Virology 534 (2019) 80–86

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

The role of cell surface expression of influenza virus neuraminidase in induction of human lymphocyte apoptosis

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ARTICLE INFO

Keywords:
Influenza virus
Neuraminidase
Human monocytes
Human macrophages
Human lymphocytes
Apoptosis

ABSTRACT

The immunopathological mechanisms as well as the role played by influenza A virus infection of human leukocytes and induction of apoptosis have not been fully elucidated. We confirm here that the percentage of cells that are infected is less than the percent of apoptotic cells. Depletion of monocytes/macrophages and depletion of cells expressing influenza neuraminidase from the cultures after exposure to virus decreased lymphocyte apoptosis. Treatment of virus-exposed leukocyte cultures with anti-neuraminidase antibodies but not with anti-hemagglutinin antibodies, reduced lymphocyte production of active caspase-3 and induction of apoptosis. Different strains of virus induced different levels of apoptosis. Variations in induction of apoptosis correlated with production and expression of viral neuraminidase by infected leukocytes. The data suggest that cell surface expression of neuraminidase plays an important role in the induction of apoptosis in human lymphocytes. The benefit, or cost, to the host of lymphocyte apoptosis warrants continued investigation.

1. Introduction

Apoptosis exerts broad functions during the response to influenza virus (IAV) and can be considered mainly an antiviral response, limiting both IAV replication and respiratory immunopathology. However, IAV can use early and late apoptosis events to increase propagation and limit early antiviral immune responses (Herold et al., 2012). IAV induces apoptosis in tissue culture cells (Hinshaw et al., 1994; Takizawa et al., 1993), human peripheral blood lymphocytes (Nichols et al., 2001) and monocytes (Fesq et al., 1994), and human NK cells (Mao et al., 2009). IAV-infected murine plasmacytoid dendritic cells have been reported to enhance mortality by eliminating virus-specific CD8 T cells via FasL-Fas-induced apoptosis (Langlois and Legge, 2010).

In earlier studies of IAV-induced apoptosis of human lymphocytes (Nichols et al., 2001), the percent of cells that were infected was shown to be less than the percent of apoptotic cells suggesting that direct effects of cell infection by the virus could not account fully for the level of cell death. Treatment of virus-exposed cultures with *anti*-TNF-α did not reduce the percent of lymphocytes that were apoptotic. In virus-exposed cultures treated with *anti*-FasL antibody, recombinant soluble human Fas, Ac-DEVD-CHO (caspase-3 inhibitor) or Z-VAD-FMK (general caspase inhibitor), apoptosis and production of the active form of caspase-3 was reduced. The apoptotic cells were Fas-high density cells while the non-apoptotic cells expressed a low density of Fas. The studies thus showed that Fas-FasL signaling plays a major role in the induction of apoptosis in lymphocytes after exposure to IAV, but the aspect of exposure to the virus that triggered the onset of apoptosis remained to be identified.

It is still not clear at this time which component of IAV induces apoptosis of the lymphocytes. UV-inactivated virus does not induce apoptosis (Morris et al., 1999) nor does heat-inactivated virus (Fesq et al., 1994) although heat-inactivated IAV does activate lymphocytes (Blazevic et al., 2000). A number of IAV proteins warrant consideration as possible triggers of apoptosis. Differential induction of apoptosis has been seen in MDCK and U-937 cells exposed to IAV strains of differing virulence. Clone 7a (virulent for humans and ferrets) induced more apoptosis than A/Fiji (attenuated for both species), and the ability of these clones to induce apoptosis was correlated with the differences in the amounts of neuraminidase (NA) activity in the two strains (Price et al., 1997). Enhanced NA production has been observed after exposure of human macrophages to an avian-human reassortant A/Kawasaki candidate vaccine that was associated with fever in recipients, suggesting a greater inflammatory immune response than elicited by other candidate vaccines (Nichols et al., 1993). It has also been reported

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https://doi.org/10.1016/j.virol.2019.06.001
Received 22 April 2019; Received in revised form 3 June 2019; Accepted 4 June 2019
Available online 10 June 2019
0042-6822/

that human influenza virus neuraminidase, but not hemagglutinin, induces murine macrophage interleukin-1 in vivo and *in vitro* (Houde and Arora, 1989).

