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Evidence for Phagocytosis of Influenza Virus-Infected, Apoptotic Cells by Neutrophils and Macrophages in Mice

Yumi Hashimoto,* Takeshi Moki,† Takenori Takizawa,‡ Akiko Shiratsuchi,*† and Yoshinobu Nakanishi2*†

Influenza virus-infected cells undergo apoptosis and become susceptible to phagocytosis by macrophages in vitro, and this leads to the propagation of the virus being inhibited. We previously showed that inhibitors of phagocytosis increased the rate of mortality among influenza virus-infected mice. However, the mode of the phagocytosis of influenza virus-infected cells in vivo has not been investigated. We, in this study, assessed this issue by histochemically analyzing bronchoalveolar lavage cells and lung tissue obtained from C57BL/6 mice infected with influenza A/WSN (H1N1) virus. Both neutrophils and macrophages accumulated in the lung soon after the viral challenge, and either type of cell was capable of phagocytosing influenza virus-infected, apoptotic cells. Changes in the level of phagocytosis and the amount of virus in lung tissue roughly correlated with each other. Furthermore, alveolar macrophages prepared from influenza virus-infected mice showed greater phagocytic activity than those from uninfected mice. The phagocytic activity of macrophages was stimulated in vitro by a heat-labile substance(s) released from influenza virus-infected cells undergoing apoptosis. These results suggested that the level of phagocytosis is augmented both quantitatively and qualitatively in the lung of influenza virus-infected animals so that infected cells are effectively eliminated. Finally, lack of TLR4 caused an increase in the rate of mortality among influenza virus-challenged mice and a decrease in the level of phagocytosis of apoptotic cells in the lung. TLR4 could thus play an important role in the host defense against influenza by positively regulating the phagocytic elimination of infected cells. The Journal of Immunology, 2007, 178: 2448–2457.

Influenza is an epidemic, acute respiratory disease that has a high rate of mortality, especially among the elderly and children, and a large number of deaths due to influenza are reported every year all over the world (1). Influenza virus is the etiological agent of this disease, and influenza A virus is the most important among the three types of the virus from a clinical point of view. Due to high genetic variability, the availability of vaccines and antiviral agents against influenza virus is limited (1). New strategies are thus needed for the development of effective antiviral agents. From this point of view, it is important to gain a deeper understanding of the mechanism of host defense against influenza virus infection.

The host immune system acts against microbial invasion by inducing both innate and adaptive responses (2, 3). Invading bacteria are killed by bactericidal substances that are produced through a humoral innate response (4). Bacteria are also eliminated by phagocytosis that occurs as a cellular response in both innate and adaptive immunity (5, 6). In contrast, some microbes including viruses are phagocytosed not directly but indirectly as microbi-infected cells. The latter mode of phagocytosis in adaptive immunity is initiated with the aid of Abs that bind to infected cells recognizing viral components expressed on the surface of host cells. The Abs are in turn recognized by a Fcγ receptor present at the surface of phagocytes (7). There is another example of the indirect phagocytosis of viruses. Infection with many types of viruses induces apoptosis in host cells, which is considered to occur as a self-defense mechanism (8); loss of host cell activity should impair the propagation of virus, but apoptosis in virus-infected cells has other roles. In general, cells undergoing apoptosis are eliminated through phagocytosis, and this is important for the maintenance of tissue homeostasis (9). Infected, apoptotic cells are thus likely to be phagocytosed and digested together with virus through the actions of degrading enzymes that exist in lysosomes of phagocytes. This mode of phagocytic elimination of invading microbes may be considered to be a cellular innate immune response. In contrast, viruses appear to resist this host action by inhibiting apoptosis using antiapoptotic proteins encoded by viral genes (10, 11).

We and other investigators previously found that cells infected with influenza virus are induced to undergo apoptosis in vitro (12–14) and in vivo (15), possibly through the action of Fas ligand (16) or TRAIL (17). Influenza virus-infected cells become susceptible to phagocytosis by macrophages in vitro (18). This phagocytosis is accomplished in a manner dependent on the membrane phospholipid phosphatidyserine, which is externalized in influenza virus-infected cells during apoptosis (18), and also on the structural modification of sugar moieties at the surface of macrophages by viral neuraminidase (19, 20). The phagocytic elimination of influenza virus-infected cells leads to the inhibition of viral growth in vitro (21). Furthermore, the administration of inhibitors of phagocytosis brings about an increase in the rate of mortality among mice that have been infected with influenza virus (22). These findings led us to speculate that the apoptosis-dependent phagocytosis of influenza virus-infected cells is a part of the self-defense
mechanism to protect animals from this infectious disease. If this is true, the development of a novel agent against influenza, such as a stimulator of phagocytosis, may be possible. However, it remains unclear which type of lung phagocytes is responsible for the phagocytosis of infected cells and how this phagocytosis is regulated. This study was undertaken to assess these issues using a mouse model.

