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

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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).

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[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.

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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

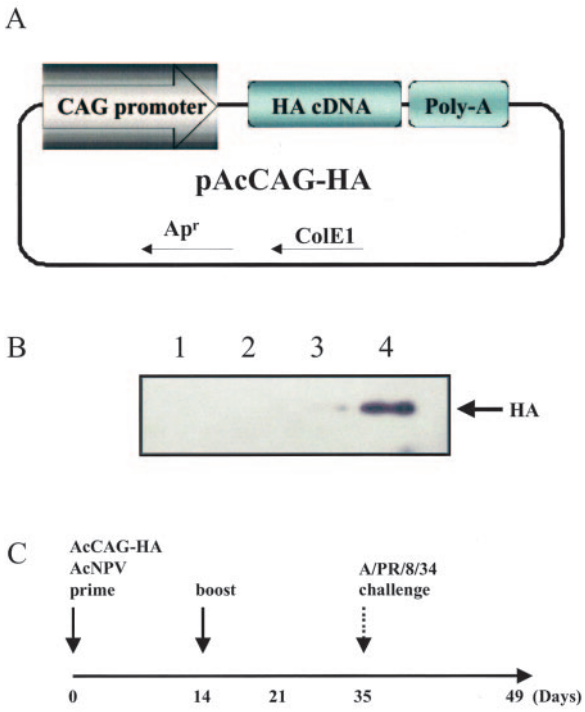

FIGURE 1. *A*, Schematic representation of the baculovirus transfer vector expressing the influenza virus hemagglutinin, pAcCAG-HA. The hemagglutinin gene of the influenza virus (A/PR/8/34) was inserted under the control of the CAG promoter. *B*, Expression of HA proteins in Huh7 cells infected with 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,

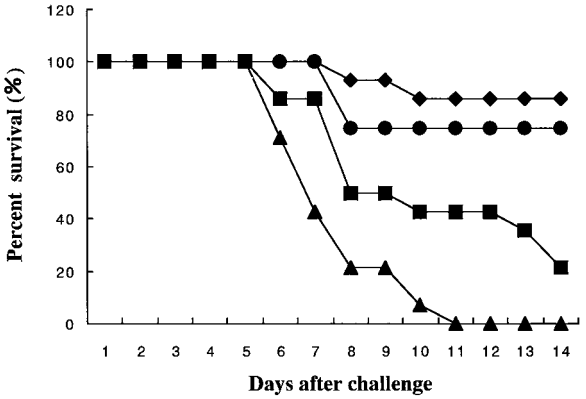

FIGURE 2. Protection from a lethal challenge of influenza virus. Mice were immunized intramuscularly (■) or intranasally (◆) with AcCAG-HA and intranasally with AcNPV (●) at a dose of 1.1×10^8 PFU. Control mice were inoculated with saline (▲). Three weeks after the second immunization, the mice were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

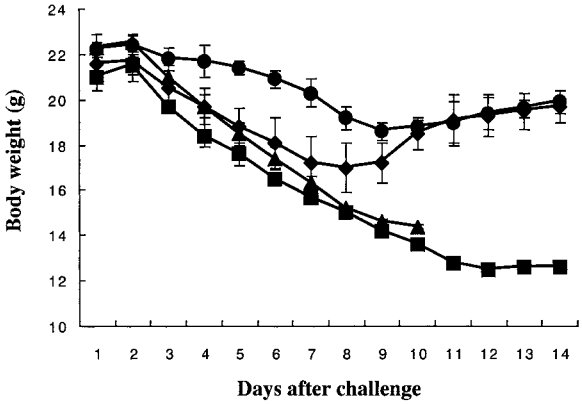

FIGURE 3. Weight loss after influenza virus challenge. Mice were immunized intramuscularly (■) or intranasally (●) with AcCAG-HA and intranasally with AcNPV (◆) at a dose of 1.1×10^8 PFU. Control mice were inoculated with saline (▲). Three weeks after the second immunization, the mice were challenged 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 ($p < 0.05$).

