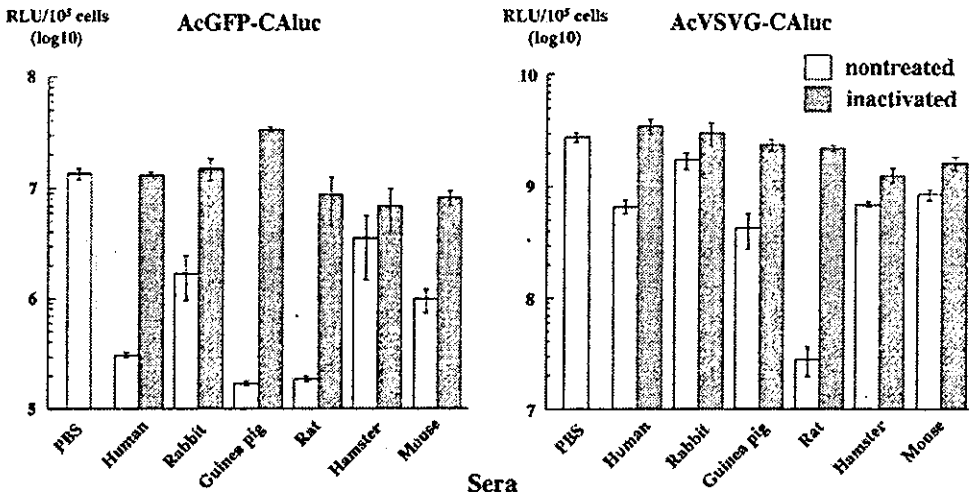

FIG. 6. Effect of VSVG incorporation into virions on inactivation of baculovirus by animal serum. Luciferase expression in HepG2 cells infected with either AcVSVG-CAluc or AcGFP-CAluc was examined after incubation with either untreated or heat-inactivated animal serum. Values for relative light units (RLU) reflect values obtained for the extracts from 10^5 cells. The results shown are the averages of three independent assays, with the error bars representing the standard deviation.

Effects of serum on gene transduction by recombinant baculoviruses. Previous studies suggest that baculoviral gene transfer into hepatocytes is strongly reduced in the presence of serum complement (11, 35). Pseudotype retroviruses possessing VSVG protein exhibited more resistance to serum components than those lacking the protein (30). To compare the susceptibility of the recombinant baculoviruses to inactivation with serum complement, AcVSVG-CAluc and AcGFP-CAluc were incubated for 1 h at 37°C with either untreated or heat-inactivated serum from various animals at a final concentration of 90%. Residual infectivity was determined following inoculation into HepG2 cells (Fig. 6). Significant reductions in luciferase expression following AcGFP-CAluc infection were observed following incubation with serum from human, guinea pig, and rat; only moderate or slight reductions in infectivity were observed following incubation with rabbit, hamster, and mouse serum. AcVSVG-CAluc, however, exhibited resistance to the serum treatment, with the exception of rat serum. Reductions in inactivation were observed in human and guinea pig serum. The reduction of gene expression following serum treatment was abolished by inactivation of the serum by incubation at 56°C for 30 min. These results indicate that incorporation of VSVG protein into baculoviruses confers resistance against inactivation by serum complement; the serum from rabbit, hamster, and mouse possesses relatively weaker inactivation effects against baculoviruses than that from human, guinea pig, and rat.

Effects of FUT-175 on serum inactivation of baculovirus. Baculovirus activates the classical complement system, leading to viral inactivation through the assembly of late complement components (11, 35). To circumvent serum inactivation, we examined the effects of a complement pathway inhibitor, FUT-175, on gene transduction by AcGFP-CAluc in the presence of rat and human serum. AcGFP-CAluc was incubated with 40% rat or human serum in the presence of various concentrations of FUT-175 for 1 h at 37°C. The remaining infectious titer was determined by infection of HepG2 cells (Fig. 7). Treatment with FUT-175 suppressed serum inactivation of AcGFP-CAluc in a dose-dependent manner. Particularly, complete restoration of infectivity to control levels was obtained at a concentration of 1 μg/ml in human serum.

Gene transfer in vivo. Sarkis et al. demonstrated GFP expression in mouse and rat neural cells after immunostaining of brains injected with an unmodified baculovirus containing the GFP gene under the control of the cytomegalovirus promoter together with cobra venom factor, an inhibitor of the complement system, with a stereotaxic apparatus (37). Recently, Lehtolainen et al. showed that unmodified baculovirus transduced choroid plexus cells in rat ventricles (19). To examine the ability of the VSVG-modified baculovirus to mediate more efficient gene transduction into the cerebrum following ordinary injection routes, AcVSVG-CAGFP was injected directly into the mouse brain. GFP expression at the brain surface of the injected areas was examined by fluorescence stereomicroscopic observation 2 days after injection. GFP expression was clearly detected in areas of the brain corresponding to the injection route of AcVSVG-CAGFP with a fluorescence stereomicroscope (Fig. 8A). Cross sections of the mouse cerebrum following injection with the recombinant baculovirus clearly revealed GFP expression in the cerebral cortex. Immunohistochemical staining of sections with antibodies against GFAP and MAP2 revealed that both astrocytes and pyramidal cells were transduced, expressing GFP following injection with the baculovirus (Fig. 8B).

We also inoculated AcVSVG-CAGFP into mouse testes via the efferent ductules. GFP expression in the testes was examined by fluorescence stereomicroscopy 2 days postinfection. High levels of GFP expression were observed in the whole mouse testes and in the seminiferous tubules (Fig. 9). In sections, we observed clear GFP expression in the basal and Sertoli cells, but not in spermatocytes or sperm cells.

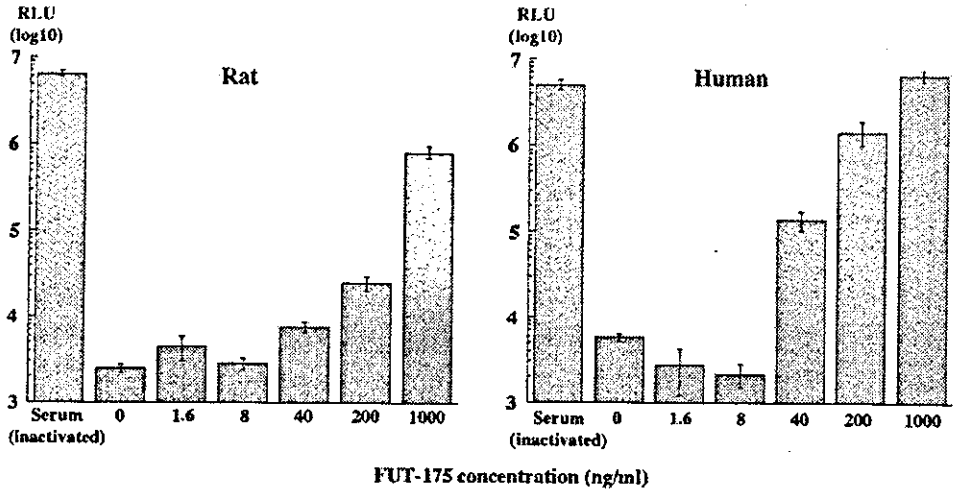

FIG. 7. Effect of FUT-175 on inactivation of baculovirus by rat and human serum. AcGFP-CAluc was incubated with medium containing 40% animal serum with various concentrations of FUT-175. Remaining baculovirus infectivity was evaluated by luciferase expression following infection of HepG2 cells. Values for relative light units (RLU) reflect values obtained for the extracts from 10^5 cells. The results shown are the averages of three independent assays, with the error bars representing the standard deviation.

DISCUSSION

Viral vectors derived from naturally occurring viruses, such as adenoviruses, adeno-associated viruses, retroviruses, and herpesviruses, are highly adapted to their natural hosts, providing the means for efficient gene transfer into cultured cells, animal models, and possibly patients (24). Baculovirus vectors, which can incorporate large DNA inserts, efficiently infect not only insect cells but also various mammalian cell lines without apparent viral replication or cytopathic effects (18, 33, 36). Furthermore, injection of baculovirus into the brain did not reveal any major safety issues, as evaluated by several clinical chemistry analyses (19). The emergence of replication-competent virus breakthroughs is a major concern discouraging the in vivo use of replication-incompetent viral vectors, such as replication-deficient adenovirus. No evidence of viral replication and transcription of the baculovirus genome, however, has been detected in mammalian cells (42).

Baculovirus vectors are used for a multitude of applications, including the production of virus-like particles and viral display systems (6). Similar to retroviral vectors, the efficiency of gene delivery into mammalian cells by baculoviruses was enhanced by the incorporation of foreign envelope proteins into virions (3, 41). In this study, the VSVG-modified baculovirus delivered reporter genes efficiently not only into neural cell lines, but also into primary rat neural cells. We also constructed an RVG-modified baculovirus for use in gene transduction into neural cells, as rabies virus is known to utilize the nicotinic acetylcholine receptor (9) and the low-affinity nerve growth factor receptor P75NTR (44) for entry. The RVG-modified virus exhibited a 10- to 500-fold-higher efficiency of gene transduction into neural cell lines than the unmodified control baculovirus. The RVG and VSVG recombinants, however, gave similar infectivities on neuronal cells in vivo. This result suggests that neural cells may express similar levels of receptors for both rabies virus and VSV.

Gene delivery to osseous tissue is essential for genetic treatment of bone diseases. Osteoblast and osteoclast cells are involved in bone formation and resorption, respectively. Disorders of these cells lead to bone diseases, such as osteopetrosis, osteosclerosis, and osteoporosis (15). Adenovirus and VSVG pseudotype retrovirus vectors can transduce foreign genes into some osteocytes in vitro and in vivo (2, 14, 21). The infectivities of VSVG-modified baculoviruses are also higher than those of the recombinant baculovirus possessing excess gp64 envelope protein. Although the infectivity of the VSVG-modified baculovirus to primary mouse osteoblast and osteoclast cells was lower than those exhibited for other cell types, these cells did not demonstrate any cytopathic effects, even at a high MOI. Furthermore, luciferase expression did not increase following infection at an MOI higher than 10^4 (data not shown), suggesting that the receptors for the VSVG-modified baculovirus on osteal cells were saturated at this point.

Baculoviruses are thought to be inactivated by serum complement in organs in direct contact with complement components (11, 35). Hüser et al. reported that the incorporation of human decay-accelerating factor into the viral envelope together with gp64 confers resistance to inactivation by serum complement, suggesting that it may be possible to circumvent complement inactivation with appropriate genetic strategies (12). The VSVG-modified baculovirus exhibited greater resistance to animal serum inactivation than the unmodified control baculovirus, similar to other pseudotype retrovirus systems (30). Barsoum et al. hypothesized that VSVG recombinant baculovirus conferred resistance to complement, imparting the ability to perform gene transduction into mouse hepatocytes following tail vein injection (3). Pieroni et al. demonstrated increased gene delivery into mouse quadriceps after direct intramuscular injection of VSVG-modified baculovirus, which partially bypasses the complement system (32). Although mouse serum had only a small effect on VSVG-modified baculovirus infectivity (Fig. 6), we could not detect either luciferase or GFP expression following injection of baculoviruses

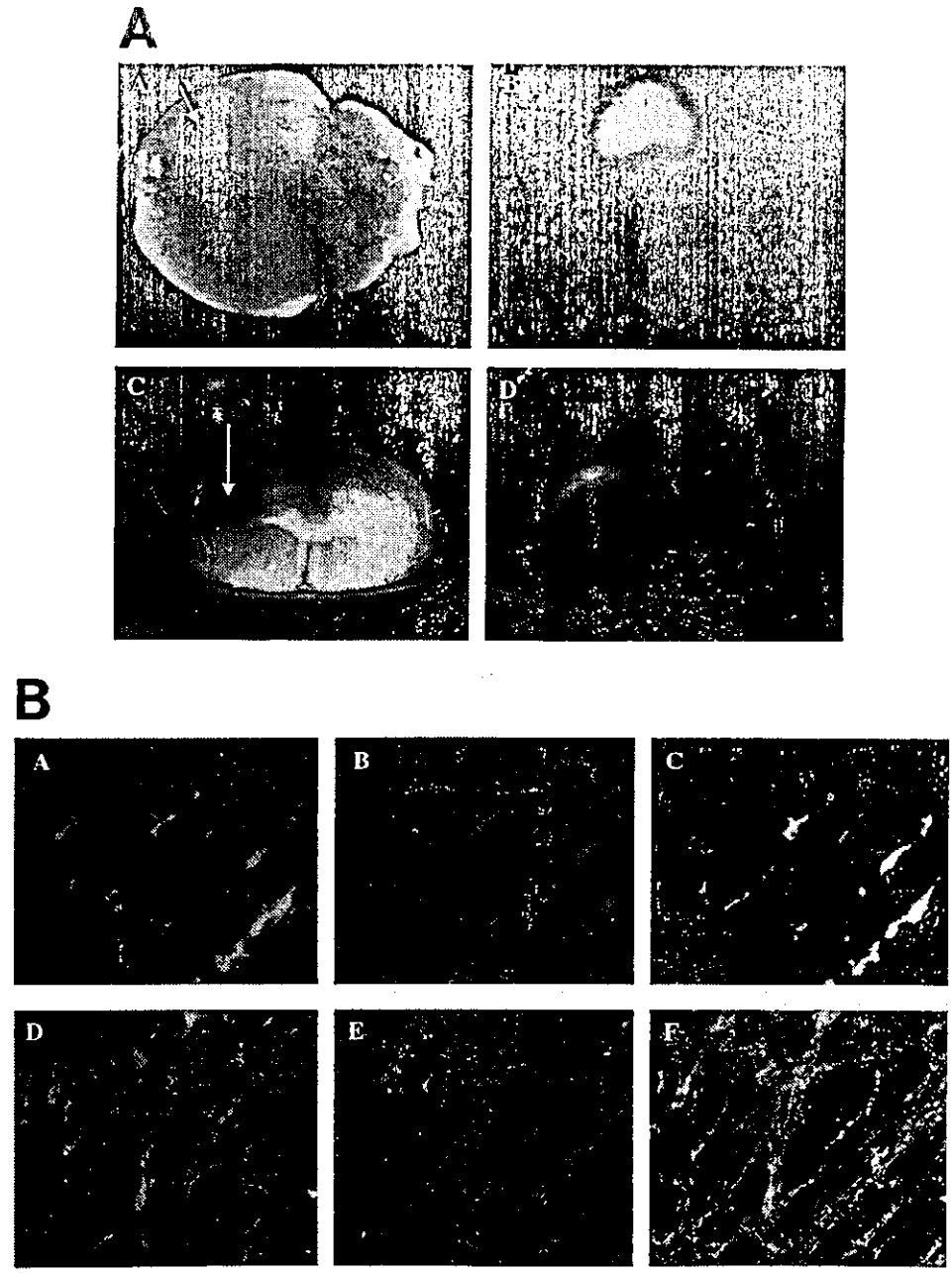

FIG. 8. GFP expression in mouse brains after cerebral injections of AcVSVG-CAGFP. Mice were injected with 4×10^7 PFU of AcVSVG-CAGFP in the right lateral ventricle. GFP expression in the brain was examined by fluorescent stereomicroscopy 2 days after injection. (A) Panels A to D are stereomicroscopic images of whole brain (A and B) and brain cross sections (C and D). Panels A and C are bright-field views, while panels B and D are fluorescent views. Arrows and dark staining indicate the injection route, as the infiltrated viral inoculum contained 0.04% trypan blue. (B) Immunohistochemical staining of the cryostat sections was examined by fluorescence microscopy following staining with antibodies specific for GFP (A and D), GFAP as a glial marker (B), or MAP2 as a neuronal marker (E). Panels C and F are merged images.

into mice by the intravenous, intraperitoneal, or intrahepatic route (data not shown). These results suggest that host factors other than the serum complement system inactivate baculoviruses in mice.