Materials and Methods

Animals, virus infection, and cell culture

All experiments involving animals were conducted according to a protocol that had been approved by the Committee on Animal Experimentation of Kanazawa University. C57BL/6 mice (females or males, 6–8 wk old, body weight 15–25 g) (3–5 mice for each experimental group) were anesthetized with ether, intranasally challenged with 60 μl (30 μl × 2) of medium containing −1 × 10^7 PFU (a dose near one-tenth of LD_{50}) of influenza A/WSN (H1N1) virus (WSN), and housed with normal lighting and feeding until analyses. Mice carrying a disrupted αv integrin gene [αvβ3 (null)] as a genetic background were kept in a specific pathogen-free facility. The αv integrin gene was disrupted in embryonic stem cells using a targeting vector containing a neomycin-resistance gene, which was selected using G418. The disrupted mice were mated with wild-type B6 mice to generate F1 mice [αvβ3 (null)/B6]. Thymocytes prepared from ddY mice (females, 8–10 wk old) were cultured with RPMI 1640 containing 10% heat-inactivated FBS at 37°C with 5% CO_{2} in air, and used as target cells in an assay of phagocytosis in vitro after they were induced to undergo apoptosis. HeLa S3 cells were maintained with Eagle’s MEM containing 10% FBS at 37°C with 5% CO_{2} in air, and used as target cells in an assay of phagocytosis in vitro after they were induced to undergo apoptosis. RAW264.7 cells, a mouse macrophage-derived cell line cultured with DMEM containing 10% heat-inactivated FBS at 37°C with 5% CO_{2} in air, and used as phagocytes in the assays of phagocytosis. To prepare bronchoalveolar lavage cells, lungs were lavaged five times with 1 ml of PBS (4.0–4.5 ml of the fluid was routinely recovered), and the lavage fluids were centrifuged to precipitate cells. Cells recovered from the lavage were smeared on silane-coated glass slides for cytochemical analyses or further processed for the preparation of alveolar macrophages (AM) as follows. The lavage cells were maintained on coverslips with RPMI 1640 containing 10% heat-inactivated FBS at 37°C with 5% CO_{2} in air, and used as target cells in an assay of phagocytosis in vitro after they were induced to undergo apoptosis. ISNT-positive cells were detected by fluorescence microscopy as done with the lavage cells, whereas cells positive in TUNEL were visualized by a coloring reaction of HRP with hydrogen peroxide and diaminobenzidine. For the identification of influenza virus-infected cells, the lung sections were subjected to immunostaining with anti-WSN Ab as follows. The sections were deparaffinized, treated with 0.3% (w/v) hydrogen peroxide and methanol for the inactivation of endogenous peroxidase, incubated with 10% FBS for blocking, and reacted with anti-WSN Ab. The samples were washed and incubated successively with biotin-labeled anti-rabbit IgG Ab and HRP-conjugated streptavidin. The signals were visualized as done for TUNEL, and the sections were counterstained with methyl green, enclosed with 50% (v/v) glycerol, and examined by microscopy. To identify influenza virus-infected, apoptotic cells, the sections were subjected to ISNT and immunohistochemistry with anti-WSN Ab, in which localization of the Ab was determined using biotinylated secondary Ab and Alexa 488-conjugated streptavidin, and the samples were examined by fluorescence microscopy. Neutrophils and macrophages were identified based on the reactivity to anti-Gr-1 (BD Biosciences) and anti-F4/80 Ab, respectively, as well as on the morphology of the shape of nuclei. The Ab reactions were done for immunohistochemistry with anti-WSN Ab using appropriate biotin-labeled secondary Abs. To examine the presence of neutrophils and macrophages that had engulfed apoptotic cells, the sections were simultaneously analyzed by immunostaining (with anti-Gr-1 or anti-F4/80 Ab) and TUNEL, in which signals were visualized by a coloring reaction of alkaline phosphatase using NBT and 5-boromo-4-chloro-3-indolyl-phosphate as substrates.

Determination of viral titers

Specimens of lung tissue obtained from mice at various time points postinfection were processed with HBSS using a Potter-type homogenizer. The homogenates were centrifuged at 300 × g for 5 min, and the supernatants were collected. Madin-Darby bovine kidney cells at confluence in culture were incubated with the lungsates as well as with a known titor of virus as a control for 1 h, and the cultures were washed with Eagle’s MEM. The cells were further maintained for ~70 h at 34°C with 5% CO_{2} in air with Eagle’s MEM containing 0.15% (w/v) L-glutamine, 1% BSA, 1% 100× MEM vitamin solution (Invitrogen Life Technologies), and 0.6% (w/v) agar. They were treated with ethanol and acetic acid (5:1 in volume) for fixation, stained with Amido Black, and examined for the number of plaques.

Induction of apoptosis

Mouse thymocytes were induced to undergo apoptosis by incubation with 10 μM dexamethasone as described previously (25). HeLa cells at subconfluence in culture were induced to undergo apoptosis in two ways, infection with influenza virus and exposure to UV. With the former procedure, HeLa cells were infected with influenza A/Udorn/72 (H3N2) virus, SP626, at a multiplicity of infection of two as described previously (12). Induction of apoptosis in virus-infected cells was monitored by cytochemically examining the occurrence of caspase 3 activation using Ab recognizing the activated form of human caspase 3 (Promega), condensation of DNA, and permeabilization with methanol. The cells were then subjected to in situ DNA synthesis (in situ nick translation; ISNT) using tetramethylrhodamine-conjugated dUTP and DNA polymerase I before being examined with a fluorescence microscope, as described previously (22). To determine the shape of nuclei, the smeared lavage cells were stained with the DNA-binding fluorescent dye Hoechst 33342 and examined by fluorescence microscopy. To examine the presence of phagocytes that had engulfed virus-infected cells and apoptotic cells, the smeared lavage cells were simultaneously subjected to immunostaining with anti-WSN Ab, ISNT, and staining with Hoechst 33342, and examined by fluorescence microscopy. For all fluorescence microscopy, samples were enclosed with 50% nonfluorescent glycerol containing 10 mM Tris-HCl buffer (pH 9.0) and 0.5% (v/v) 2-ME.