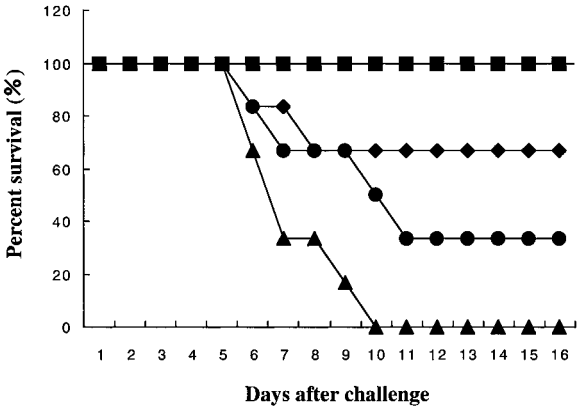

FIGURE 4. Protection from a lethal challenge of influenza virus by AcNPV. AcNPV was inoculated intranasally (■) at a dose of 1.1×10^8 PFU. Recombinant murine IFN-α (●) and poly:(I–C) (◆) were inoculated i.p. at doses of 1 and 100 μg, respectively. Control mice were inoculated with saline (▲). Each group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

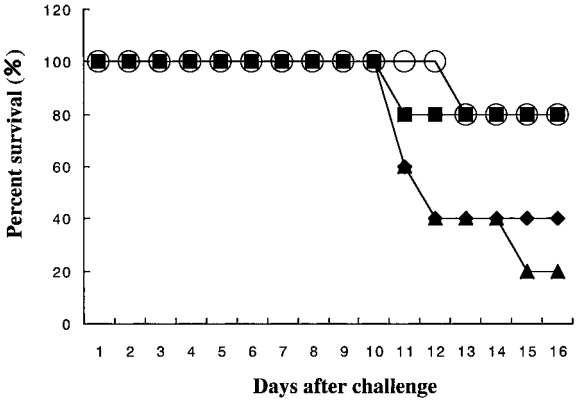

FIGURE 5. Protection from a lethal challenge of different serotypes of influenza virus by AcNPV. AcNPV was intranasally inoculated 24 h before the influenza virus challenge, and the mice were challenged with a lethal dose of influenza virus A/Guizhou (■) and B/Ibaraki virus (○). Infection control mice 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).

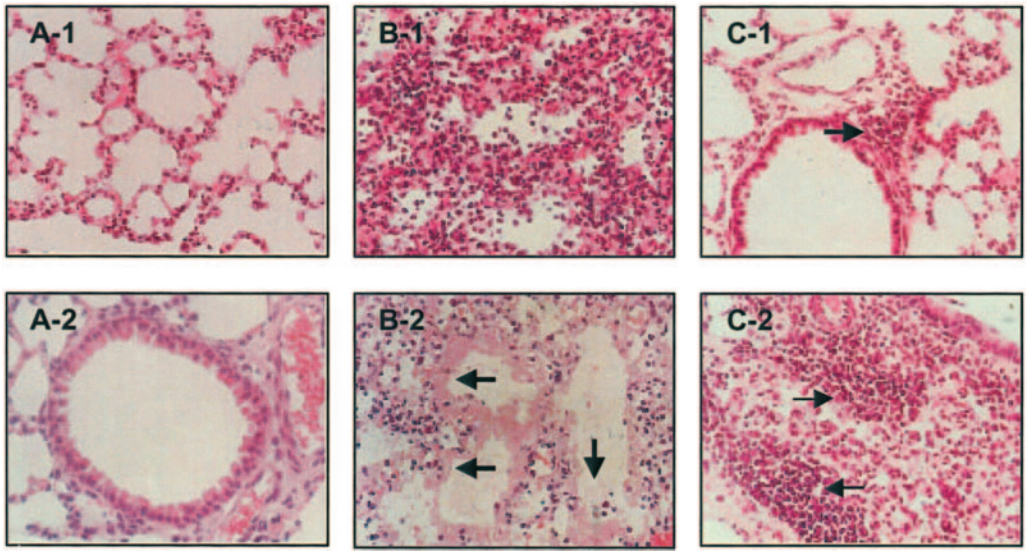

FIGURE 6. Effects of pretreatment with AcNPV on the lung morphology in mice infected with influenza virus. Seven days after influenza challenge, lungs were removed, sectioned, and stained with H&E. *A*, Uninfected control; *B*, the lung of a mouse infected with influenza virus without any treatment. 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.