We employed the synthetic protease inhibitor FUT-175 to prevent complement activation during baculovirus infection. FUT-175, which inhibits the complement pathways, has been used clinically for more than 20 years in Japan for treatment of patients with acute pancreatitis and disseminated intravascular coagulation. No serious side effects have been associated with its use (13, 23, 31). FUT-175 specifically binds the Bb fragment of factor B, an important enzyme in the alternative complement pathway. FUT-175 is also incorporated into the active site of the intermediary C1r form, inhibiting both the alterna-

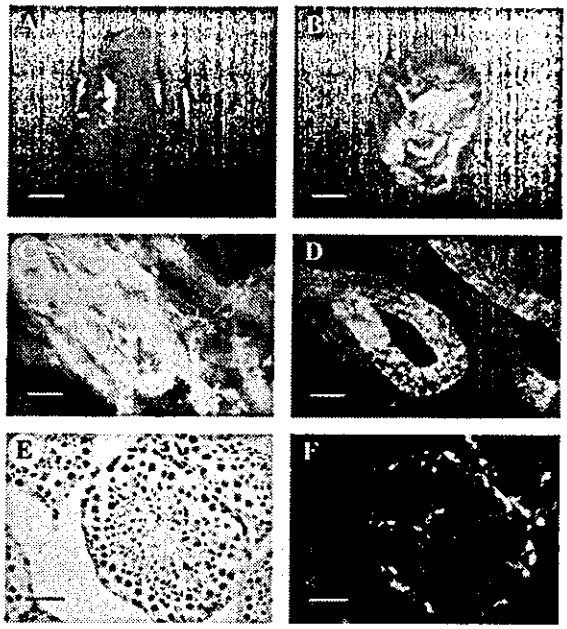

FIG. 9. GFP expression in mouse testis after inoculation of AcVSVG-CAGFP. Mice were injected with 2×10^8 PFU of AcVSVG-CAGFP via the efferent ductules. GFP expression in the testes was examined by fluorescent stereomicroscopy 2 days after injection. Panels A and B are stereomicroscopic images of whole testis. Panels C and D are images of the efferent ductules, while panels E and F are microscopic images of cross sections of the seminiferous tubules. The left-hand panels are bright-field views. The right-hand panels are fluorescent views. Panel E is the same section as panel F stained with hematoxylin. The scale bars in the upper, middle, and lower panels represent 1 mm, 200 nm, and 50 nm, respectively.

tive and classical pathways of the complement system (13, 26). This compound also protects retroviral vectors against serum inactivation (23). The infectivity of the unmodified control baculovirus could be recovered in the presence of FUT-175 in a dose-dependent manner, suggesting that FUT-175 prevents activation of complement-mediated inactivation. Although it is difficult to prevent the activation of the entire complement system in vivo with only FUT-175, due to its short half-life, in vivo clinical application of FUT-175 in combination with the VSVG-modified baculovirus system may prove highly effective.

Previous studies demonstrated that recombinant baculoviruses, including envelope-modified viruses, can transfer reporter genes into human liver segments ex vivo (35) and rat hepatocytes (12), rabbit carotid artery (1), mouse skeletal muscle (32), rodent glial cells (37), and mouse retinal cells in vivo (8). Gene transfer by baculovirus vectors in vivo has not been successful, however, in organs directly exposed to the complement system. In this study, we demonstrated efficient gene transduction of GFP into mouse brain and testes by direct injection of a GFP-encoding VSVG-modified baculovirus. For gene transfer into testes, foreign gene delivery has been mediated by previous viral vectors, such as adenovirus (4), and with nonviral vectors through both lipofection (16) and electroporation (25). Furthermore, we demonstrated that baculovirus vectors are capable of delivering foreign genes to the interstitial compartment of the adult mouse testes. Although histochemical studies of the infected testis indicated that expression of the introduced gene extended into the innermost region of the testes, the GFP-expressing cells were confined to the spermatogenic cells and Sertoli cells within the seminiferous tubules.

Although the reason underlying the lack of gene expression in sperm cells is not known, construction of a recombinant baculovirus possessing a ligand specific for sperm cells will determine the ability of baculoviral vectors for gene transduction into these cells. For gene transfer into the central nervous system, Sarkis et al. demonstrated reporter gene expression in the brains of mice and rats following direct injection with unmodified baculovirus. Using a stereotaxic apparatus as a precaution to avoid hemorrhage, they obtained results suggesting that the baculovirus was not inactivated by the complement system within the brain (37). In this study, we could detect GFP expression in the mouse cerebral cortex by fluorescence stereomicroscopy following injection of the VSVG-modified baculovirus into the brain. These results indicate that the VSVG-modified baculovirus is a promising vector for gene delivery into the brain.

Gronowski et al. demonstrated that baculovirus is able to stimulate interferon production from both human and mouse cells in vitro. Pretreatment with baculovirus also confers protection against lethal challenge of mice with encephalomyocarditis virus (7). Inhibition of activation by either antibodies against gp64 or UV inactivation suggested that virus-dependent processing, in addition to the interaction of gp64 with cell surface molecules, is required for the reaction. Induction of the innate immune response following administration of baculovirus is important for future applications of baculovirus vectors in vivo, not only for gene therapy but also for vaccine trials.

In summary, we have investigated the feasibility of gene transfer with recombinant baculoviruses in vitro and in vivo. Further studies examining the transcription of baculovirus genes in mammalian cells will be required for certification of safety for in vivo use. In addition, the development of a vector capable of targeting specific organs is needed for future in vivo applications of the baculovirus vector in the treatment of acquired or inherited diseases in humans.

ACKNOWLEDGMENTS

We thank S. Ogawa for excellent technical assistance and M. A. Whitt for critical reading of the manuscript and helpful discussions.

This work was supported in part by grants-in-aids from the Ministry of Health, Labor and Welfare to Y.M.

REFERENCES

1. **Airenne, K. J., M. O. Hiltunen, M. P. Turunen, A-M. Turunen, O. H. Laitinen, M. S. Kulomaa, and S. Ylä-Herttuala.** 2000. Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. Gene Ther. 7:1499–1504.
2. **Baltzer, A. W., J. D. Whalen, M. Stefanovic-Racic, B. Ziran, P. D. Robbins, and C. H. Evans.** 1999. Adenoviral transduction of human osteoblastic cell cultures: a new perspective for gene therapy of bone diseases. Acta Orthop. Scand. 70:419–424.
3. **Barsoum, J., R. Brown, M. McKee, and F. M. Boyce.** 1997. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum. Gene Ther. 8:2011–2018.
4. **Blanchard, K. T., and K. Boekelheide.** 1997. Adenovirus-mediated gene transfer to rat testis in vivo. Biol. Reprod. 56:495–500.
5. **Boyce, F. M., and N. L. R. Bucher.** 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. USA 93:2348–2352.
6. **Grabherr, R., W. Ernst, C. Oker-Blom, and I. Jones.** 2001. Developments in the use of baculoviruses for the surface display of complex eukaryotic proteins. Trends Biotechnol. 19:231–236.

7. Gronowski, A. M., D. M. Hilbert, K. C. F. Sheehan, G. Garotta, and R. D. Schreiber. 1999. Baculovirus stimulates antiviral effects in mammalian cells. J. Virol. 73:9944–9951.
8. Haeseleer, F., Y. Imanishi, D. A. Saperstein, and K. Palczewski. 2001. Gene transfer mediated by recombinant baculovirus into mouse eye. Investig. Ophthalmol. Vis. Sci. 42:3294–3300.
9. Hanham, C. A., F. Zhao, and G. H. Tignor. 1993. Evidence from the anti-idiotypic network that the acetylcholine receptor is a rabies virus receptor. J. Virol. 67:530–542.
10. Hofmann, C., V. Sandig, G. Jennings, M. Rudolph, P. Schlag, and M. Strauss. 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. USA 92:10099–10103.
11. Hofmann, C., and M. Strauss. 1998. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene Ther. 5:531–536.
12. Hüser, A., M. Rudolph, and C. Hofmann. 2001. Incorporation of decay-accelerating factor into the baculovirus envelope generates complement-resistant gene transfer vectors. Nat. Biotechnol. 19:451–455.
13. Ikari, N., Y. Sakai, Y. Hitomi, S. Fujii. 1983. New synthetic inhibitor to the alternative complement pathway. Immunology 49:685–691.
14. Kalajzic, I., M. L. Stover, P. Liu, Z. Kalajzic, D. W. Rowe, and A. C. Lichtler. 2001. Use of VSV-G pseudotyped retroviral vectors to target murine osteoprogenitor cells. Virology 284:37–45.
15. Karsenty, G. 1999. The genetic transformation of bone biology. Genes Dev. 13:3037–3051.
16. Kim, J. H., H. S. Jung-Ha, H. T. Lee, and K. S. Chung. 1997. Development of a positive method for male stem cell-mediated gene transfer in mouse and pig. Mol. Reprod. Dev. 46:515–526.
17. Koseki, T., Y. Gao, N. Okahashi, Y. Murase, T. Tsujisawa, T. Sato, K. Yamato, and T. Nishihara. 2002. Role of TGF-beta family in osteoclastogenesis induced by RANKL. Cell. Signal. 14:31–36.
18. Kost, T. A., and J. P. Condreay. 2002. Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol. 20:173–180.
19. Lehtolainen. P., K. Tyynelä, J. Kannasto, K. J. Airenne, and S. Ylä-Herttuala. 2002. Baculoviruses exhibit restricted cell type specificity in rat brain: a comparison of baculovirus- and adenovirus-mediated intracerebral gene transfer in vivo. Gene Ther. 9:1693–1699.
20. Matsuura, Y., R. D. Possee, H. A. Overton, and D. H. L. Bishop. 1987. Baculovirus expression vector: the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. 68:1233–1250.
21. Mehrara, B. J., P. B. Saadeh, D. S. Steinbrech, M. Dudziak, J. A. Spector, J. A. Greenwald, G. K. Gittes, and M. T. Longaker. 1999. Adenovirus-mediated gene therapy of osteoblasts in vitro and in vivo. J. Bone Mineral Res. 14:1290–1301.
22. Miller, L. K., and P. D. Friesen. 2001. Insect viruses, p. 599–628. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott-Raven, Philadelphia, Pa.
23. Miyao, Y., K. Ikenaka, H. Kishima, M. Tamura, K. Nakamura, M. Kurumi, T. Hayakawa, and K. Shimizu. 1997. FUT-175, a synthetic inhibitor of the complement pathway, protects against the inactivation of infectious retroviruses by human serum. Hum. Gene Ther. 8:1575–1583.
24. Mulligan, R. C. 1993. The basic science of gene therapy. Science 260:926–932.
25. Muramatsu, T., O. Shibata, S. Ryoki, Y. Ohmori, and J. Okumura. 1997. Foreign gene expression in the mouse testis by localized in vivo gene transfer. Biochem. Biophys. Res. Commun. 233:45–49.
26. Niinobe, M., Y. Ueno, Y. Hitomi, and S. Fujii. 1984. Detection of intermediary C1r with complete active site, with a synthetic proteinase inhibitor. FEBS Lett. 172:159–162.
27. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–200.
28. Ogawa, T., J. M. Arechaga, M. R. Avarbock, R. L. Brinster. 1997. Transplantation of testis germinal cells into mouse seminiferous tubules. Int. J. Dev. Biol. 41:111–122.
29. Okahashi, N., Y. Murase, T. Koseki, T. Sato, K. Yamato, and T. Nishihara. 2001. Osteoclast differentiation is associated with transient upregulation of cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. J. Cell. Biochem. 80:339–345.
30. Ory, D. S., B. A. Neugeboren, and R. C. Mulligan. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc. Natl. Acad. Sci. USA 93:11400–11406.
31. Pfeifer, P. H., M. S. Kawahara, and T. E. Hugli. 1999. Possible mechanism for in vitro complement activation in blood and plasma samples: FUT-175/EDTA controls in vitro complement activation. Clin. Chem. 45:1190–1199.
32. Pieroni, L., D. Maione, and N. La Monica. 2001. In vivo gene transfer in mouse skeletal muscle mediated by baculovirus vectors. Hum. Gene Ther. 12:871–881.
33. Pieroni, L., and N. La Monica. 2001. Towards the use of baculovirus as a gene therapy vector. Curr. Opin. Mol. Ther. 3:465–467.
34. Ruggieri, A., T. Harada, Y. Matsuura, and T. Miyamura. 1997. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 229: 68–76.
35. Sandig, V., C. Hofmann, S. Steinert, G. Jennings, P. Schlag, and M. Strauss. 1996. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum. Gene Ther. 7:1937–1945.
36. Sandig, V., and M. Strauss. 1996. Liver-directed gene transfer and application to therapy. J. Mol. Med. 74:205–212.
37. Sarkis, C., C. Serguera, S. Petres, D. Buchet, J.-L. Ridet, L. Edelman, and J. Mallet. 2000. Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. Proc. Natl. Acad. Sci. USA 97:14638–14643.
38. Shoji, I., H. Aizaki, H. Tani, K. Ishii, T. Chiba, I. Saito, T. Miyamura, and Y. Matsuura. 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J. Gen. Virol. 78:2657–2664.
39. Song, S. U., S.-H. Shin, S.-K. Kim, G.-S. Choi, W.-C. Kim, M.-H. Lee, S.-J. Kim, I.-H. Kim, M.-S. Choi, Y.-J. Hong, and K.-H. Lee. 2003. Effective transduction of osteogenic sarcoma cells by a baculovirus vector. J. Gen. Virol. 84:697–703.
40. Tamura, Y., M. Hirado, K. Okamura, Y. Minato, and S. Fujii. 1977. Synthetic inhibitors of trypsin, plasmin, kallikrein, thrombin, C1r, and C1 esterase. Biochim. Biophys. Acta 484:417–422.
41. Tani, H., M. Nishijima, H. Ushijima, T. Miyamura, and Y. Matsuura. 2001. Characterization of cell-surface determinants important for baculovirus infection. Virology 279:343–353.
42. Tjia, S. T., G. M. zu Altenschildesche, and W. Doerfler. 1983. Autographa californica nuclear polyhedrosis virus (AcNPV) DNA does not persist in mass cultures of mammalian cells. Virology 125:107–117.
43. Tuchiya, K., Y. Matsuura, A. Kawai, A. Ishihama, and S. Ueda. 1992. Characterization of rabies virus glycoprotein expressed by recombinant baculovirus. Virus Res. 25:1–13.
44. Tuffereau, C., J. Bénéjean, D. Blondel, B. Kieffer, and A. Flamand. 1998. Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. EMBO J. 17:7250–7259.
45. Yap, C. C., K. Ishii, Y. Aoki, H. Aizaki, H. Tani, H. Shimizu, Y. Ueno, T. Miyamura, and Y. Matsuura. 1997. A hybrid baculovirus-T7 RNA polymerase system for recovery of an infectious virus from cDNA. Virology 231:192–200.