Analysis of lung tissue

Two and three mice were analyzed at day 2 and days 0 and 4 postinfection, respectively. Lungs were dissected out from mice, fixed with Bouin’s solution, and embedded in paraffin. Sections with a thickness of 5 μm were prepared on silane-coated glass slides and subjected to further histochemical analyses. In each analysis, regions containing the airway and the alveolar ducts were separately examined. We first examined a set of five lung lobes of a randomly chosen mouse by staining with H&E and TUNEL, and found that the patterns of H&E staining and distribution of TUNEL signals were almost the same among the lobes. We thus further analyzed any one lobe in the anticipation that all lung lobes should give similar results. To identify apoptotic cells, the sections were subjected to ISNT or TUNEL using a commercial kit (ApopTag; Chemicon) for the detection of cells containing fragmented DNA. ISNT-positive cells were detected by fluorescence microscopy as done with the lavage cells, whereas cells positive in TUNEL were visualized by a coloring reaction of HRP with hydrogen peroxide and diaminobenzidine. For the identification of influenza virus-infected cells, the lung sections were subjected to immunostaining with anti-WSN Ab as follows. The sections were deparaffinized, treated with 0.3% (w/v) hydrogen peroxide and methanol for the inactivation of endogenous peroxidase, incubated with 10% FBS for blocking, and reacted with anti-WSN Ab. The samples were washed and incubated successively with biotin-labeled anti-rabbit IgG Ab and HRP-conjugated streptavidin. The signals were visualized as done for TUNEL, and the sections were counterstained with methyl green, enclosed with Entellan, and examined by microscopy. To identify influenza virus-infected, apoptotic cells, the sections were subjected to ISNT and immunohistochemistry with anti-WSN Ab, in which localization of the Ab was determined using biotinylated secondary Ab and Alexa 488-conjugated streptavidin, and the samples were examined by fluorescence microscopy. Neutrophils and macrophages were identified based on the reactivity to anti-Gr-1 (BD Biosciences) and anti-F4/80 Ab, respectively, as well as on the morphology of the shape of nuclei. The Ab reactions were done for immunohistochemistry with anti-WSN Ab using appropriate biotin-labeled secondary Abs. To examine the presence of neutrophils and macrophages that had engulfed apoptotic cells, the sections were simultaneously analyzed by immunostaining (with anti-Gr-1 or anti-F4/80 Ab) and TUNEL, in which signals were visualized by a coloring reaction of alkaline phosphatase using NBT and 5-boromo-4-chloro-3-indolyl-phosphate as substrates.

Abbreviations used in this paper: WSN, influenza A/WSN (H1N1) virus; AM, alveolar macrophage; ISNT, in situ nick translation; z-VAD-fmk, benzoxoxy-carbonyl-Val-Ala-Asp-fluoromethylketone.
chromatin using Hoechst 33342, and surface exposure of phosphatidylserine using fluorescence-labeled annexin V. For UV-induced apoptosis, the cells with PBS were irradiated with UV-C (253 nm) at 100 J/m² for 6 h, and the induction of apoptosis was examined by determining the extent of chromatin condensation. To examine the occurrence of a necrotic change, HeLa cells infected with influenza virus or irradiated with UV were analyzed for the staining with propidium iodide, a membrane nonpermeabilizing fluorochrome. These cells undergoing apoptosis with no evidence of necrosis were used as target cells in assays of phagocytosis while culture supernatants were collected and used to treat phagocytes. To obtain supernatants of cell cultures where apoptosis was inhibited, HeLa cells were infected with influenza virus in the presence of the pan-caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) (20 μM). Under these conditions, apoptosis was almost completely inhibited (18).

**Assay of phagocytosis in vitro**

An assay of phagocytosis in vitro was conducted essentially as described previously (18, 25). For the phagocytosis of apoptotic thymocytes by AM prepared from mice not infected with influenza virus, dexamethasone-treated mouse thymocytes were mixed with the macrophages (4–6 × 10⁴) (at a ratio of 100 target cells to one macrophage), and the mixture was incubated at 37°C for 2 h. The samples were agitated by pipetting with PBS to remove thymocytes free from or lightly attached to macrophages. To monitor the removal of unengulfed thymocytes, macrophages were incubated with apoptotic thymocytes that had been labeled with PKH2, a membrane-integrating fluorochrome (Zynaxis), washed with PBS by pipetting, surface labeled with biotin followed by the addition of Alexa 546-conjugated streptavidin, and finally examined by fluorescence microscopy. This

