situation where it was delivered 4 days prior to the delivery of pMEa. We found that co-inoculation of pGM-CSF along with pMEa, both by IM and ID routes, augmented anti-JEV antibody titers. Inoculation of pGM-CSF 4 days ahead of pMEa also enhanced anti-JEV antibody titers and this enhancement was similar to that achieved by the co-inoculation of the plasmids. Thus DNA vaccine co-inoculation with pGM-CSF may be the preferred protocol, as it would mean one less injection.

IL-2 is another cytokine that has been extensively explored as an adjuvant since IL-2 activates macrophage functions and stimulates the release of secondary mediators such as GM-CSF (14). Inoculation of IL-2 within a few days after the priming with the antigen appears to be optimal for the enhancement of immune responses (3, 20, 23). However, Geissler et al. achieved enhanced immune response to hepatitis C core protein by co-administration of IL-2-encoding plasmid and the hepatitis C core DNA expression plasmid (6). In the present study, we administered pIL-2 at the same time or 4 days post-administration of pMEa. The IM co-administration of pIL-2 and pMEa did not affect anti-JEV antibody titers; however when pIL-2 was given 4 days after pMEa, the antibody titers were higher than those obtained in mice immunized with pMEa alone. Administration of pIL-2 at the same time as the pMEa by the ID route did not affect anti-JEV antibody titers (data not shown). This was not entirely unexpected, as ID delivery of DNA using the gene gun is known to induce Th2 kind of immune responses where IL-2 may not have any significant role.

Similar to anti-JEV antibody titers by ELISA, the JEV-neutralizing antibody titers were also enhanced when cytokine-encoding plasmids were used for immunization in conjunction with pMEa although the increase in neutralizing antibody titers was not significant statistically due to the large variation recorded in titers. The small increase in anti-JEV antibody titers in mice immunized IM with pMEa along with the cytokine-encoding plasmids did not result in an enhancement of the level of protection of mice against lethal JEV challenge. This may perhaps be due to the supralethal dose (100 LD_{50}) that was used for the challenge experiments. However, in the case of ID delivery of the DNA, co-immunization with pMEa and pGM-CSF led to an enhanced level of protection in immunized mice against lethal JEV challenge. This may be because of a larger increase in anti-JEV antibody titers in the case of ID-delivered pMEa and the cytokine-encoding plasmids when compared with the titers obtained from pMEa and the cytokine-encoding plasmids delivered IM. It may be noted that the level of protection of mice after ID plasmid delivery was lower than that obtained after the IM plasmid delivery. This may simply be related to the lower amounts of DNA used for the ID immunization (0.5 μg) than those used in IM immunization (50 μg). Significantly, ID co-administration of small amounts of pMEa along with pGM-CSF resulted in protection levels that were similar to those obtained from IM inoculation of the higher amounts of pMEa. Thus ID co-administration of pMEa and pGM-CSF by gene gun may be a preferred method of immunization against JEV requiring only small amounts of DNA.

This work was supported by the Department of Biotechnology, Govt. of India grant no. BT/Med/JEV/02/02.

References

1) Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D., and Stockinger, B. 1999. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J. Exp. Med. **189**: 169–178.
2) Barouch, D.H., Craiu, A., Kuroda, M.J., Schmitz, J.E., Zheng, X.X., Santra, S., Frost, J.D., Krivulka, G.R., Lifton, M.A., Crabbs, C.L., Heidecker, G., Perry, H.C., Davies, M.E., Xie, H., Nickerson, C.E., Steenbeke, T.D., Lord, C.I., Montefiori, D.C., Strom, T.B., Shiver, J.W., Lewis, M.G., and Letvin, N.L. 2000. Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys. Proc. Natl. Acad. Sci. U.S.A. **97**: 4192–4197.
3) Barouch, D.H., Santra, S., Steenbeke, T.D., Zheng, X.X., Perry, H.C., Davies, M.E., Freed, D.C., Craiu, A., Strom, T.B., Shiver, J.W., and Letvin, N.L. 1998. Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration. J. Immunol. **161**: 1875–1882.