Baculovirus Induces an Innate Immune Response and Confers Protection from Lethal Influenza Virus Infection in Mice[1]

Takayuki Abe,* Hitoshi Takahashi,*† Hiroyuki Hamazaki,* Naoko Miyano-Kurosaki,*† Yoshiharu Matsuura,‡ and Hiroshi Takaku[2]*†

A recombinant baculovirus expressing the hemagglutinin gene of the influenza virus, A/PR/8/34 (H1N1), under the control of the chicken β-actin promoter, was constructed. To determine the induction of protective immunity in vivo, mice were inoculated with the recombinant baculovirus by intramuscular, intradermal, i.p., and intranasal routes and then were challenged with a lethal dose of the influenza virus. Intramuscular or i.p. immunization with the recombinant baculovirus elicited higher titers of antihemagglutinin Ab than intradermal or intranasal administration. However, protection from a lethal challenge of the influenza virus was only achieved by intranasal immunization of the recombinant baculovirus. Surprisingly, sufficient protection from the lethal influenza challenge was also observed in mice inoculated intranasally with a wild-type baculovirus, as evaluated by reductions in the virus titer, inflammatory cytokine production, and pulmonary consolidations. These results indicate that intranasal inoculation with a wild-type baculovirus induces a strong innate immune response, which protects mice from a lethal challenge of influenza virus. ***The Journal of Immunology,*** **2003, 171: 1133–1139.**

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV)[3] has long been used as a biopesticide and as a tool for efficient recombinant protein production in insect cells (1, 2). Its host specificity was originally thought to be restricted to cells derived from arthropods. However, several groups recently reported that AcNPV, containing an appropriate eukaryotic promoter, efficiently transfers and expresses foreign genes in several types of mammalian cells (3–9) and animal models (10–13). The advantageous characteristics of AcNPV for its use in gene therapy applications are its low cytotoxicity in mammalian cells even at a high multiplicity of infection (m.o.i.), an inherent incapability to replicate in mammalian cells, and the absence of preexisting Abs against baculovirus in animals.

In general, viral infection with mammalian cells results in the production of various cytokines, including members of the IFN family. dsRNA, produced during the replication of many RNA and DNA viruses, induces αβ IFNs in various cell types. Gronowski et al. (14) reported that live baculovirus induced IFN production in mammalian cells, including mouse embryo fibroblast cells, and provided in vivo protection of mice from encephalomyocarditis virus (ECMV) infection. However, the precise mechanisms underlying IFN induction and protection from the lethal EMCV infection remain unclear. In addition, Beck et al. (15) reported that baculovirus infection represses phenobarbital-mediated gene induction and stimulates TNF-α, IL-1α, and IL-1β expression in primary cultures of rat hepatocytes.

The present study examined the induction of protective immunity in mice immunized with a recombinant baculovirus expressing the influenza virus hemagglutinin (HA) against a lethal influenza infection. Protection from the lethal influenza virus challenge was observed not only in mice intranasally immunized with the recombinant baculovirus but also in those immunized with a wild-type baculovirus. Inoculation of baculovirus also induced the secretion of inflammatory cytokines, such as TNF-α and IL-6, in a murine macrophage cell line, RAW264.7. These results indicate that baculovirus induces an innate immunity that confers protection from a lethal influenza virus challenge in mice.

Materials and Methods

Preparation of baculoviruses

AcNPV and the recombinant baculovirus were propagated in *Spodoptera frugiperda* 9 cells in TNM-FH medium (BD PharMingen, San Diego, CA) containing 100 μg/ml kanamycin and 10% FBS. The baculovirus transfer vector, pAcCAG-HA, was constructed by inserting the full-length HA cDNA (kindly provided by Dr. S. Nakada, Yamanouchi Pharmaceutical, Tokyo, Japan) into the cloning site of the baculovirus transfer vector, pAcCAGMCS (6). The recombinant baculovirus containing the influenza virus HA genome (AcCAG-HA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD PharMingen) after cotransfection into *S. frugiperda* 9 cells, as described previously (1). AcCAG-HA and AcNPV were purified as follows: culture supernatants were harvested 3–4 days after infection and cell debris were removed by centrifugation at 5,000 rpm for 15 min at 4°C. The virus was pelleted by centrifugation at 25,000 rpm (57,000 × *g*) for 90 min at 4°C, resuspended in 1 ml of PBS, loaded on a 10–60% (w/v) sucrose gradient, and centrifuged at 25,000 rpm (57,000 × *g*) for 90 min at 4°C. The virus band was collected, resuspended in PBS, and centrifuged at 25,000 rpm (57,000 × *g*) for 90 min at 4°C. The virus pellet was resuspended in PBS, and the infectious titers were determined by a plaque assay as described previously (1). All tissue culture media and media components used in this study were free of endotoxin. The endotoxin label in these viruses was free endotoxin (<0.01 endotoxin units/ml), as determined using a Pyrodick endotoxin measure kit (Seikagaku, Tokyo, Japan).

*Department of Industrial Chemistry and †High Technology Research Center, Chiba Institute of Technology, Tsudanuma, Narashino, Chiba, Japan; and ‡Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

Received for publication August 29, 2002. Accepted for publication May 23, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a Grant-in-Aid for High Technology Research from the Ministry of Education, Science, and Culture, Japan, and by a Grant-in-Aid for Scientific Research on Priority Areas, No. 09309011, from the Ministry of Education, Science and Culture, Japan.

[2] Address correspondence and reprint requests to Dr. Hiroshi Takaku, Department of Industrial Chemistry and High Technology Research Center, Chiba Institute of Technology, Tsudanuma, Narashino, Chiba 275-0016, Japan. E-mail address: takaku@ic.-it-chiba.ac.jp

[3] Abbreviations used in this paper: AcNPV, *Autographa californica* nuclear polyhedrosis virus; m.o.i., multiplicity of infection; ECMV, encephalomyocarditis virus; HA, hemagglutinin; MR, mannose receptor; TLR, Toll-like receptor.

0022-1767/03/$02.00

Influenza virus

Influenza virus A/PR/8/34 (H1N1) was grown in Madin-Darby canine kidney cells cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing glucose (1000 mg/L), L-glutamine (3 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% (v/v) heat-inactivated FBS for 3 days. Culture supernatants were harvested and stored at −80°C. The virus titer was determined by a plaque assay, as previously described (16). To determine the virus titer in the lung, the mice were sacrificed under light diethyl ether anesthesia. Lung homogenates were prepared and serially diluted in saline, and viral titers were determined by plaque assays.

Detection of HA protein in virus-infected cultured cells

The human hepatoma cell line, Huh7, was maintained in DMEM containing 10% heat-inactivated FBS and infected at an m.o.i. of 20 or 200 with AcCAG-HA and AcNPV. At 24 h postinfection, the cells were lysed with lysis buffer containing 50 mM Tris-HCl, pH 6.8, 0.1 M dithiothreitol, 2% SDS, and 10% glycerol. Cell extracts were separated by SDS-PAGE, and proteins were blotted onto a polyvinylidene difluoride membrane (Roche Molecular Biochemicals, Mannheim, Germany). The HA protein was detected on the blot following sequential incubations with a 1/200 dilution of an anti-A/PR/8/34 mouse polyclonal Ab (sera obtained from mice immunized with purified influenza viruses) and a 1/1000 dilution of an anti-mouse IgG Ab conjugated with HRP (Amersham Biosciences, Piscataway, NJ), using an ECL detection system (Amersham Biosciences).

Immunization and challenge

The baculoviruses (AcCAG-HA or AcNPV) (1.1×10^8 PFU/mouse) were inoculated twice, 2 wk apart, into the abdominal epidermis of BALB/c female mice (6-wk-old) obtained from Charles River Breeding Laboratories (Kanagawa, Japan). A lethal challenge with influenza virus consisting of 5.6×10^5 PFU of mouse-adapted A/PR/8/34 influenza virus (100 LD_{50}) in 50 μl of saline was administered intranasally 3 wk after the second immunization. This infection caused rapid, widespread viral replication in the lung and death within 5 to 7 days. The survival rates of mice immunized with AcNPV or AcCAG-HA were compared after the challenge. Furthermore, AcNPV or AcCAG-HA was also administered intranasally or intramuscularly (1.1×10^8 PFU/mouse) 24 h before challenge with the influenza virus, under light diethyl ether anesthesia.

Ab assay

The Ab titers against HA were measured using an ELISA. ELISA was performed sequentially from the solid phase (96-well Nunc Maxisorp P/N; Nalge Nunc International, Rochester, NY) with a ladder of reagents consisting of 1); HA molecules purified from the A/PR/8/34 influenza virus; 2), a serum sample; 3), goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) conjugated with biotin; 4), streptavidin-conjugated with alkaline phosphatase (Kirkegaard & Perry Laboratories); and finally, *p*-nitrophenyl phosphate (Moss, Pasadena, MD). The chromogen produced was measured for absorbance at 405 nm with a microplate autoreader (Titertec Multican Labsystem, Oy, Helsinki, Finland).

Inflammatory cytokine production

The amounts of one of the inflammatory cytokines, IL-6, in the lung homogenates were determined by sandwich ELISA using an OptEIA mouse IL-6 Set (BD PharMingen). A 96-well ELISA plate (Nunc Maxisorp P/N; Nalge Nunc International) was coated with the capture Ab, the anti-mouse IL-6 mAb, diluted in coating buffer (0.2 M sodium phosphate, pH 6.5) overnight at 4°C. After washing with PBS containing 0.05% Tween 20 and blocking with PBS containing 10% FBS for 2 h at room temperature, 100 μl of 10-fold-diluted lung homogenates or serially diluted mouse rIL-6 was incubated for 2 h at room temperature. After washing, a total of 100 μl of biotinylated mouse IL-6 mAb and avidin-HRP conjugate was placed in each well, and the plates were incubated for 1 h at room temperature. After extensive washing, 100 μl of substrate solution was added, and after incubating for 30 min at room temperature, the OD was measured at 450 to 540 nm on a microplate reader (Titertec Multican Labsystem) following the addition of 50 μl of stop solution (1 M H_3PO_4). The IL-6 content was calculated using a standard curve of mouse rIL-6 with the substrate solution as the blank.

Histopathologic examination

For microscopic evaluation of the pathologic changes in the lung, mice were killed under light anesthesia with inhaled diethyl ether on day 7 following the influenza virus infection. The lungs were removed and inflated with 10% formalin in PBS. After fixation, the lungs were embedded in paraffin, sectioned, and stained with H&E.

Stimulation of mouse macrophage RAW264.7 cells by AcNPV for cytokine production

To determine the effects of infection with AcNPV on cytokine production, the mouse macrophage cell line, RAW264.7, was seeded in 6-well plates (2×10^6 cells/well). After 24 h of cultivation, RAW264.7 cells were inoculated with various concentrations of AcNPV or bacterial LPS (Sigma-Aldrich). The amounts of inflammatory cytokines (IL-6 and TNF-α) in the culture supernatants were quantified by a sandwich ELISA using an OptEIA mouse IL-6 and TNF-α set (BD PharMingen).

Flow cytometry

The expression of CD69, MHC class I and II, and mature macrophage Ag receptors on RAW264.7 cells stimulated with AcNPV or LPS was analyzed by flow cytometry (BD Biosciences, San Jose, CA) after staining with specific Abs purchased from BD PharMingen (CD69, MHC class I and II Abs) and Yamasa (Chiba, Japan) (mature macrophage Ag receptor Ab, clone F4/80).

Statistics

Data were presented as means ± SEM. The Student *t* test was used to analyze virus titers and cytokine production. A p value of <0.05 was considered to indicate significance.

Results

Construction of a recombinant baculovirus expressing the influenza virus HA protein

To determine the efficacy of immunization with a recombinant baculovirus, we constructed a recombinant baculovirus expressing

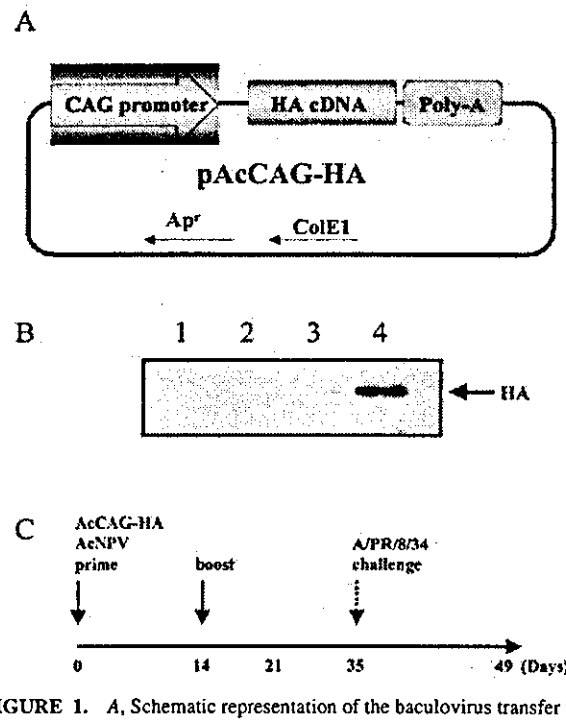

FIGURE 1. *A*, Schematic representation of the baculovirus transfer vector expressing the influenza virus hemagglutinin, pAcCAG-HA. The hemagglutinin gene of the influenza virus (A/PR/8/34) was inserted under the control of the CAG promoter. *B*, Expression of HA proteins in Huh7 cells infected with the recombinant baculovirus, AcCAG-HA. Cell extracts were separated by 7.5% SDS-PAGE and analyzed by immunoblotting using a mouse polyclonal Ab against the influenza virus. *Lane 1*, Mock-infected cell lysate; *lane 2*, infected with AcNPV at an m.o.i. of 200; *lanes 3* and *4*, infected with AcCAG-HA at an m.o.i. of 20 and 200, respectively. The arrow indicates HA proteins, *C*, Mice were immunized twice, 2 wk apart, with AcCAG-HA or AcNPV at a dose of 1.1×10^8 PFU per mouse, by intramuscular, intradermal, i.p., or intranasal routes. Three weeks after the second immunization, mice were challenged with a lethal dose (5.6×10^5 PFU) of influenza virus.