**FIGURE 1.** Pathogenesis of influenza in the mouse. Mice were intranasally challenged with influenza virus on day 0 and analyzed at the indicated time points postinfection. A, Survival rate and body weight. The total number of mice analyzed is 104. B, The level of influenza virus-infected, apoptotic cells determined with bronchoalveolar lavage cells. The set of micrographs shows an example of a cell positive for both fragmented DNA (ISNT) and influenza virus proteins (anti-WSN), and phase contrast and fluorescence views of the same cell are exhibited. Scale bar, 10 μm. Numerical data are shown below the micrographs. C, The levels of influenza virus-infected cells and apoptotic cells determined with lung sections. The micrographs show the results of immunohistochemistry with anti-WSN Ab (left), TUNEL (middle), and the simultaneous analysis by ISNT and immunohistochemistry with anti-WSN Ab (right). Positive signals are seen in brown in the left and middle panels. Phase contrast and fluorescence views of the same microscopic field are shown in the right panels, and the micrograph at the bottom right shows a magnified portion of the adjacent micrograph. The arrowheads in the right panel point to a cell containing both fragmented DNA and viral proteins. Scale bars, 50 μm (for anti-WSN and TUNEL) and 10 μm (for ISNT and anti-WSN). Numerical data from immunohistochemistry and TUNEL are shown below the micrographs. D, Viral titer in the lung. **Assay of phagocytosis in vitro**

An assay of phagocytosis in vitro was conducted essentially as described previously (18, 25). For the phagocytosis of apoptotic thymocytes by AM prepared from mice not infected with influenza virus, dexamethasone-treated mouse thymocytes were mixed with the macrophages (4–6 × 10⁴) (at a ratio of 100 target cells to one macrophage), and the mixture was incubated at 37°C for 2 h. The samples were agitated by pipetting with PBS to remove thymocytes free from or lightly attached to macrophages. To monitor the removal of unengulfed thymocytes, macrophages were incubated with apoptotic thymocytes that had been labeled with PKH2, a membrane-integrating fluorochrome (Zynaxis), washed with PBS by pipetting, surface labeled with biotin followed by the addition of Alexa 546-conjugated streptavidin, and finally examined by fluorescence microscopy. This
FIGURE 2. Accumulation of phagocytes in the lung after infection with influenza virus. Mice were challenged with influenza virus on day 0 and analyzed at the indicated time points postinfection. A, The levels of neutrophils, macrophages, and lymphocytes in bronchoalveolar lavage. Each cell was identified based on the presence of cell type-specific esterase and the shape of nucleus. B, The levels of neutrophils and macrophages in the lung. Lung sections were subjected to immunohistochemistry with anti-Gr-1 (for neutrophils) or anti-F4/80 (for macrophages) Ab. The set of micrographs show a typical result with the airway region. Scale bars, 50 μm. Numerical data with the airway region and the alveolus are shown on the right.

procedure makes all target cells marked with PKH2, whereas thymocytes present outside the macrophages are labeled with both PKH2 and Alexa 546. We found that cells labeled with the two fluorochromes existed before a wash but were almost absent after a wash in the macrophage cultures (data not shown). These data indicated that we determined the extent of phagocytosis of apoptotic cells in vitro under the condition where target cells bound to phagocytes were removed. The macrophages were treated with PBS containing 2% paraformaldehyde, 0.1% glutaraldehyde, and 0.05% Triton X-100 for fixation, stained with hematoxylin, and examined by light microscopy for the presence of engulfed thymocytes. For the phagocytosis of apoptotic thymocytes by AM prepared from influenza virus-challenged mice, dexamethasone-treated mouse thymocytes were surface labeled with biotin and incubated with macrophages as described above. The mixture was washed with PBS, and the remaining cells were fixed with 4% paraformaldehyde, membrane permeabilized with cold methanol, and treated with 10% FBS for blocking. The samples were successively incubated with anti-F4/80 Ab, Alexa 488-conjugated anti-rat IgG Ab, Alexa 546-conjugated streptavidin, and Hoechst 33342, and finally examined by fluorescence microscopy. Cells positive for both Alexa 488 (signal from F4/80) and Alexa 546 (signal from target thymocytes) were considered to be AM that had phagocytosed thymocytes. For the phagocytosis of HeLa cells by AM prepared from mice not infected with influenza virus, HeLa cells (infected or not infected with influenza virus) were surface labeled with biotin and incubated with macrophages at a ratio of one macrophage to five target cells. The mixture was incubated at 37°C for 2 h and washed with PBS to remove unincorporated target cells. The remaining cells were treated with PBS containing 2% paraformaldehyde, 0.1% glutaraldehyde, and 0.05% Triton X-100 for fixation, and then treated with methanol for membrane permeabilization. The samples were supplemented with FITC-conjugated avidin and examined under a fluorescence microscope. For the phagocytosis of latex beads by mouse AM or RAW264.7 cells, phagocytes were incubated with FITC-labeled latex beads (diameter = 1.58 μm; Polysciences) at 37°C, washed, and examined by fluorescence microscopy. To examine the effect of culture supernatants, phagocytes were preincubated with the supernatants (25–100% in volume) at 37°C overnight, washed with fresh medium, and analyzed for the phagocytic activity in vitro as described above. To test the dependence of phagocytosis on phosphatidylserine, phagocytosis reactions were conducted in the presence (1 mM lipids) of phosphatidylserine-containing liposomes (phosphatidylserine:phosphatidylcholine = 3:7) or liposomes consisting of phosphatidylcholine alone as a negative control, as described previously.