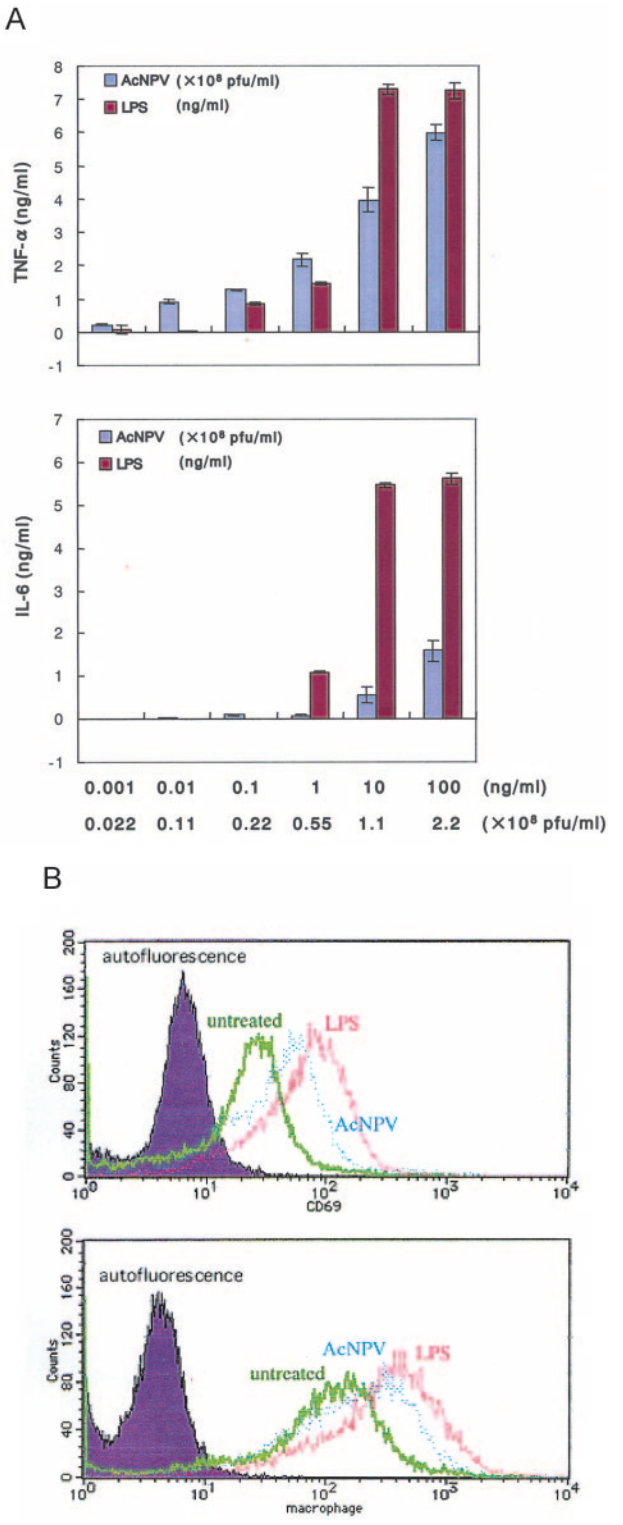

FIGURE 7. Activation of the mouse macrophage cell line, RAW264.7, treated with AcNPV or bacterial LPS. *A*, RAW264.7 cells were incubated with different concentrations of AcNPV or bacterial LPS for 24 h. Production levels of TNF-α and IL-6 were determined by sandwich ELISA. Data represent the mean concentrations 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 RNase 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.

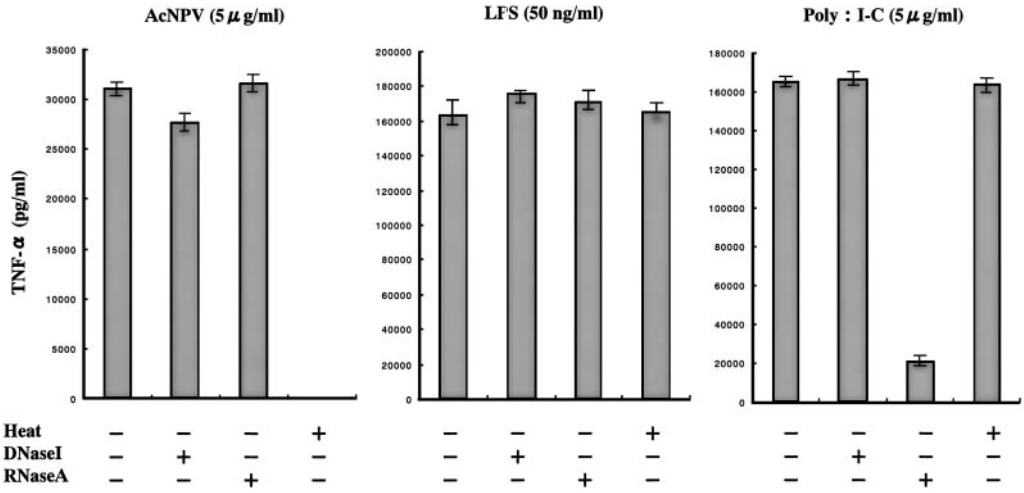

FIGURE 8. Effect of nucleases and heat treatments on the activation of macrophages by AcNPV. AcNPV (5 μg/ml), LPS (50 ng/ml), and poly:(I–C) (5 μg/ml) were treated with RNase A (50 μg/ml) and DNase I (75 U/ml) for 30 min at 37°C or heating for 30 min at 56°C. These treated compounds were added to RAW264.7 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 $\pm$ 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.

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