Table I. *Ab titers of nasal and serum anti-A/PR8 HA IgG, IgA in mice immunized via various routes with wild-type or recombinant baculovirus expressing HA protein*[a]

Immunization	No. of Mice	A/PR8 HA-Reactive IgG Serum (μg/ml)	A/PR8 HA-Reactive IgA Nasal Wash (ng/ml)
Control	3	<0.1	<0.1
Infection control	27	<0.1	<0.1
AcNPV (i.n.)	8	<0.1	<0.1
AcCAG-HA			
i.m.	14	52.6 ± 16.8	<0.1
i.n.	14	19.2 ± 12.9	<0.1
i.d.	14	11.0 ± 2.3	NT
i.p.	14	29.7 ± 8.8	NT

[a] Mice were immunized by inoculation via various routes with a wild-type or the recombinant baculovirus on days 0 and 14. Three weeks later, they were challenged with a lethal dose of influenza virus. On day 38 (3 days after challenge), serum samples and nasal wash samples for Ab titration were obtained. Values represent mean ± SD of each group. i.m., Intramuscular; i.n., intranasal; i.d., intradermal; NT, not tested.

the HA gene of the influenza virus under the control of the CAG promoter (pAcCAG-HA, Fig. 1*A*). The CAG promoter is used in a wide variety of mammalian cell lines and exhibits stronger expression than the CMV and respiratory syncytial virus promoters (Niwa et al.) (17). Expression of HA protein due to infection with the recombinant baculovirus was examined by Western blot analyses using the polyclonal Ab raised against the HA protein. In Huh7 cells infected with AcCAG-HA, the expression of the HA protein was detected in a dose-dependent manner, whereas there was no band observed in cells infected with AcNPV (Fig. 1*B*). Mice were immunized twice, 2 wk apart, with AcNPV or AcCAG-HA at a dose of 1.1×10^8 PFU per mouse, by intramuscular, intradermal, i.p., or intranasal routes (Fig. 1*C*). Three weeks after the second immunization, the mice were challenged with a lethal dose (5.6×10^5 PFU) of influenza virus.

Protection from a lethal challenge of influenza virus in mice immunized with the recombinant baculovirus

The efficacy of immunization with AcCAG-HA was evaluated by the induction of IgG and IgA against the HA protein and the mouse survival rates. Control mice were immunized with AcNPV. Serum samples and nasal wash samples were obtained on days 14 and 21 after the first immunization and 3 days after influenza challenge,

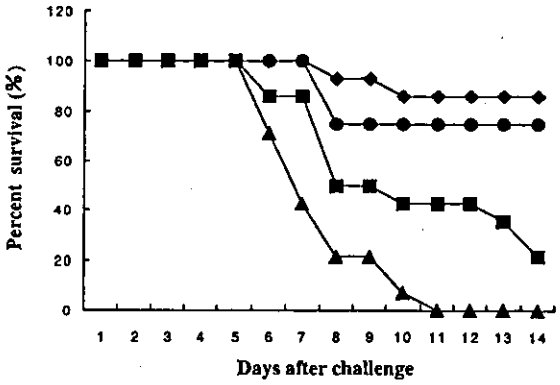

FIGURE 2. Protection from a lethal challenge of influenza virus. Mice were immunized intramuscularly (■) or intranasally (◆) with AcCAG-HA and intranasally with AcNPV (●) at a dose of 1.1×10^8 PFU. Control mice were inoculated with saline (▲). Three weeks after the second immunization, the mice were challenged with a lethal dose (5.6×10^5 PFU) of influenza virus. Survival rates were recorded until day 14 after the influenza challenge.

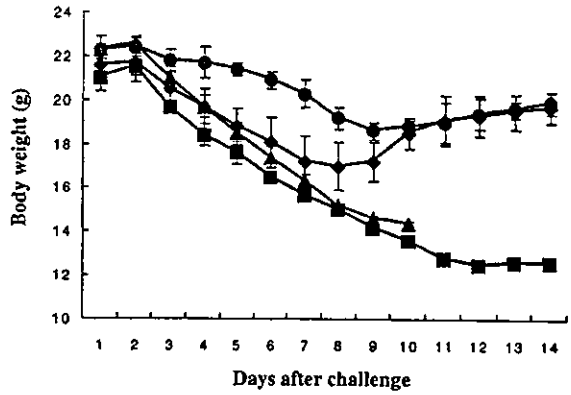

FIGURE 3. Weight loss after influenza virus challenge. Mice were immunized intramuscularly (■) or intranasally (●) with AcCAG-HA and intranasally with AcNPV (◆) at a dose of 1.1×10^8 PFU. Control mice were inoculated with saline (▲). Three weeks after the second immunization, the mice were challenged with a lethal dose (5.6×10^5 PFU) of influenza virus and monitored daily for weight loss.

and examined for a specific IgG and IgA response against HA by ELISA. As shown in Table I, intramuscular or i.p. immunization of mice with the recombinant baculovirus induced a higher Ab response than immunization by the other routes. There was a significant correlation between the inoculation doses of AcCAG-HA (10^5–10^8 PFU per mouse) and the induction of Abs against HA (data not shown). Although intranasal immunization with AcCAG-HA induced a low, but detectable, IgG response, there was no anti-HA IgA response detected in the nasal wash. Mice immunized twice with recombinant baculovirus (1.1×10^8 PFU per mouse/inoculation) were challenged on day 21 after the second immunization with 50 μl of influenza virus A/PR/8/34 (5.6×10^5 PFU/mouse) under light diethyl ether anesthesia. As shown in Fig. 2, intramuscular immunization with AcCAG-HA induced a specific Ab response, but actual protection was only achieved by intranasal immunization. Mice immunized by the other inoculation routes lacked protection (data not shown), in spite of the induction of an Ab response. Moreover, the mice immunized intranasally with AcNPV, but not those inoculated by other routes (data not shown), were also protected against the influenza virus challenge. Typical weight loss curves are shown in Fig. 3. Mice immunized intranasally with AcCAG-HA or AcNPV were conferred protection

Table II. *Protection against influenza virus challenge in mice immunized via various routes with wild-type or recombinant baculovirus expressing HA protein*[a]

Immunization	No. of Mice	Lung Virus Titer ($\times 10^5$ PFU/ml)	IL-6 (ng/ml)
Control	3	<0.1	<0.1
Infection control	27	3.8 ± 0.4	11.8 ± 2.7
AcNPV (i.n.)	8	0.7 ± 0.6[b]	2.6 ± 0.7[b]
AcCAG-HA			
i.m.	14	1.1 ± 0.5	6.1 ± 3.9
i.n.	14	0.4 ± 0.1[b]	1.3 ± 0.7[b]
i.d.	14	1.0 ± 0.4	5.5 ± 2.3
i.p.	14	1.0 ± 0.3	8.5 ± 5.7

[a] Mice were immunized by inoculation via various routes with a wild-type or the recombinant baculovirus on days 0 and 14. Three weeks later, they were challenged with a lethal dose of influenza virus. On day 38 (3 days after challenge), lung homogenates for cytokine production and virus titration were obtained. Values represent mean ± SD of each group. Values represent mean ± SD of each group. i.m., Intramuscular; i.n., intranasal; i.d., intradermal.

[b] Significant difference ($p < 0.05$).

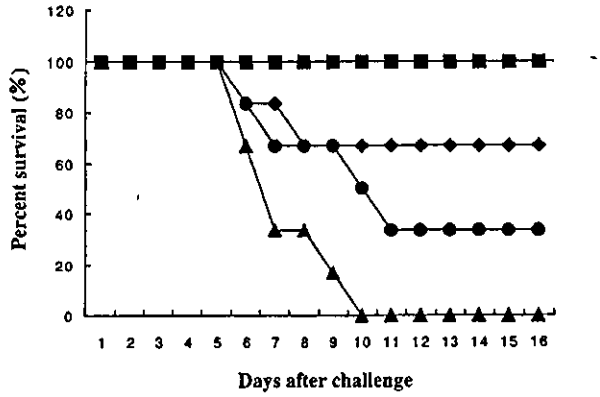

FIGURE 4. Protection from a lethal challenge of influenza virus by AcNPV. AcNPV was inoculated intranasally (■) at a dose of 1.1×10^8 PFU. Recombinant murine IFN-α (●) and poly:(I–C) (◆) were inoculated i.p. at doses of 1 and 100 μg, respectively. Control mice were inoculated with saline (▲). Each group of six mice was challenged with a lethal dose of influenza virus 24 h after the inoculation. Survival rates were recorded until day 14 after the challenge.

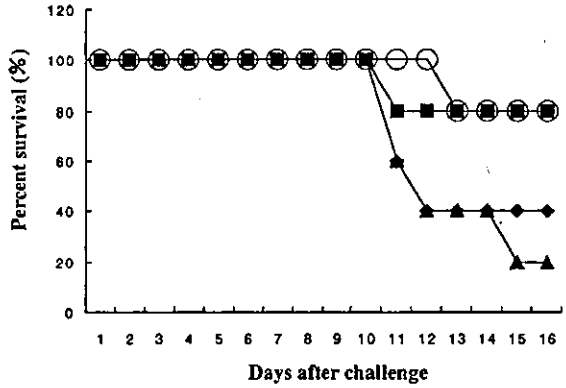

FIGURE 5. Protection from a lethal challenge of different serotypes of influenza virus by AcNPV. AcNPV was intranasally inoculated 24 h before the influenza virus challenge, and the mice were challenged with a lethal dose of influenza virus A/Guizhou (■) and B/Ibaraki virus (○). Infection control mice were challenged with A/Guizhou (▲) or B/Ibaraki (◆) without preinoculation of AcNPV. Each group of five mice was challenged with a lethal dose of influenza virus at 24 h after the inoculation. Survival rates were recorded until day 14 after the challenge.

against lethal challenge of the infection and lost significantly less weight. Animals immunized by other routes or saline sustained significant weight loss, approaching 30–40% of their initial weight before they died. The virus titer and the inflammatory cytokines in the lung 3 days postchallenge were determined to assess the protection of the mice from an acute lung influenza infection. Intranasal immunization of mice with either AcCAG-HA or AcNPV induced a significant reduction in virus titers and IL-6 production in the lung, as compared to those immunized by other routes and nonimmunized mice (Table II). These results indicate that an innate immune response was induced by inoculation with the baculovirus in mice.

Induction of an innate immune response in mice by AcNPV

To determine the antiviral effects induced by the baculovirus inoculation in more detail, protection from influenza virus infection was compared to that induced by treatment with purified murine IFN-α or poly:(I–C) (Fig. 4). AcNPV was intranasally administered at 24 h before the influenza virus challenge. Surprisingly, mice inoculated intranasally with AcNPV were completely protected, as indicated by their negligible weight loss after challenge, lack of changes in activity and grooming, and 100% survival rate. All of the control mice inoculated with saline died within 10 days. The mice treated i.p. with 1 μg of murine IFN-α and 100 μg of poly:(I–C) exhibited 33.3 and 66.6% survival, respectively. These results indicate that intranasal inoculation with a wild-type baculovirus 24 h before the challenge confers complete protection in mice from a lethal influenza virus infection. The protective efficacy of AcNPV was further evaluated by the challenge of other serotypes of influenza viruses. Mice inoculated intranasally with AcNPV also exhibited protection from a lethal challenge of different serotypes of influenza viruses, A/Guizhou (H3N2) and B/Ibaraki (Fig. 5).

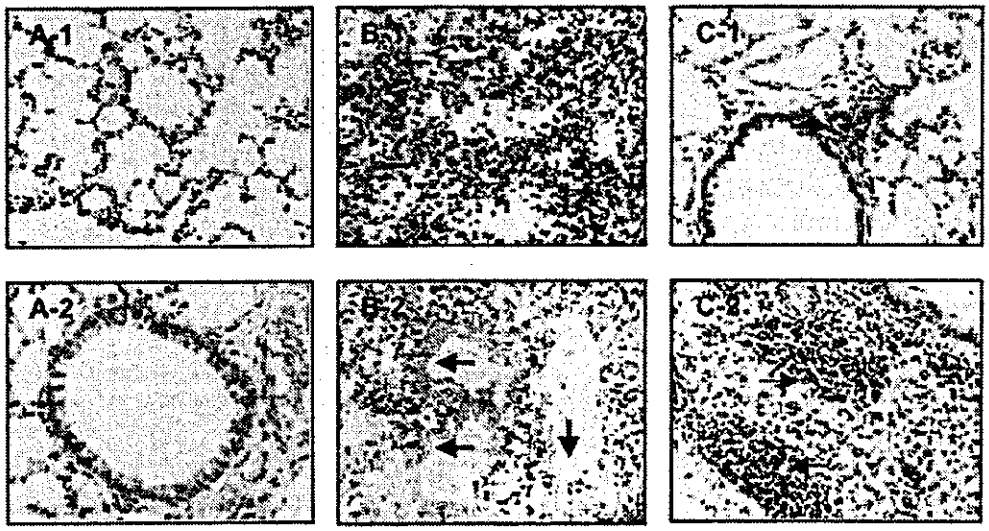

FIGURE 6. Effects of pretreatment with AcNPV on the lung morphology in mice infected with influenza virus. Seven days after influenza challenge, lungs were removed, sectioned, and stained with H&E. *A*, Uninfected control; *B*, the lung of a mouse infected with influenza virus without any treatment. The infiltration of mononuclear cells and polymorphonuclear cells, as well as hydatid cambium, within the perialveolar space was observed (indicated arrow); *C*, the lung of a mouse infected with influenza virus pretreated with the intranasal administration of AcNPV at a dose of 1.1×10^8 PFU. The mice pretreated with AcNPV exhibited a marked infiltration of monocytes, consisting of macrophages (arrow) and exhibited less lung damage.