In all phagocytosis reactions in vitro, the number of phagocytes that had engulfed target cells was determined and shown relative to the total number of phagocytes in percentage terms as the phagocytic index.

Data processing and statistical analysis
In the analysis of bronchoalveolar lavage cells and assays of phagocytosis in vitro, data are representative of at least three independent experiments that yielded similar results. For the analysis of lung tissue, sections prepared from two to three mice at each time point were histochemically analyzed. Randomly chosen two to four microscopic fields (each field contained −300 cells at a magnification of ×400) for each section were examined, and the data with a total of six microscopic fields for each time point were numerically processed. In the analysis of phagocytosis with lung tissue, at least 100 phagocytes were examined. Data from quantitative analyses are expressed as the mean ± SD (n > 3). Statistical analyses were performed using Student’s t test, and p values of <0.05 were considered significant.

Results
Accumulation of virus-infected, apoptotic cells and phagocytes in the lung after infection with influenza virus
We first examined the pathogenic effect of influenza virus in mice. Mice were intranasally infected with WSN, and changes in the rate of survival and body weight were determined. The mice started to die 4 days after infection, whereas a change in body weight became evident as early as day 1 and continued to gradually decrease until day 8 postinfection (Fig. 1A). We therefore examined mice for the accumulation of phagocytes and the occurrence of the phagocytosis of virus-infected cells in the lung at day 2 and 4–5 postinfection as representative of mice before the onset of influenza and with the disease in progression, respectively. Cells obtained from bronchoalveolar lavage and sections of lung tissue were examined for the presence of cells that were infected with influenza virus, undergoing apoptosis, or characteristic of phagocytes. Bronchoalveolar lavage cells were first analyzed for the occurrence of viral infection...
and apoptosis based on the presence of viral proteins and fragmented DNA, respectively. Cells positive for viral proteins became detectable soon after viral infection and existed either free from or seemingly within other cells (see below). Influenza virus-infected, apoptotic cells, which were almost absent at day 0, already existed at day 2, and persisted at least until day 5 (Fig. 1B). Approximately 30% of all infected cells were undergoing apoptosis at day 2, and ~80 and 50% of apoptotic cells at day 2 and 5, respectively, were positive for the viral proteins. The analysis of lung sections also showed an increase of cells containing influenza virus proteins and fragmented DNA after infection. Both virus-infected cells and apoptotic cells became detectable in either the airway region or the alveolus of lung tissue at day 2 and decreased during the next 2 days (Fig. 1C). Influenza virus-infected, apoptotic cells were present in the lung, and ~60% of apoptotic cells were infected with influenza virus. We then determined how the amount of influenza virus in the lung changes during the course of infection. To do so, extracts of lung tissue were subjected to a plaque-forming assay. There were no significant changes in the weight and protein content of the lung before and after infection with influenza virus (data not shown). We found that the level of influenza virus was high at day 2 but decreased during the next 2 days as the disease progressed (Fig. 1D). Finally, the level of phagocytes, i.e., neutrophils and macrophages, was determined. Each type of phagocyte present in bronchoalveolar lavage was identified based on the presence of the cell type-specific esterase and the shape of nucleus (Fig. 2A). Neutrophils were almost undetectable before infection, but they appeared in the lung soon after infection and slightly decreased in number thereafter. In contrast, macrophages that already existed in the lung increased in number after the infection, and this increase persisted until day 5. Lymphocytes were a minor population in the lung, and continued to accumulate during the period examined. Both

**FIGURE 3.** Occurrence of the phagocytosis of influenza virus-infected cells in the lung. Mice were challenged with influenza virus on day 0 and analyzed at the indicated time points postinfection. A, Presence of neutrophils and macrophages that have phagocytosed influenza virus-infected, apoptotic cells in bronchoalveolar lavage. Bronchoalveolar lavage cells were simultaneously analyzed by ISNT and immunocytochemistry with anti-WSN Ab. The set of micrographs shows an example of a neutrophil or a macrophage that contains both fragmented DNA and influenza virus proteins. Phase contrast and fluorescence views of the same cell are shown at each row. Scale bar, 10 μm. Numerical data are shown on the right. B, Detection of neutrophils and macrophages that have phagocytosed apoptotic cells in the lung. Lung sections were simultaneously analyzed by TUNEL and immunohistochemistry with anti-Gr-1 or anti-F4/80 Ab followed by counterstaining with methyl green. An example of a neutrophil or a macrophage that contains a TUNEL-positive additional nucleus is shown on the left. Positive signals in TUNEL and immunohistochemistry are seen in blue and brown, respectively. The arrowheads point to phagocytes that have phagocytosed apoptotic cells. Scale bars, 10 μm. Numerical data with the airway region and the alveolus are shown on the right.
types of phagocytes were immunohistochemically identified in the sections of lung tissue (Fig. 2B, left panels). Both neutrophils and macrophages accumulated in the airway region and the alveolus after infection with influenza virus, and their levels were higher in the airway region than in the alveolus (Fig. 2B, right panels).

These results indicated that 1) the presence of virus-infected cells becomes evident in the lung soon after infection with influenza virus; 2) most apoptotic cells are infected with influenza virus; 3) the viral titer increases before the onset of influenza and decreases as the disease progresses; and 4) both neutrophils and macrophages accumulate in the lung soon after infection.