4) Boyer, J.D., Cohen, A.D., Ugen, K.E., Edgeworth, R.L., Bennett, M., Shah, A., Schumann, K., Nath, B., Javadian, A., Bagarazzi, M.L., Kim, J., and Weiner, D.B. 2000. Therapeutic immunization of HIV-infected chimpanzees using HIV-1 plasmid antigens and interleukin-12 expressing plasmids. AIDS **14**: 1515–1522.
5) Burger, J.A., Mendoza, R.B., and Kipps, T.J. 2001. Plasmids encoding granulocyte-macrophage colony-stimulating factor and CD154 enhance the immune response to genetic vaccines. Vaccine **19**: 2181–2189.
6) Geissler, M., Gesien, A., Tokushige, K., and Wands, J.R. 1997. Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. J. Immunol. **158**: 1231–1237.
7) Harrison, R.A., Richards, A., Laing, G.D., and Theakston, R.D. 2002. Simultaneous GeneGun immunisation with plasmids encoding antigen and GM-CSF: significant enhancement of murine antivenom IgG1 titres. Vaccine **20**: 1702–1706.
8) Kaur, R., Sachdeva, G., and Vrati, S. 2002. Plasmid DNA immunization against Japanese encephalitis virus: immunogenicity of membrane-anchored and secretory envelope protein. J. Infect. Dis. **185**: 1–12.
9) Kaur, R., and Vrati, S. 2003. Development of a recombinant vaccine against Japanese encephalitis. J. Neurovirol. **9**: 421–431.
10) Konishi, E., Yamaoka, M., Khin, S.W., Kurane, I., Takada, K., and Mason, P.W. 1999. The anamnestic neutralizing antibody response is critical for protection of mice from challenge following vaccination with a plasmid encoding the Japanese encephalitis virus premembrane and envelope genes. J. Virol. **73**: 5527–5534.
11) Leachman, S.A., Tigelaar, R.E., Shlyankevich, M., Slade, M.D., Irwin, M., Chang, E., Wu, T.C., Xiao, W., Pazhani, S., Zelterman, D., and Brandsma, J.L. 2000. Granulocyte-macrophage colony-stimulating factor priming plus papillomavirus E6 DNA vaccination: effects on papilloma formation and regression in the cottontail rabbit papillomavirus-rabbit model. J. Virol. **74**: 8700–8708.
12) Lee, S.W., Cho, J.H., and Sung, Y.C. 1998. Optimal induction of hepatitis C virus envelope-specific immunity by bicistronic plasmid DNA inoculation with the granulocyte-macrophage colony-stimulating factor gene. J. Virol. **72**: 8430–8436.
13) Moore, A.C., Kong, W.P., Chakrabarti, B.K., and Nabel, G.J. 2002. Effects of antigen and genetic adjuvants on immune responses to human immunodeficiency virus DNA vaccines in mice. J. Virol. **76**: 243–250.
14) Oppenheim, J.J., Zachariae, C.O., Mukaida, N., and Matsushima, K. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu. Rev. Immunol. **9**: 617–648.
15) Plesner, A.M., and Ronne, T. 1997. Allergic mucocutaneous reactions to Japanese encephalitis vaccine. Vaccine **15**: 1239–1243.
16) Putnak, R., Porter, K., and Schmaljohn, C. 2003. DNA vaccines for flaviviruses. Adv. Virus Res. **61**: 445–468.
17) Sakaguchi, M., Yoshida, M., Kuroda, W., Harayama, O., Matsunaga, Y., and Inouye, S. 2000. Systemic immediate-type reactions to gelatin included in Japanese encephalitis vaccines. Vaccine **15**: 121–122.
18) Sin, J.I., Kim, J.J., Ugen, K.E., Ciccarelli, R.B., Higgins, T.J., and Weiner, D.B. 1998. Enhancement of protective humoral (Th2) and cell-mediated (Th1) immune responses against herpes simplex virus-2 through co-delivery of granulocyte-macrophage colony-stimulating factor expression cassettes. Eur. J. Immunol. **28**: 3530–3540.
19) Sin, J.I., Sung, J.H., Suh, Y.S., Lee, A.H., Chung, J.H., and Sung, Y.C. 1997. Protective immunity against heterologous challenge with encephalomyocarditis virus by VP1 DNA vaccination: effect of coinjection with a granulocyte-macrophage colony stimulating factor gene. Vaccine **15**: 1827–1833.
20) Tuteja, R., Li, T.C., Takeda, N., and Jameel, S. 2000. Augmentation of immune responses to hepatitis E virus ORF2 DNA vaccination by codelivery of cytokine genes. Viral Immunol. **13**: 169–178.
21) Vrati, S., Agarwal, V., Malik, P., Wani, S.A., and Saini, M. 1999. Molecular characterization of an Indian isolate of Japanese encephalitis virus that shows an extended lag phase during growth. J. Gen. Virol. **80**: 1665–1671.
22) World Health Organization. 1998. Japanese encephalitis vaccines. Wkly. Epidemiol. Rec. **73**: 334–344.
23) Xiang, Z., and Ertl, H.C. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. Immunity **2**: 129–135.
24) Xin, K.Q., Hamajima, K., Sasaki, S., Honsho, A., Tsuji, T., Ishii, N., Cao, X.R., Lu, Y., Fukushima, J., Shapshak, P., Kawamoto, S., and Okuda, K. 1998. Intranasal administration of human immunodeficiency virus type-1 (HIV-1) DNA vaccine with interleukin-2 expression plasmid enhances cell-mediated immunity against HIV-1. Immunology **94**: 438–444.