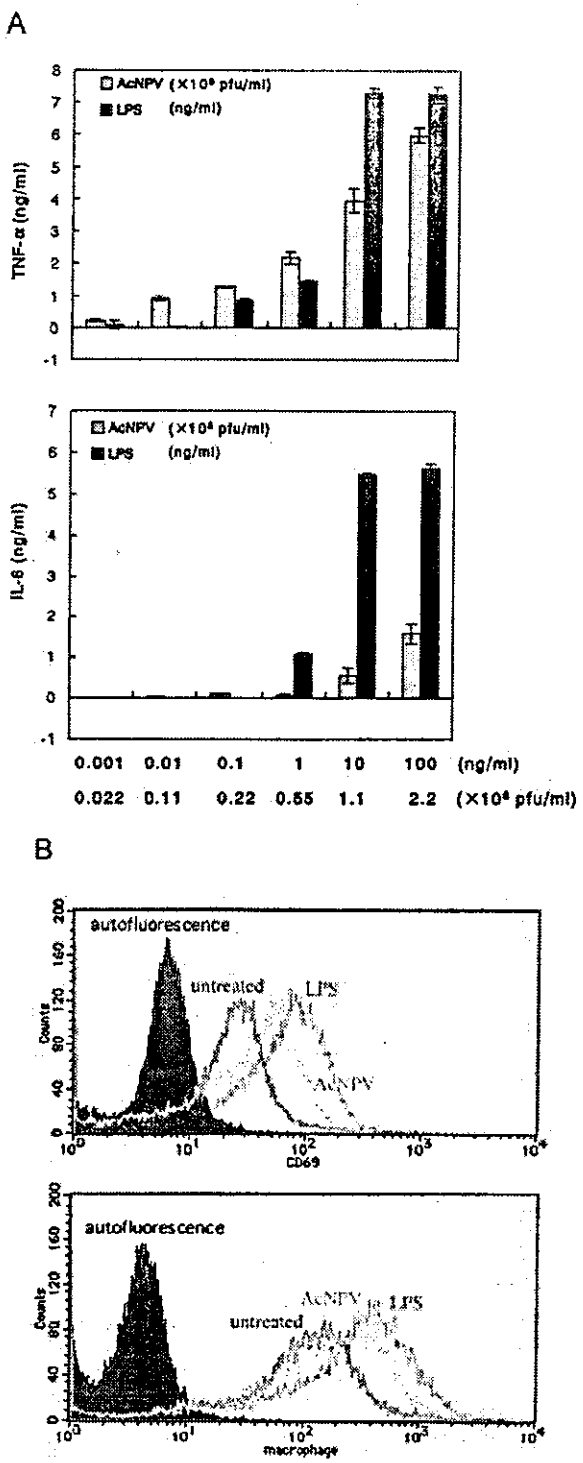

FIGURE 7. Activation of the mouse macrophage cell line, RAW264.7, treated with AcNPV or bacterial LPS. *A*, RAW264.7 cells were incubated with different concentrations of AcNPV or bacterial LPS for 24 h. Production levels of TNF-α and IL-6 were determined by sandwich ELISA. Data represent the mean concentrations of TNF-α or IL-6 in the supernatant ± SD of three independent experiments, each performed in triplicate. *B*, Effect of treatment of mouse macrophage cells with AcNPV or LPS on the expression of CD69 and mature macrophage Ag receptor. RAW264.7 cells were double-stained with FITC-labeled anti-CD69 and PE-labeled anti-mature macrophage Ag receptor Abs, and were analyzed by flow cytometry. The *x* and *y* axes are the relative fluorescence intensity and the numbers of cells, respectively. Filled histograms are unstained cells. Green lines are unstimulated cells. Blue and red lines are cells treated with AcNPV and LPS, respectively.

Morphologic changes in the lungs of mice infected with influenza virus after intranasal inoculation with AcNPV

The histologic changes in the lungs of mice challenged with influenza virus after intranasal inoculation with AcNPV are shown in Fig. 6. AcNPV was intranasally inoculated 24 h before the influenza challenge. Neither inflammatory cells nor damaged tissues were observed in the lungs of naive controls (Fig. 6, *A-1* and *A-2*). There was marked damage on the lung surfaces of mice treated with saline alone 7 days after infection with influenza virus (data not shown). Neutrophil infiltration and marked congestion within the peribronchiolar and perialveolar spaces were apparent (Fig. 6*B-1*). Moreover, infiltration of mononuclear cells and polymorphonuclear cells, as well as hydatid cambium, within the perialveolar space was observed (Fig. 6*B-2*). In contrast, mice preinoculated with AcNPV exhibited a marked infiltration of monocytes consisting of macrophages (Fig. 6, *C-1* and *C-2*) and less damage on the lung surface after challenge (data not shown) and the peribronchiolar and perialveolar spaces contained fewer neutrophils (data not shown). These results indicate that monocytes consisting of macrophages were strongly induced by preinoculation with AcNPV and these activated immunocompetent cells suppressed the spread of the influenza virus infection in lung tissue.

AcNPV induces inflammatory cytokine production in a mouse macrophage cell line

These in vivo experiments revealed that not only the recombinant baculovirus but also a wild-type virus, AcNPV induces an innate immune response in mice. To determine whether treatment with AcNPV stimulates and activates an innate immune response in vitro, the mouse macrophage cell line, RAW264.7, was inoculated with AcNPV and production of inflammatory cytokines, such as TNF-α and IL-6, was examined. Bacterial LPS, a well-known inducer of macrophage activation, was used as a positive control. High levels of TNF-α and IL-6 production were detected in RAW264.7 cells treated with AcNPV or bacterial LPS in a dose-dependent manner (Fig. 7*A*). To determine the macrophage activation, the cell surface expression of CD69 and mature macrophage Ag receptor in RAW264.7 cells treated with AcNPV or bacterial LPS was examined by flow cytometry. Treatment with AcNPV also induced the expression of both mature macrophage Ag receptor and CD69 in RAW264.7 cells, but to a lesser extent than induction with bacterial LPS (Fig. 7*B*). In contrast, there was no significant difference in the expression of MHC class I and II molecules on RAW264.7 cells after treatment with AcNPV and bacterial LPS (data not shown). These in vivo and in vitro data indicate that AcNPV stimulates a strong, innate immune response in mice and induces inflammatory cytokine production in macrophages.

Effect of nucleases and heat treatments on the activation of macrophages by AcNPV

To determine the components responsible for the activation of macrophages by AcNPV, effects of treatment with RNase A, DNase I, and heating during induction were determined. The stimulation of macrophages by AcNPV was unaffected by treatment with RNasé A or DNase I, but was completely abrogated by heating for 30 min at 56°C (Fig. 8). In contrast, the stimulation by poly:(I–C) was destroyed by RNase A treatment, but not by DNase I treatment or heating. Stimulation by LPS was resistant to all of the treatments. These data indicate that activation of macrophages by AcNPV is mediated by heat-labile viral components and is not due to contamination of the LPS.

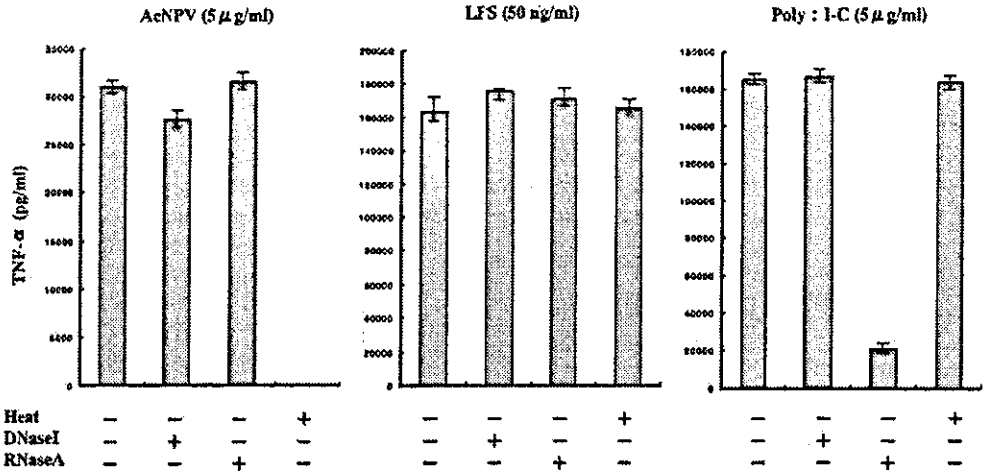

FIGURE 8. Effect of nucleases and heat treatments on the activation of macrophages by AcNPV. AcNPV (5 μg/ml), LPS (50 ng/ml), and poly:(I–C) (5 μg/ml) were treated with RNase A (50 μg/ml) and DNase I (75 U/ml) for 30 min at 37°C or heating for 30 min at 56°C. These treated compounds were added to RAW264.7 and incubated for 24 h. Production of TNF-α was determined by a sandwich ELISA. Data represent the mean concentrations of TNF-α in the supernatant ± SD of three independent experiments, each performed in triplicate.

Discussion

The results of this study provide evidence that live baculovirus stimulates and activates an innate immune response, such as by macrophage cells in vitro and in vivo. Intramuscular and i.p. immunization of mice with baculovirus induced higher levels of a specific Ab response, but protection against influenza virus challenge was achieved only by intranasal immunization. Intranasal inoculation of the recombinant baculovirus expressing HA induced only a low level of IgA in the nasal wash. Moreover, mice immunized intranasally with a wild-type baculovirus used as the control viral vector were also provided protection similar to that conferred by the recombinant virus against the influenza virus challenge. These results indicate that inoculation with baculoviruses imparts nonspecific antiviral activity to mice. Gronowski et al. (14) reported that live baculovirus exhibited antiviral activity in mammalian cells and also in EMCV-infected mice. They suggested that IFN induction requires an interaction between the baculovirus envelope protein, gp64, and the receptors on the responding cell membrane. However, the mechanism underlying the baculovirus-induced IFN expression in vitro and in vivo remains unclear.

The baculovirus envelope glycoprotein, gp64, is a major component of the envelope of the budded virus and is involved in virus entry into the host cells by endocytosis (18–24). Previous studies indicated that recombinant proteins expressed by baculovirus in insect cells do not pass through *N*-linked oligosaccharides to form complexes containing outer-chain galactose and sialic acid residues. The gp64 envelope protein contains mannose, fucose, and *N*-acetyl glucosamine, but no detectable galactose or terminal sialic acid residues (25). The glycans linked to recombinant glycoproteins produced by an insect cell system differ from those found on native mammalian products. In this report, we demonstrated that AcNPV stimulates innate immunity, such as that exerted by macrophages in vivo. The lungs of AcNPV-treated mice exhibited a marked infiltration of monocytes, consisting of macrophages, after influenza infection. Thus, the effect of the baculovirus treatment on lung consolidation depends on the inhibition of virus growth in the lung tissues. Moreover, mouse macrophage RAW264.7 cells infected with AcNPV increased their expression of activation ligands (CD69 and mature macrophage Ag receptor) and produced inflammatory cytokines, such as TNF-α and IL-6. These data strongly support the conclusion that AcNPV has immunostimulatory capacities to promote the release of inflammatory cytokines from RAW264.7 macrophages. The mannose receptor (MR) recognizes a range of carbohydrates present on the surface and cell walls of micro-organisms. The MR is primarily expressed on macrophages and dendritic cells, and is involved in MR-mediated endocytosis and phagocytosis. In addition, the MR has a key role in host defense and induces innate immunity. Therefore, it is possible that AcNPV-inoculated mice were directly stimulated by the mannose-bearing gp64 envelope proteins interacting with MRs expressed on the surfaces of dendritic cells and macrophages present within special mucosal sites, such as nasal-associated lymphoid tissues and the lung tissues.

In a recent study, the activation of innate immunity was closely linked to the defensive and secondary adaptive immune responses of the host. Members of the Toll-like receptor (TLR) family are essential components in this process (26–28). Ten TLRs have been identified in mammalian systems; the current paradigm is that individual TLRs have distinct ligands (28). TLR4 is a receptor for the LPS from Gram-negative bacteria, TLR2 controls cellular responsiveness to a variety of bacterial cell wall components, including lipoteichoic acid, peptidoglycans, and bacterial outer-membrane lipoproteins, and TLR5 mediates bacterial flagellin-induced cell activation (29–31). Members of the TLR family have some common structural features, including an extracellular domain consisting of a signal peptide, multiple leucine-rich repeats, and a cytoplasmic Toll interleukin-1 receptor domain.

In this study, AcNPV was shown to be a potent stimulant of immune cells. It is possible that the baculovirus envelope protein, gp64, with its high mannose content, recognizes a TLR molecule and thus activates the immune response.

Acknowledgments

We thank Drs. H. Tani and S. Nakada for helpful discussion and for providing plasmids.

References

1. Matsuura, Y., R. D. Possee, H. A. Overton, and D. H. L. Bishop. 1987. Baculovirus expression vector: the requirement for high level expression of proteins, including glycoproteins. *J. Gen. Virol. 68:1233.*
2. Luckow, V. A., and M. D. Summers. 1988. Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. *Virology 167:56.*