Evidence for phagocytosis of influenza virus-infected cells in the lung

We next examined whether influenza virus-infected cells are phagocytosed in mice, and, if so, which type of phagocyte, neutrophils or macrophages, is responsible. Some neutrophils with crescent-shaped, branched nuclei and macrophages with oval nuclei in bronchoalveolar lavage contained additional nuclei with fragmented DNA, and such extra nuclei seemed to be surrounded by influenza virus proteins (Fig. 3A, left panels). We considered that those neutrophils and macrophages had phagocytosed influenza virus-infected, apoptotic cells. The level of phagocytosis continued to increase during the period of investigation (Fig. 3A, right panel). Phagocytosis of apoptotic cells by either neutrophils or macrophages was also evident in both the airway region and the alveolus of the lung (Fig. 3B). These results collectively indicated that influenza virus-infected cells undergoing apoptosis are phagocytosed in the lung of virus-challenged mice, and that both neutrophils and macrophages are responsible for this phagocytosis.

Presence of a factor(s) stimulating phagocytic activity of AM in culture supernatants of influenza virus-infected cells

We then asked whether any qualitative changes occur to the lung phagocytes after infection with influenza virus, in addition to an increase in their number (see Fig. 2). For this purpose, the phagocytic activity of macrophages prepared from bronchoalveolar lavage of mice infected or not infected with virus was determined in vitro using mouse thymocytes undergoing dexamethasone-induced apoptosis as targets. We found that AM prepared from mice at day 3 postinfection possessed greater phagocytic activity than those from mice not infected with virus (Fig. 4A). We then examined the possibility that influenza virus-infected cells produce a factor(s) that stimulates the phagocytic activity of macrophages. To do so, AM prepared from mice not infected with influenza virus were preincubated with culture supernatants of either influenza virus-infected or mock-infected HeLa cells and examined for the phagocytic activity. Influenza virus-infected HeLa cells showed biochemical features typical of apoptotic cells, with no evidence for an increase in the permeability of the plasma membrane (Fig. 4B). Culture supernatants of virus-infected cells stimulated the phagocytosis of influenza virus-infected HeLa cells by AM in a dose-dependent manner, but culture supernatants of noninfected cells did not have any effect (Fig. 4C, left panel). This effect of the culture supernatants was also observed in the reaction with latex beads as targets as well as in the reaction with a cell line derived from mouse macrophages as phagocytes (Fig. 4C, middle and right panels), suggesting that culture supernatants of influenza virus-infected cells contain a factor(s) that stimulates phagocytosis irrespective of the type of targets and phagocytes.

We previously reported that the phagocytosis of influenza virus-infected cells by mouse peritoneal macrophages in vitro was dependent on phosphatidylserine that had been exposed on the surface of target cells during apoptosis (18). We thus tested the dependence on phosphatidylserine of the phagocytosis of influenza virus-infected HeLa cells by AM prepared from mice not infected

\[ \text{AM} / \text{thymocytes} \]

\[ \begin{array}{c|c|c|c}
\text{Phagocytosis index} & \text{mock} & \text{Flu} \\
\hline
0.0 & 20 & 22 \\
0.2 & 22 & 27 \\
0.4 & 30 & 36 \\
0.6 & 30 & 41 \\
0.8 & 30 & 47 \\
1.0 & 30 & 59 \\
\end{array} \]

\[ \text{AM} / \text{HeLa} \]

\[ \begin{array}{c|c|c|c}
\text{Phagocytosis index} & \text{mock} & \text{Flu} \\
\hline
0.0 & 20 & 22 \\
0.2 & 22 & 27 \\
0.4 & 30 & 36 \\
0.6 & 30 & 41 \\
0.8 & 30 & 47 \\
1.0 & 30 & 59 \\
\end{array} \]

\[ \text{AM} / \text{beads} \]

\[ \begin{array}{c|c|c|c}
\text{Phagocytosis index} & \text{mock} & \text{Flu} \\
\hline
0.0 & 20 & 22 \\
0.2 & 22 & 27 \\
0.4 & 30 & 36 \\
0.6 & 30 & 41 \\
0.8 & 30 & 47 \\
1.0 & 30 & 59 \\
\end{array} \]

\[ \text{RAW} / \text{beads} \]

\[ \begin{array}{c|c|c|c}
\text{Phagocytosis index} & \text{mock} & \text{Flu} \\
\hline
0.0 & 20 & 22 \\
0.2 & 22 & 27 \\
0.4 & 30 & 36 \\
0.6 & 30 & 41 \\
0.8 & 30 & 47 \\
1.0 & 30 & 59 \\
\end{array} \]