3. Hofmann, C., V. Sandig, G. Jennings, M. Rudolph, P. Schlag, and M. Strauss. 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc. Natl. Acad. Sci USA 92:10099.*
4. Boyce, F. M., and N. L. R. Bucher. 1996. Baculovirus mediated gene transfer into mammalian cells. *Proc. Natl. Acad. Sci USA 93:2348.*
5. Sandig, V., C. Hofmann, S. Steinert, G. Jennings, P. Schlag, and M. Strauss. 1996. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. *Hum. Gene Ther. 7:1937.*
6. Shoji, I., H. Aizaki, H. Tani, K. Ishii, T. Chiba, I. Saito, T. Miyamura, and Y. Matsuura. 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. *J. Gen. Virol. 78:2657.*
7. Barsoum, J., R. Brown, M. Mckee, and F. M. Boyce. 1997. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. *Hum. Gene Ther. 8:2011.*
8. Condreay, J. P., S. M. Witherspoon, W. C. Clay, and T. A. Kost. 1999. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc. Natl. Acad. Sci. USA 96:127.*
9. Tani, H., M. Nishijima, H. Ushijima, T. Miyamura, and Y. Matsuura. 2001. Characterization of cell-surface determinants important for baculovirus infection. *Virology 279:343.*
10. Sarkis, C., C. Serguera, S. Petres, D. Buchet, J. L. Ridet, L. Edelman, and J. Mallet. 2000. Efficient transduction of neural cells in vitro and in vivo by baculovirus-derived vector. *Proc. Natl. Acad. Sci. USA 97:14638.*
11. Airenne, K. J., M. O. Hiltunen, M. P. Turunen, A. M. Turunen, O. H. Laitinen, M. S. Kulomaa, and S. Y. Herttuala. 2000. Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. *Gene Ther. 7:1499.*
12. Pieroni, L., D. Maione, and N. L. Monica. 2001. In vivo gene transfer in mouse skeletal muscle mediated by baculovirus vectors. *Hum. Gene Ther. 12:871.*
13. Huser, A., M. Rudolph, and C. Hofmann. 2001. Incorporation of decay-accelerating factor into the baculovirus envelope generates complement resistant gene transfer vectors. *Nat. Biotechnol. 19:451.*
14. Gronowski, A. M., D. M. Hilbert, K. C. F. Sheehan, G. Garotta, and R. D. Schreiber. 1999. Baculovirus stimulates antiviral effect in mammalian cells. *J. Virol. 73:9944.*
15. Beck, N. B., J. S. Sidhu, and C. J. Omiecinski. 2000. Baculovirus vectors repress phenobarbital-mediated gene induction and stimulate cytokine expression in primary cultures of rat hepatocytes. *Gene Ther. 7:1274.*
16. Tobita, K., A. Sugiura, C. Enomoto, and M. Furuyama. 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med. Microbiol. Immunol. 162:9.*
17. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene 108:193.*
18. Wang, P., D. A. Hammer, and R. R. Granados. 1997. Binding and fusion of *Autographa californica* nucleopolyhedrovirus to cultured insect cells. *J. Gen. Virol. 78:3081.*
19. Blissard, G. W., and J. R. Wenz. 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH dependent membrane fusion. *J. Virol. 66:6829.*
20. Jarvis, D. L., and A. Garcia Jr. 1994. Biosynthesis and processing of the *Autographa californica* nuclear polyhedrosis virus gp64 protein. *Virology 205:300.*
21. Markovic, I., H. Pulyaeva, A. Sokoloff, and L. V. Chernomordik. 1998. Membrane fusion mediated by baculovirus gp64 involves assembly of stable gp64 trimers into multiprotein aggregates. *J. Cell. Biol. 143:1155.*
22. Monsma, S. A., and G. W. Blissard. 1995. Identification of membrane fusion domain and an oligomerization domain in the baculovirus gp64 envelope fusion protein. *J. Virol. 69:2583.*
23. Monsma, S. A., A. G. P. Oomens, and G. W. Blissard. 1996. The gp64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *J. Virol. 70:4607.*
24. Oomens, A. G. P., and G. W. Blissard. 1999. Requirement for gp64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. *Virology 254:297.*
25. Jarvis, D. L., and E. E. Finn. 1995. Biochemical analysis of the *N*-glycosylation pathway in baculovirus-infected lepidopteran insect cells. *Virology 212:500.*
26. Ulevitch, R. J. 1999. Toll gates for pathogen selection. *Nature 401:755.*
27. Levashina, E. A., E. Langley, C. Green, D. Gubb, M. Ashburner, J. A. Hoffmann, and J. M. Reichhart. 1999. Constitutive activation of Toll-mediated antifungal defense in serpin-deficient *Drosophila. Science 285:1917.*
28. Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature 406:782.*
29. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in TLR4 gene. *Science 282:2085.*
30. Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature 395:284.*
31. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature 410:1099.*

〔ウイルス　第53巻　第2号，pp. 185-193，2003〕

特集　ウイルスベクター：最新の話題

6．バキュロウイルスベクター
—哺乳動物細胞への遺伝子導入—

谷　英樹，阿部　隆之，林　昌宏，望月　理加，山岸　潤也，
北川　善紀，渡辺　理恵，宮本　大伸，森石　恆司，松浦　善治

はじめに

遺伝子治療は細胞医療や再生医療とともに，現行療法の限界を超える画期的な治療法として期待され，欧米を中心に活発な研究開発が行われている．外来遺伝子の導入方法としてはウイルスベクターやリポソームなどの非ウイルスベクターがあるが，効率的にはウイルスベクターに勝るものはない．ウイルスベクターとしては，アデノウイルスやレトロウイルスなどが開発され，すでに一部で臨床試験が行なわれている．レトロウイルスは遺伝子を染色体内に導入する為，導入遺伝子の挿入部位によっては宿主細胞を癌化させる欠点が指摘されており，フランスでの臨床試験で白血病の発症が報告されている．一方，アデノウイルスはほとんどのヒトが既に中和抗体を持っており，投与量が多くなる欠点が指摘されている．また，他のヒト病原ウイルスでは弱毒化や無毒化が施されているとはいえ，安全性の問題が皆無とは言えない．さらに，現行のベクター技術では目的の遺伝子を目的の細胞にいかにして効率的よく導入するかという点に多くの問題点が残されている．このような問題点を克服するには新しいウイルスベクターの開発が必須である．

昆虫を宿主とするバキュロウイルスは，約130kbpの環状2本鎖DNAをゲノムとして持ち，感染すると全蛋白の30～40%が多角体蛋白質になるほどの強力な多角体プロモーターを有している．この性質を利用して，本ウイルスは昆虫細胞を用いた目的蛋白質の高度産生法として普及している[20~23]．ところが近年，バキュロウイルスが昆虫細胞のみならず，広範な哺乳動物細胞にも複製することなく，効率よく外来遺伝子を導入できることが判明し，新しい遺伝子導入ベクターとしても脚光を浴びる事となった[6,15,19,26,28,32,35]．本稿では，バキュロウイルスベクターについて概説し，バキュロウイルスによる哺乳動物細胞への遺伝子導入と宿主応答について紹介したい．

1．組換えウイルスの作製

哺乳動物細胞で遺伝子を発現できるバキュロウイルスは，組換えに用いるトランスファーベクターが異なる点と，ウイルスが哺乳動物細胞内で複製しないため，感染時に比較的高力価のウイルス（moi 20-100）を接種する必要がある点を除けば，従来の昆虫細胞に感染させるバキュロウイルスと基本的には同じである．組換えウイルスの作製方法としては，従来の致死欠損ウイルスDNAとトランスファーベクターを用いた相同組換え法の他に，全バキュロウイルスゲノムを組み込んだバクミドにトランスポゾンを利用して，大腸菌体内で効率よく外来遺伝子を挿入できる方法が開発されている．このシステムでは組換えウイルス遺伝子を持ったバクミドを菌体から抽出し，昆虫細胞にトランスフェクトするだけで容易に組換えウイルスを回収できる．大きな外来遺伝子を導入する場合にはこちらのシステムを用いる方がよい．昆虫細胞の取り扱いや詳細な組換えウイルスの作製法は他の総説を参照して頂きたい[21,25,32,35]．

2．哺乳動物細胞への遺伝子導入

バキュロウイルスを哺乳動物細胞に接種すると，細胞内に取り込まれることは以前より報告されていたが[37]，多角体プロモーターは哺乳動物細胞内で機能しないこともあり外来遺伝子の発現は確認されていなかった．ところが，数

大阪大学微生物病研究所エマージング感染症研究センター
（〒565-0871 吹田市山田丘3番1号）
Baculovirus vector—Gene transfer into mammalian cells—
Hideki Tani, Takayuki Abe, Chang Kwang Limn, Rika Mochizuki, Junya Yamagishi, Yoshinori Kitagawa, Rie Watanabe, Kohji Moriishi, Yoshiharu Matsuura
Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan
TEL：81-6-6879-8340
FAX：81-6-6879-8269
E-mail：matsuura@biken.osaka-u.ac.jp

図1A

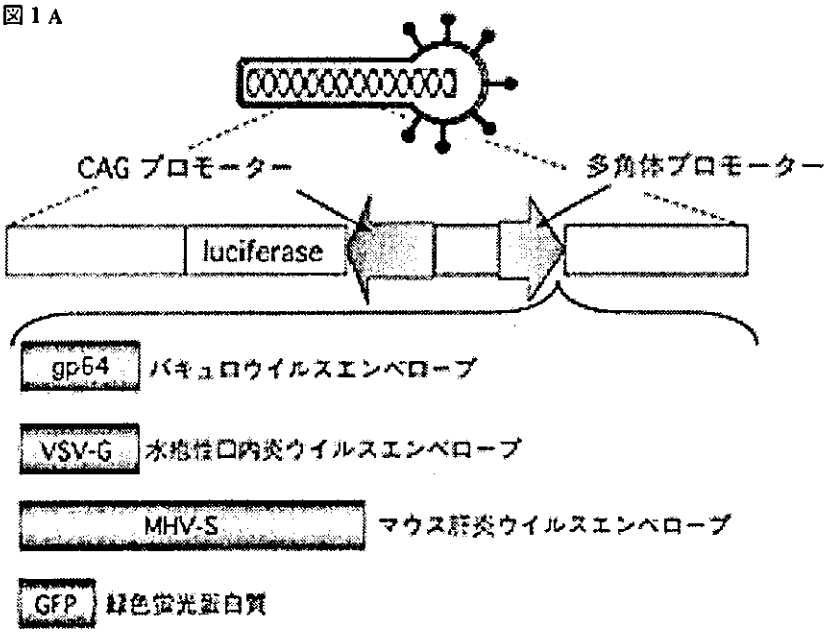

図1B

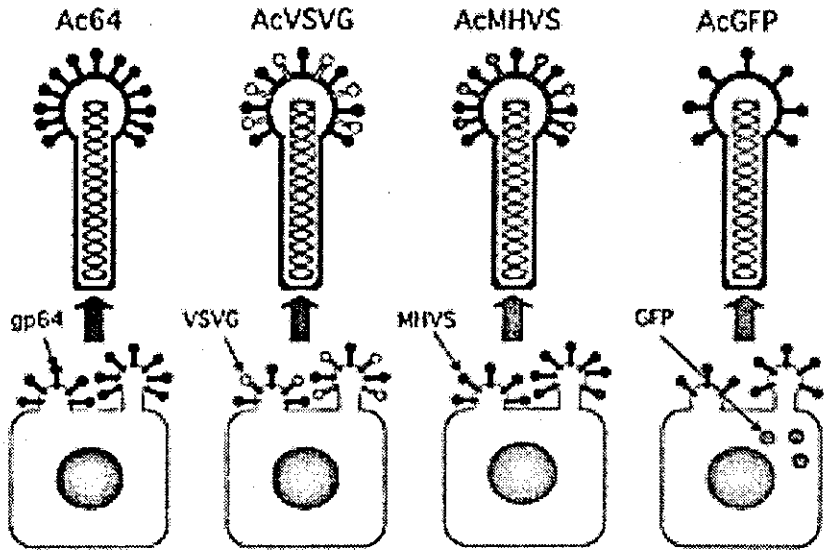

図1 シュードタイプバキュロウイルスの作製

A) トランスファーベクター：昆虫細胞で他種のウイルスエンベロープを発現させるためにポリヘドリンプロモーターの下流に各種ウイルスのエンベロープ遺伝子を，哺乳動物細胞での発現解析のためにCAGプロモーターとリポーターとして蛍のルシフェラーゼ遺伝子を挿入したベクターを作製した．これらのベクターと感染性のウイルスDNAを昆虫細胞へ導入して相同組換えにより，組換えウイルスを作製した．B) 予想される組換えウイルスの模式図：昆虫細胞表面から出芽してくるウイルスは細胞表面に発現しているウイルスエンベロープ蛋白質を取り込んでくるため，図のような形態をとっているものと思われる．実際に粒子中にこれらのウイルスエンベロープ蛋白質が取り込まれていることが確認できた．Ac64はgp64蛋白質を過剰に被ったバキュロウイルス，AcVSVGとAcMHVSはgp64蛋白質以外に，VSVGとMHVSをそれぞれ被ったシュードタイプバキュロウイルス，AcGFPは対照のバキュロウイルス．

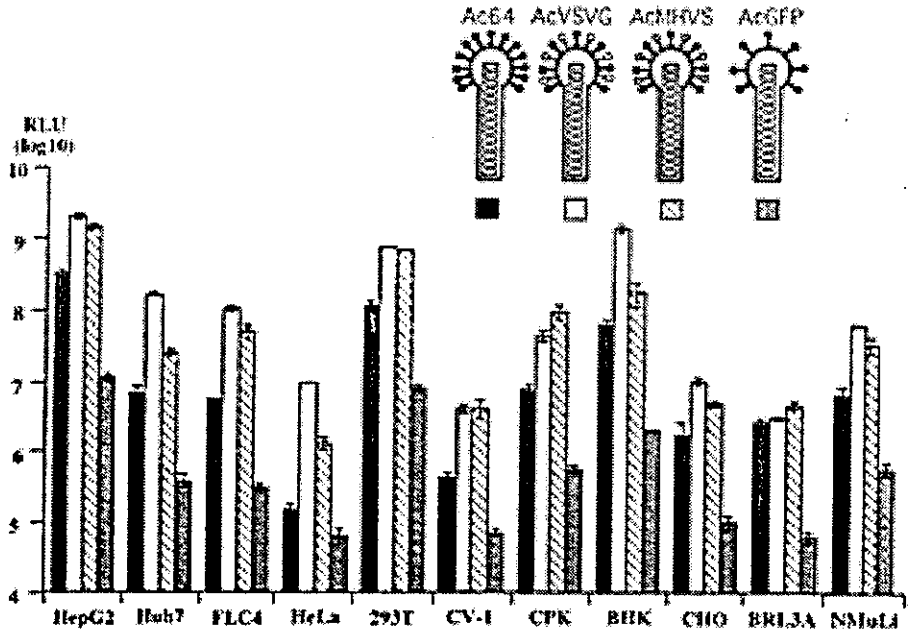

図2　哺乳動物細胞における遺伝子導入効率
作製した組換えウイルスを各種細胞にmoi 50で接種し，24時間後に感染細胞を回収し，ルシフェラーゼの活性を測定した．いずれの細胞においても，組換えウイルスが高い遺伝子導入活性を示した．

年前に哺乳動物細胞で機能するプロモーターを組み込んだバキュロウイルスが，肝細胞株特異的に外来遺伝子を効率よく発現出来るという報告が相次いでなされた[6,15]．その後，多くのグループから様々な哺乳動物細胞株へも遺伝子導入が可能であることが示され[3,7,24,31,36,41]，現在では培養細胞株だけでなく各種初代培養細胞などへの遺伝子導入も可能であることが明らかにされている．

3．遺伝子導入効率の改良

バキュロウイルスは肝細胞などの一部の細胞株では極めて高効率で遺伝子導入することが出来るが，全ての哺乳動物細胞株に高率よく遺伝子導入出来るわけではない．細胞への遺伝子導入効率を上げるためのいくつかの手法が報告されている．ヒストン脱アセチル化阻害剤であるトリコスタチンAやブチル酸を用いてバキュロウイルスによって細胞内に導入された発現ユニットの転写効率を上げる方法や[7,29]，他のウイルスの外被タンパクを被った組換えウイルスの利用等が報告されている[3,34]．特にシュードタイプウイルスの作製でよく用いられる，水疱性口内炎ウイルスのエンベロープ蛋白質（VSVG）を被った組換えウイルスは従来のバキュロウイルスよりも哺乳動物細胞における感染性が高いことが報告されている[3]．我々もバキュロウイルスのgp64蛋白質，VSVG，あるいは，マウス肝炎ウイルス（MHV）のエンベロープ蛋白質であるMHVSを多角体プロモーターの下流に，また，リポーター遺伝子をCAGプロモーターの下流にそれぞれ組み込みこんだウイルスを作製した（図1）．これらのウイルスを各種哺乳動物細胞に接種したところ，いずれの細胞でも感染価を上げるに従ってリポーター活性（ルシフェラーゼ活性）が上昇し，コントロールウイルスに比べてgp64，VSVG，およびMHVSを被った組換えバキュロウイルスは，～500倍も高い値を示した（図2）．また，これらの組換えウイルスによる遺伝子導入は，gp64とVSVGあるいはMHVSの抗体で中和されることから，これらのウイルスはそれぞれのエンベロープ蛋白質を介して感染し，遺伝子を細胞に導入することができるものと考えられる[34]．

4．バキュロウイルスの哺乳動物細胞への侵入機構

多くのウイルスは宿主細胞の表面に存在する特異的レセプターに結合することにより感染を開始する．従って，レセプターはウイルスの宿主や組織へのトロピズムを決定する上で重要な分子となっている．バキュロウイルスはエンベロープ蛋白質gp64を介して宿主に感染していると考えられているものの，自然宿主である昆虫細胞ばかりでなく，哺乳動物細胞においても未だ宿主レセプターや感染機構についての解析は進んでいない．これまでにプロテアーゼで処理した細胞にはバキュロウイルスが結合しないことから，細胞表面の蛋白性分子が感染に関与しているとの報告があるが，詳細は不明であった[39,40]．我々は上記の各種組換えバキュロウイルスを用いて，バキュロウイルスの哺乳動物細胞への感染に関与する細胞因子の解析を行った．gp64を過剰に粒子表面に被ったウイルスが，他のウイル

図3A

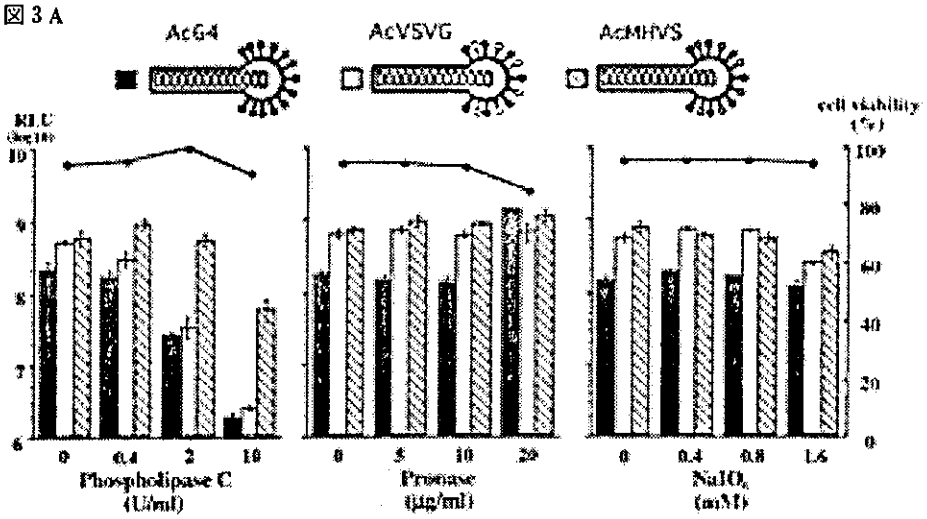

図3B

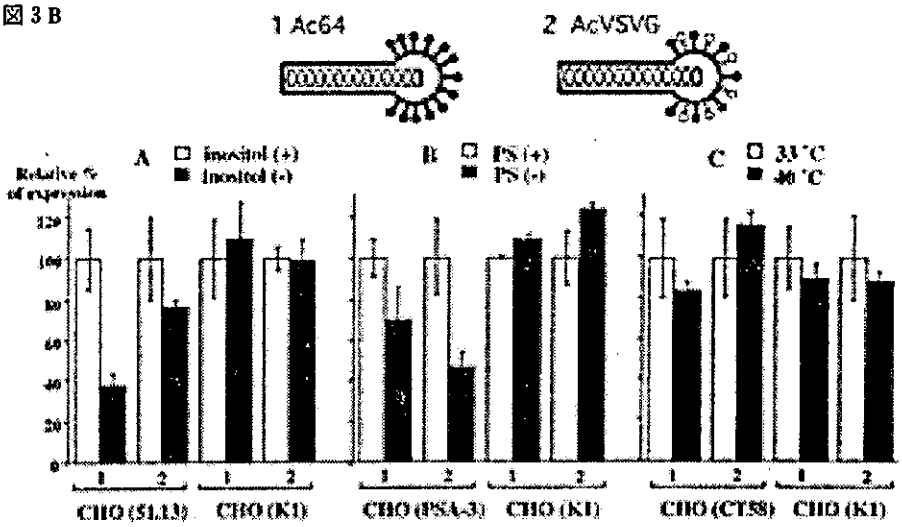

図3 バキュロウイルスの侵入に関与する細胞因子

A) HepG2細胞をフォスフォリパーゼC，プロナーゼ，あるいは$NaIO_4$で処理した後，種々の組換えウイルスを感染させ24時間後に感染細胞を回収し，ルシフェラーゼの活性を測定した．グラフ上方の実線は生存細胞の比率を示している．これらの薬剤処理条件では細胞の生存率にほとんど影響は認められなかった．B) 脂質合成欠損変異CHO細胞株をそれぞれの条件下で培養し，脂質の発現の有無を確認後，バキュロウイルスによる遺伝子導入を試みた．A) フォスファチジルイノシトール合成欠損株（51.13），B) フォスファチジルセリン合成欠損株（PSA-3），および，C) フォスファチジルコリン合成欠損株（CT58）を合成可能条件（□）と合成不可条件（■）で培養し，Ac64 (1) とAcVSVG (2) を接種した．Ac64はフォスファチジルイノシトール合成阻害細胞で，AcVSVGはフォスファチジルセリン合成阻害細胞でそれぞれ遺伝子導入の阻害傾向が観察された．一方，フォスファチジルコリンの発現は両ウイルスとも遺伝子導入に差異は認められなかった．

スエンベロープ蛋白質を被った組換えウイルスと同様に哺乳動物細胞に効率よく感染することから，動物細胞の表面にバキュロウイルスのgp64蛋白質を認識する何らかのリセプターが存在することが推測された．そこで，最も感受性が高かったヒト肝癌由来細胞（HepG2細胞）を，蛋白質を分解するプロナーゼ，糖質を分解する過ヨウ素酸，リン脂質を分解するフォスフォリパーゼCでそれぞれ処理し，ウイルスに対する感受性を検討した（図3A）．その結果，プロナーゼや過ヨウ素酸処理では変化は認められないが，フォスフォリパーゼCの処理によって濃度依存的に感染効率が減少したことから，バキュロウイルスは何らかのリン脂質を介して哺乳動物細胞へ感染しているものと

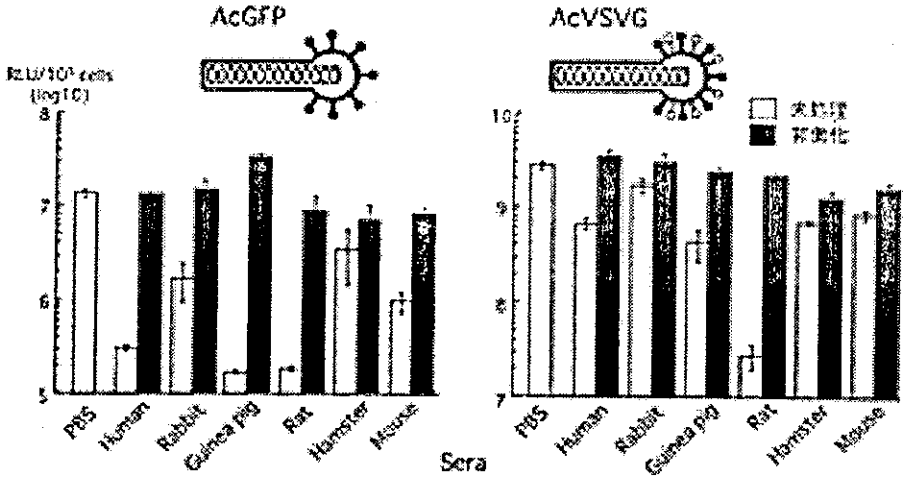

図4　バキュロウイルス感染における血清の影響
各種動物血清成分（ヒト，ウサギ，モルモット，ラット，ハムスター，マウス）を非働化したもの（■）と未処理のもの（□）をそれぞれバキュロウイルスと反応させた後，HepG 2細胞へ接種し，遺伝子導入への影響を調べた．

考えられた．次に，精製脂質を組換えウイルスに反応させ，HepG 2細胞に感染させたところ，濃度依存的にフォスファチジン酸（PA）とフォスファチジルイノシトール（PI）で感染性の阻害が観察された．また，特定の培養条件下で脂質の合成をコントロールできるハムスター変異細胞株を用いて脂質の発現と感染性の相関を調べたところ，親株およびフォスファチジルセリンやフォスファチジルコリンの欠損状態に比べ，PIが合成できない状態ではウイルスの感染性が低いことが示された（図3B）．このことから，バキュロウイルスが哺乳動物感染に感染するには，細胞表面のリン脂質の一つであるPIが深く関与していることが示唆された[34]．

5．*In vivo* での遺伝子導入

バキュロウイルスの *in vivo* における遺伝子導入は，血液中の補体成分によってウイルスが不活化されるため，それをなんとか克服する必要がある．これまでに補体の活性化を阻害する蛇毒因子や可溶型補体レセプター因子を用いてウイルスの不活化を抑制したり[14,16]，補体制御因子であるDAFと融合させた組換えgp64蛋白質を持ったバキュロウイルスの作製[17]等が試みられているが，動物個体への遺伝子導入は満足の行くものではなかった．様々な動物血清に対するバキュロウイルスの感受性を調べてみたところ，非働化していないヒト，モルモットおよびラット血清はバキュロウイルスを不活化させたが，VSVGを被った組換えバキュロウイルスでは対照ウイルスに比べて血清成分に対して抵抗性を示した（図4）．また，補体経路の活性化を抑制できる薬剤であるFUT-175（6-amidino-2-naphthyl 4-guanidinobenzoate，フサン）は，濃度依存的に血清成分によるバキュロウイルスの不活化を阻害した．今後は，組換えバキュロウイルスとFUT-175のような薬剤との併用による方法により，動物個体への遺伝子導入も可能になるものと思われる．これまでに，バキュロウイルスによる *in vivo* での遺伝子導入はいくつか成功例が報告されており，マウスの筋肉細胞[27]，脳神経細胞[29]，網膜細胞[13]，ウサギの内皮細胞[2]などへのウイルスの直接導入による外来遺伝子の発現が確認されている．我々も組換えウイルスを用いてマウスの脳組織（図5A）および精巣のセルトリ細胞（図5B）での遺伝子発現を認めている[33]．

6．ターゲッティング可能なバキュロウイルスベクターの開発

上述のように他のウイルス蛋白質を組み込むことによって広範な細胞に高率よく遺伝子導入できるバキュロウイルスの開発とともに，ウイルス粒子表面に任意の蛋白質のみを提示させることによって，狙った細胞だけに遺伝子を導入できるターゲッティングベクターの開発も重要である．gp64蛋白質は動物細胞に普遍的に存在するリン脂質を認識して侵入するため，このままでは遺伝子導入に特異性を持たせることは困難である．そこで，バキュロウイルスのgp64遺伝子を欠損させ，ウイルス粒子表面に任意の蛋白質を自在に提示させることによって，狙った細胞だけに目的遺伝子を導入可能なターゲティングベクターの作製系を構築した．まず，ウイルスゲノムからgp64遺伝子のみを欠損させ，この欠損ウイルスゲノムをgp64蛋白質を発現している昆虫細胞に導入すると，gp64蛋白質がトランスに供給されるため，欠損ウイルスはこの細胞においてのみ増殖可能である．このウイルスをバキュロウイルスの粒子表面に提示したい蛋白質を発現している昆虫細胞に感染させることにより，gp64蛋白質の代わりに目的蛋白質のみ

図5A

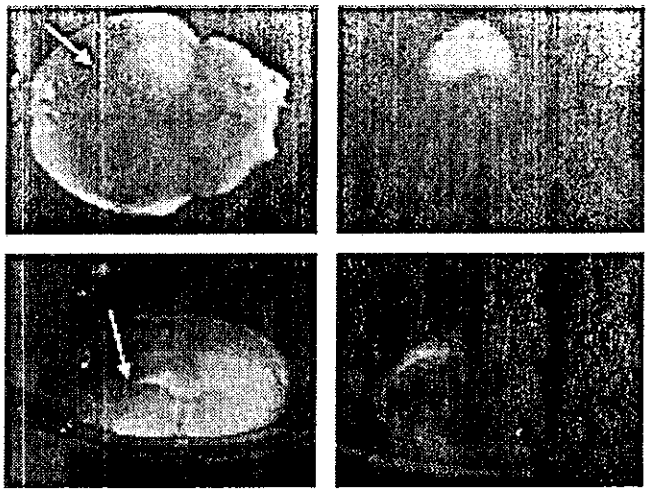

図5B

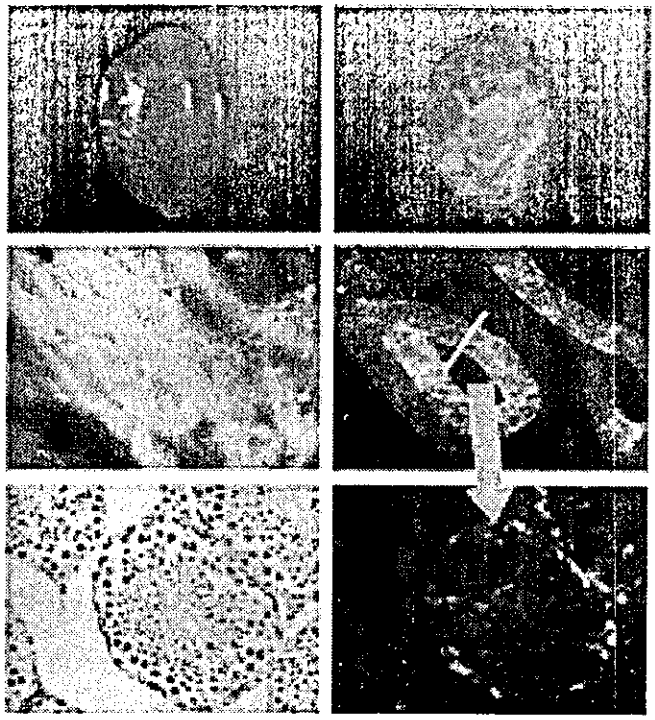

図5 *In vivo* におけるバキュロウイルス遺伝子導入
A）マウス脳への導入：AcVSVG-CAGFP（4X10⁷ PFU）を大脳皮質内へ直接接種し，2日後に実体蛍光顕微鏡下でGFPの発現を観察した．接種部位にGFPの発現が観察された（上右）．断面でも蛍光が確認できる（下右）．B）マウス精巣への導入：AcVSVG-CAGFPを2X10⁸ PFUで精巣管に沿って精巣に直接接種し，2日後に実体蛍光顕微鏡下でGFPの発現を確認した．精巣全体像(上)，精巣管(中)，精巣管の断面図（下）を示す．左は光学顕微鏡像，右は蛍光顕微鏡像．下左図はヘマトキシリン染色像．