\[ \text{FIGURE 4.} \text{ Stimulation of the phagocytic activity of macrophages by culture supernatants of influenza virus-infected cells. A, The level of phagocytosis in vitro of apoptotic thymocytes by AM prepared from mice not infected with influenza virus or at day 3 of infection. B, Apoptosis in influenza virus-infected HeLa cells. HeLa cells infected (Flu) or mock-infected (mock) with influenza virus were examined for the occurrence of apoptosis and the permeability of the plasma membrane. Ratios of cells positive for activated caspase 3, condensed chromatin, and bound annexin V (AnnV) as well as of cells stained with propidium iodide (PI), an indicator for the membrane permeability, are shown. \( r < 0.05 ; ** , r < 0.01 ; *** , r < 0.001 . \) C, Stimulation of phagocytosis by culture supernatants of influenza virus-infected cells. The phagocytes (AM of uninfected mice (AM) in the left and middle panels; RAW264.7 cells (RAW) in the right panel) were preincubated with culture supernatants of influenza virus-infected (Flu) or mock-infected (mock) HeLa cells. They were then subjected to in vitro phagocytosis reactions with influenza virus-infected HeLa cells (left panel) or latex beads (middle and right panels) as targets. In the left panel, increasing amounts of supernatants (shown as the ratio in volume) were added to macrophage cultures. The amount of supernatants used in the experiments shown as middle and right panels was 50% in volume. \( * , r < 0.05 ; ** , r < 0.01 ; *** , r < 0.001 . \) D, The level of phagocytosis of influenza virus-infected (Flu) or mock-infected (mock) HeLa cells by AM of uninfected mice (AM) in the presence or absence of the indicated liposomes. In the panel shown on the right, the macrophages were preincubated with culture supernatants (Sup) (50% in volume) of influenza virus-infected (Flu) or mock-infected (mock) HeLa cells. PS, Phosphatidylserine-containing liposomes; PC, liposome consisting of phosphatidylcholine alone. \( * , r < 0.01 . \)"
with virus. Phagocytosis reactions in vitro were conducted in the presence and absence of phosphatidylserine-containing liposomes or control liposomes that consisted of phosphatidylcholine alone. The phagocytosis was observed only when virus-infected HeLa cells were used as targets, and was inhibited when phosphatidylserine-containing liposomes were present (Fig. 4D, left panel). These results indicated that apoptosis-dependent phagocytosis of HeLa cells by AM was mediated by phosphatidylserine. We next examined whether culture supernatants of virus-infected cells stimulate the phosphatidylserine-mediated phagocytosis. The presence of phosphatidylserine-containing liposomes in the reaction with macrophages treated with the culture supernatants reduced the level of phagocytosis (Fig. 4D, right panel), suggesting that the phosphatidylserine-mediated phagocytosis of apoptotic cells is enhanced when macrophages are preincubated with culture supernatants of influenza virus-infected cells.

**Partial characterization of phagocytosis-stimulating factor**

We next partially characterized a factor(s) that stimulates the phagocytic activity of macrophages. We first examined which event, infection or apoptosis, is more important for this activity. For this purpose, HeLa cells were infected with influenza virus in the presence or absence of the pancaspase inhibitor z-VAD-fmk, and the resulting culture supernatants were examined for their effects on the activity of macrophages. We found that the presence of the apoptosis inhibitor almost completely abolished the stimulatory effect of culture supernatants (Fig. 5A). Furthermore, culture supernatants of HeLa cells undergoing UV-induced apoptosis had no effect on the phagocytic activity of AM (Fig. 5B). These results indicated that the phagocytosis-stimulating factor(s) is produced and released from cells that are undergoing apoptosis induced by certain insults including infection with influenza virus. Next, culture supernatants of virus-infected cells were heated at 90°C before they were used to treat macrophages. The data showed that the heated samples lost the activity of stimulating phagocytosis (Fig. 5C), indicating that the factor responsible for the activity is heat labile.

**Increased mortality of TLR4-lacking mice after infection with influenza virus**

We previously reported that TLR4, an innate immune receptor against bacterial invasion (4), acts to delay the degradation of engulfed cells in macrophages (25). We thus examined whether or
A duplicate of those shown in Fig. 4 apoptotic thymocytes by AM prepared from mice infected (on day 3) or not infected with influenza virus. The data with wild-type mice are a duplicate of those shown in Fig. 2A. B, The levels of neutrophils and macrophages in the airway region of lung tissue. Lung sections were subjected to immunohistochemistry with anti-Gr-1 (for neutrophils) or anti-F4/80 (macrophages) Ab. The data with wild-type mice are a duplicate of those shown in Fig. 2B. C, The level of the phagocytosis of apoptotic cells by neutrophils or macrophages determined with bronchoalveolar lavage cells or the airway region of lung tissue. Apoptotic cells were identified by ISNT and TUNEL with lavage cells and lung sections, respectively. The data for the airway region with wild-type mice are a duplicate of those shown in Fig. 2C. D, The level of the phagocytosis of apoptotic thymocytes by AM prepared from mice infected (on day 3) or not infected with influenza virus. The data with wild-type mice are a duplicate of those shown in Fig. 2D.

Discussion

The data obtained in this study provide, for the first time, evidence for the phagocytic elimination of influenza virus-infected cells in vivo. Our previous studies in vitro showed that influenza virus-infected cells are phagocytosed by macrophages in a manner dependent on apoptosis (18), and that this leads to the inhibition of viral propagation (21). These facts suggest that influenza virus-infected cells are subjected to apoptosis-dependent phagocytosis and degraded together with invading virus within phagocytes. Both neutrophils and macrophages appeared to be responsible for this phagocytosis. This is, to our knowledge, the first paper to show the phagocytosis of apoptotic cells by neutrophils in vivo. Both types of phagocytes accumulate in lung tissue soon after viral infection, and this is most likely an innate immune response to the invasion of microbes (4). We showed in this study that the phagocytic activity of AM is stimulated in influenza virus-infected mice, and that a heat-labile factor(s) produced by infected cells is responsible for this stimulation. Kobayashi and colleagues (27) reported that accumulating neutrophils enhance the digestion of engulfed apoptotic cells in macrophages. These data collectively suggest that both the quantity and quality of phagocytes in lung tissue are augmented in influenza virus-infected animals to maximize the efficiency of the phagocytic elimination of infected cells.

Although we showed that AM phagocytose influenza virus-infected cells in a phosphatidylserine-mediated manner in vitro, it is unclear whether the same is true for phagocytosis in vivo. Our recent study showed that the intranasal administration of annexin V, which specifically binds to phosphatidylserine and prevents phosphatidylserine-exposing cells from being phagocytosed, augments the rate of mortality among influenza virus-infected mice (22). This suggests that influenza virus-infected cells undergo phosphatidylserine-mediated phagocytosis in the lung. If so, neutrophils and macrophages should possess a phosphatidylserine-recognizing phagocytosis receptor. There are two distinct ways in which phosphatidylserine present at the surface of apoptotic cells...
is recognized by phagocytes; directly by membrane-bound receptors and indirectly with the aid of phosphatidylserine-binding serum proteins. The receptor involved in the former includes lectin-like oxidized low-density lipoprotein receptor 1 of endothelial cells (28) and class B scavenger receptor type I of testicular Sertoli cells (29), but the identity of such a receptor expressed in "professional" phagocytes, i.e., macrophages, has been unknown (30, 31). The receptor in the latter mode of recognition is represented by the Axl/Mer/Tyro3 receptor-tyrosine kinase family of proteins that bind to the product of growth arrest-specific gene 6 (32) and protein S (33), and also by integrin αβ3 as a receptor of milk fat globule epidermal growth factor protein 8 (34) and the product of developmental endothelial locus-1 (35). However, it is not known whether such serum proteins exist in the lung. It is therefore necessary to identify and characterize a phagocytosis receptor of phagocytes in the lung, which binds to phosphatidylserine at the surface of influenza virus-infected, apoptotic cells and induces the engulfment of those cells. If the phagocytosis of influenza virus-infected cells in the lung is accomplished in the manner described above, this phenomenon can be considered an innate immune response because Abs and thus complement components that are activated through the classical pathway are apparently not involved. It is well established that virus-infected cells are attacked by lymphocytes such as CTLs, which recognize viral Ag expressed at the surface of infected cells. The invading influenza virus is thus most probably eliminated through both innate and adaptive immune responses.

AM appear to be activated upon infection with influenza virus so that they serve as more effective phagocytes. Our data showed that this activation is likely to be endowed by a heat-labile substance(s) that is produced and released by apoptotic cells. Surface-taint proteins appear to stimulate the phagocytosis of apoptotic neutrophils by AM (36), but the production of those proteins is apparently not restricted to apoptotic cells. Uchide et al. (37) reported that culture supernatants of influenza virus-infected cells induce the differentiation of monocytes to macrophages accompanied by an increase in the level of class A scavenger receptors. However, the cells whose phagocytic activity was stimulated by culture supernatants of influenza virus-infected cells in the present study were macrophages rather than monocytes. It is generally acknowledged that apoptosing cells produce various substances during the apoptotic process: some are synthesized through gene expression and others via the modification of pre-existing molecules. Examples of the latter are caspase-cleaved protein products and lysosphospholipids produced by caspase-activated phospholipases (38). In fact, a recently published paper reported caspase-cleaved proteins as candidates for molecules responsible for the differentiation of monocytes to macrophages (39). However, the situation in our case is somewhat complicated, because production of the stimulatory factor is seemingly restricted to cells undergoing apoptosis induced by particular types of stimuli, including infection with influenza virus. This indicates that the mechanism of its production is not as simple as the assumption that a protein acquires the phagocytosis-stimulating activity after cleavage by a caspase. The infection with influenza virus activates various signaling pathways that lead to the production of molecules with a wide range of activity (40). Further studies are needed before we identify the stimulatory factor and solve the mechanism of its production in influenza virus-infected cells.

We showed for the first time the involvement of TLR4 in the pathogenesis of influenza. Previous studies reported that TLR4 is not involved in immunity to influenza A virus (41, 42). However, there is no discrepancy between our findings and the others, because the rate of mortality among virus-infected mice was not examined in the previous studies. An increase in the rate of mortality was the most significant change caused by a loss of the expression of TLR4 in influenza virus-infected mice. Out data suggested that TLR4 is involved in the phagocytic elimination of influenza virus-infected, apoptotic cells, in some way other than directly enhancing the activity of phagocytes. Cluff et al. (43) recently reported that the administration of synthetic TLR4 agonists endows mice with resistance to influenza virus challenge. Collectively, TLR4 could play an important role in the host defense against influenza through activating innate immunity, including the phagocytic elimination of infected cells, in a ligand-dependent manner. It is unlikely that influenza virus-infected cells express LPS, a ligand for TLR4, and the mechanism by which TLR4 protects animals from infection with influenza virus is not known at present.

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Disclosures

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