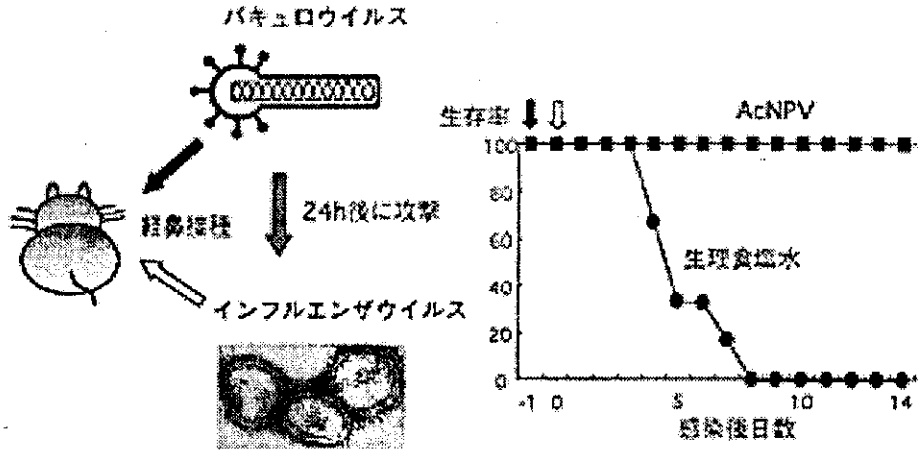

図6 バキュロウイルスによる自然免疫誘導
バキュロウイルス（10⁸ PFU）をマウスに鼻腔内接種に接種し，24時間後に致死量のインフルエンザウイルス（A/RR/8/34）で攻撃した．バキュロウイルス接種群（■）とコントロールとして等量の生理食塩水（●）を鼻腔内接種したマウスの感染後，14日目までの生存率．

を被ったシュードタイプウイルスを作製できる．gp64の代わりにVSVGを被ったウイルスによる遺伝子導入は，抗gp64抗体では阻止できず，抗VSVG抗体で中和されたことから，設計どおりに粒子表面に発現させたVSVGを介して遺伝子が導入されていることが確認された．また，ウイルスのエンベロープ蛋白質だけでなく，逆にウイルスのリセプター分子や癌抗原に対する単鎖抗体を粒子表面に提示すれば，ウイルスに感染してエンベロープ蛋白質を発現している細胞や癌細胞だけにチミジンキナーゼ等の自殺遺伝子を導入し，プロドラッグとの併用によって目的の細

胞だけを生体から排除することが可能となると思われる．

7．バキュロウイルスによる宿主応答

初代ラット培養細胞にバキュロウイルスを接種するとTNF-α，IL-1α，IL-1βの誘導が惹起されることが報告されており[4]，さらに，バキュロウイルスの感染細胞のエンベロープ蛋白質画分をマウスの腹腔内に投与することによりインターフェロンの誘導が見られ，脳心筋炎ウイルスの致死感染からマウスが防御されることが報告されている[12]．また，阿部らはバキュロウイルスを24時間前に鼻腔内投与したマウスがA型およびB型インフルエンザウイルスの致死量の鼻腔内攻撃から防御されることを明らかにした（図6）[1]．これまで多くのウイルスエンベロープ蛋白質が自然免疫を誘導できることが報告されており，これまでのバキュロウイルスでの成績もエンベロープ蛋白質が自然免疫を誘導することを支持するものであった[12]．しかしながら，我々はバキュロウイルスによる自然免疫の誘導がエンベロープ蛋白質によるものではなく，細胞内に取り込まれたウイルスゲノムがToll-like receptor 9（TLR 9）を介してシグナルを核に伝達していることを明らかにした．免疫担当細胞においてはエンベロープ蛋白質の細胞融合活性によって細胞内に侵入したバキュロウイルスは，主に分解経路に輸送され，分解されたゲノムDNAが，細胞内に局在するTLR 9にシグナルを伝達しているものと思われる（阿部，論文投稿中）．この成績は，バキュロウイルスが遺伝子導入ベクターとしてだけでなく，接種経路によってはアジュバント活性を併せ持った新しいワクチンベクターとしての可能性を示唆するものである．この様に免疫担当細胞に対しては，細胞に侵入したバキュロウイルスゲノムがTLR 9を介して免疫遺伝子の発現を誘導するが，外来遺伝子の発現は確認できない．一方，一般の細胞ではウイルスゲノムは核に移行し，効率よく外来遺伝子が転写翻訳されるものと思われる．バキュロウイルスを接種しても細胞傷害性が低いことは，バキュロウイルスの遺伝子治療用ベクターとしての有利な特徴のひとつであると考えられる．そこで，遺伝子治療用ベクターとしての安全性を評価するため，哺乳動物細胞にバキュロウイルスを感染させた際に転写される，バキュロウイルス遺伝子を網羅的に解析した．バキュロウイルスをHepG 2細胞に接種した場合，150個の全バキュロウイルス遺伝子のうち3種の遺伝子の発現が転写レベルで僅かに検出さるのみであった．この成績から，哺乳動物細胞においてはバキュロウイルス自身のゲノムの発現がほとんどなく，これが細胞傷害性が低いことの一因であると推測される．

おわりに

バキュロウイルスが哺乳動物細胞に遺伝子を導入できることが明らかとなり，これまでに多くのグループによって効果的な利用法が検討されてきた．特に，肝細胞に高効率に遺伝子導入出来ることを利用して，B型肝炎ウイルス[8]やC型肝炎ウイルス[10,30,38]の基礎研究への応用にも用いられている．昆虫細胞で各種ウイルスのウイルス様粒子（VLP）を大量に産生することが可能であることから，粘膜免疫の誘導を目的としたVLPベクターや，バキュロウイルスの粒子表面に効率よく外来蛋白質を提示させるウイルスディスプレイシステム[5,9,11]の開発も期待される領域である．また，昆虫細胞に哺乳動物細胞の糖鎖転移酵素遺伝子を導入したヒト化昆虫細胞の樹立も報告されており，ヒト型糖鎖を保持した組換え蛋白質の産生も期待される[19]．様々な可能性を秘めたバキュロウイルスベクターの今後の進展に期待したい．

文　献

1）Abe, T., Takahashi, H., Hamazaki, H., Miyano-Kurosaki, N., Matsuura, Y., and Takaku, H.(2003). Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. J. Immunol. **171**, 1133-1139.

2）Airenne, K. J., Hiltunen, M. O., Turunen, M. P., Turunen, A-M., Laitinen, O. H., Kulomaa, M. S., and Yla-Herttuala, S.(2000). Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. Gene Ther. **7**, 1499-1504.

3）Barsoum, J., Brown, R., McKee, M., and Boyce, F. M.(1997). Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum. Gene Ther. **8**, 2011-2018.

4）Beck, N. B., Sidhu, J. S., and Omiecinski, C. J.(2000). Baculovirus vectors repress phenobarbital-mediated gene induction and stimulate cytokine expression in primary cultures of rat hepatocytes. Gene Ther. **7**, 1274-1283.

5）Boublik, Y., Di-Bonito, P., and Jones, I. M.(1995). Eukaryotic virus display：engineering the major surface glycoprotein of the Autographa californica nuclear polyhedrosis virus (AcNPV) for the presentation of foreign proteins on the virus surface. Bio/Technology **13**, 1079-1084.

6）Boyce, F. M., and Bucher, N. L. R.(1996). Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. USA **93**, 2348-2352.

7）Condreay, J. P., Witherspoon, S. M., Clay, W. C., and Kost T. A.(1999). Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc. Natl. Acad. Sci. USA **96**, 127-132.

8）Delaney, W. E. 4th, and Isom, H. C.(1998). Hepatitis B virus replication in human HepG 2 cells mediated by hepatitis B virus recombinant baculovirus. Hepatology **28**, 1134-1146.

9）Ernst, W., Grabherr, R., Wegner, D., Borth, N., Grassauer, A., Katinger, H.(1998). Baculovirus surface display：construction and screening of a eukaryotic epi-

tope library. Nucleic Acids Res. **26**, 1718–1723.
10) Fipaldini, C., Bellei, B., amd La Monica, N.(1999). Expression of hepatitis C virus cDNA in human hepatoma cell line mediated by a hybrid baculovirus–HCV vector. Virology **255**, 302–311.
11) Grabherr, R., Ernst, W., Oker-Blom, C., and Jones, I. (2001). Developments in the use of baculoviruses for the surface display of complex eukaryotic proteins. Trends in Biotechnology, **19**, 231–236.
12) Gronowski, A. M., Hilbert, D. M., Sheehan, K. C. F., Garotta, G., and Schreiber, R. D.(1999). Baculovirus stimulates antiviral effects in mammalian cells. J. Virol. **73**, 9944–9951.
13) Haeseleer, F., Imanishi, Y., Saperstein, D. A., and Palczewski, K.(2001). Gene transfer mediated by recombinant baculovirus into mouse eye. Invest. Ophthalmol. Vis. Sci. **42**, 3294–3300.
14) Hofmann, C., Huser, A., Lehnert, W., Strauss, M. (1999). Protection of baculovirus–vectors against complement–mediated inactivation by recombinant soluble complement receptor type 1. Biol. Chem. **380**, 393–395.
15) Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P., and Strauss, M.(1995). Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. USA **92**, 10099–10103.
16) Hofmann, C., and Strauss, M.(1998). Baculovirus–mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene Ther. **5**, 531–536.
17) Huser, A., Rudolph, M., and Hofmann, C.(2001). Incorporation of decay–accelerating factor into the baculovirus envelope generates complement–resistant gene transfer vectors. Nat. Biotechnol. **19**, 451–455.
18) Jarvis, D. L.(2003). Developing baculovirus–insect cell expression systems for humanized recombinant glycoprotein production. Virology **310**, 1–7.
19) Kost, T. A., and Condreay, J. P.(2002). Recombinant baculoviruses as mammalian cell gene–delivery vectors. Trends in Biotechnology, **20**, 173–180.
20) Luckow, V. A., and Summers, M. D.(1988). Trends in the development of baculovirus expression vectors. Biotechnology **6**, 47–55.
21) Matsuura, Y., Possee, R. D., Overton, H. A., and Bishop, D. H. L.(1987). Baculovirus expression vectors：the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. **68**, 1233–1250.
22) 松浦善治：バキュロウイルスベクターを用いた遺伝子発現，(1992)．蛋白核酸酵素，**37**，211–222.
23) 松浦善治：バキュロウイルス発現系，(1994)．「遺伝子導入と発現・解析法」(横田 崇，新井賢一編) 羊土社，東京
24) 松浦善治，勝二郁夫，葉 真珠，谷 英樹，石井孝司，宮村達男：バキュロウイルスを用いた哺乳動物細胞への遺伝子導入とその応用，(1997)．ウイルス，**47**，247–256.
25) 松浦善治，谷 英樹：昆虫培養細胞（バキュロウイルスベクター），(2001)．生物化学実験法45組換えタンパク質生産法（塚越規弘編著）学会出版センター，東京
26) Pieroni, L., and La Monica, N.(2001). Towards the use of baculovirus as a gene therapy vector. Curr. Opin. Mol. Ther. **3**, 465–467.
27) Pieroni, L., Maione, D., and La Monica, N.(2001). *In vivo* gene transfer in mouse skeletal muscle mediated by baculovirus vectors. Hum. Gene Ther. **12**, 871–881.
28) Sandig, V., and Strauss, M.(1996). Liver–directed gene transfer and application to therapy. J. Mol. Med. **74**, 205–212.
29) Sarkis, C., Serguera, C., Petres, S., Buchet, D., Ridet, J., Edelman, L., and Mallet, J.(2000). Efficient transduction of neural cells *in vitro* and *in vivo* by a baculovirus –derived vector. Proc. Natl. Acad. Sci. USA **97**, 14638–14643.
30) Shimoike, T., Mimori, S., Tani, H., Matsuura, Y., and Miyamura, T.(1999). Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. J. Virol. **73**, 9718–9725.
31) Shoji I., Aizaki H., Tani H., Ishii K., Chiba T., Saito I., Miyamura T., and Matsuura Y.(1997). Efficient gene transfer into various mammalian cells including non–hepatic cells by baculovirus vectors. J. Gen. Virol. **78**, 2657–2664.
32) 勝二郁夫，谷 英樹，松浦善治，バキュロウイルスを用いた哺乳動物細胞への遺伝指導入，(1997)．実験医学「遺伝子導入＆発現解析実験法」，(斎藤 泉，管野純夫編)，羊土社，東京
33) Tani, H., Limn, C. K., Yap, C. C., Onishi, M., Nozaki, M., Nishimune, Y., Okahashi, N., Kitagawa, Y., Watanabe, R., Mochizuki, R., Moriishi, K., and Matsuura, Y.(2003). *In vitro* and *In vivo* gene delivery by recombinant baculoviruses. J. Virol. **77**, 9799–9808.
34) Tani, H., Nishijima, M., Ushijima, H., Miyamura, T., and Matsuura, Y.(2001). Characterization of cell–surface determinants important for baculovirus infection. Virology **279**, 343–353.
35) 谷 英樹，松浦善治，バキュロウイルスを用いた哺乳動物細胞への遺伝子導入，(2001)．細胞，33，30–34.
36) van Loo, N–D., Fortunati, E., Ehlert, E., Rabelink, M., Grosveld, F., and Scholte, B. J.(2001). Baculovirus infection of nondividing mammalian cells：mechanisms of entry and nuclear transport of capsids. J. Virol. **75**, 961–970.
37) Volkman, L. E., and Goldsmith P. A.(1983). *In vitro* survey of Autographa california nuclear polyhedrosis virus interaction with nontarget vertebrate host cells. Appl. Env. Microbiol. **45**, 1085–1093.
38) Wang, T. H., Rijnbrand, R. C., and Lemon, S. M.(2000). Core protein–coding sequence, but not core protein, modulates the efficiency of cap–independent translation directed by the internal ribosome entry site of hepatitis C virus. J. Virol. **74**, 11347–11358.
39) Wang, P., Hammer, D. A., and Granados, R. R.(1997). Binding and fusion of Autographa californica nucleopolyhedrovirus to cultured insect cells. J. Gen. Virol. **78**, 3081–3089.
40) Wickham, T. J., Shuler, M. L., Hammer, D. A., Granados, R. R., and Wood, H. A.(1992). Equilibrium and ki-