We now examine in detail the potential role of NA, or IAV-infected cells expressing cell surface NA, in induction of apoptosis. We examined the effects upon $CD3^+$ T lymphocytes since such cells are most important in limiting infection and securing recovery of the host. We suggest that close cell-cell contact with IAV-infected NA-expressing monocytes/macrophages may play an important role in induction of apoptosis in human $CD3^+$ lymphocytes.

2. Materials and methods

2.1. Virus

The following IAVs were used in these studies: A/Marton/43 H1N1, A/Bethesda/85 H3N2 (wild type, termed wt/Bethesda), A/Mallard/NY/6750/78 x A/Bethesda/85 (avian-human reassortant, termed ah/Bethesda), A/Ann Arbor/6/60 x A/Bethesda/85 (cold adapted, termed ca/Bethesda), A/Kawasaki/87 H1N1 (wt/Kawasaki), A/Mallard/NY/6750/78 x A/Kawasaki/87 (ah/Kawasaki), and A/Ann Arbor/6/60 x A/Kawasaki/87 (ca/Kawasaki). The vaccine strains contained the wild type HA and NA. The vaccine strains were a kind gift from Dr. Brian Murphy (NIAID, Bethesda, MD). The A/Marton/43 strain was a clinical isolate used in many previous studies by ourselves as well as others (Kilbourne et al., 2002), and it was used for all exposures to virus unless indicated otherwise.

The viruses were grown in allantoic cavities of 10-day old embryonated hen's eggs. The allantoic fluid was pooled after collection and frozen at −70 °C until titered to 10^7 or 10^8 when assayed in Madin-Darby Canine Kidney (MDCC) cells (ATCC, Rockville, Maryland) or used for exposure of PBMC (Roberts, Jr. and Nichols, 1989). For sham-exposures, allantoic fluid from uninfected eggs was collected, pooled, and frozen at −70 °C until used.

2.2. Cell collection, separation, and exposure to viruses

Peripheral blood mononuclear cells (PBMC, containing both monocytes/macrophages and lymphocytes) were obtained from the peripheral blood of healthy volunteer donors by Ficoll-Hypaque sedimentation (Boyum, 1968). Informed consent for withdrawal of blood was obtained from all donors, and these studies were approved by the Institutional Review Board of the University of Texas Medical Branch. Donors ranged in age from 18 to 45 years of age. Equal numbers of male and female subjects were used as volunteer donors. It was expected that all donors had experienced past in vivo exposure to IAV. All experiments used concomitant assays of autologous cell preparations, and results are based on six or more experiments for the different analyses unless stated otherwise in the figure legends.

Purified monocytes/macrophages were obtained by adherence of PBMC in plastic culture dishes for 24 h, followed by extensive washing to remove nonadherent cells, and scraping and collection of adherent cells with a rubber policeman (Roberts et al., 1979; Roberts and Steigbigel, 1978). Viability of cells was determined by ability to exclude trypan blue dye (Roberts and Steigbigel, 1978), and purity was determined by phenotyping and flow cytometry (Roberts, Jr. and Nichols, 1989).

Monocytes/macrophages were depleted from cultures by adherence after 1 h of serum-free cell culture followed by staining of residual monocyte-macrophages with anti-CD14 antibody and gating for cell sorting on CD14-negative cells. The sorted cells were collected, resuspended in warm culture medium and reincubated at 37 °C in medium supplemented with 10% heat-inactivated defined fetal calf serum (DFCS) (Hyclone).

PBMC were exposed or sham-exposed to influenza virus at a multiplicity of infection (MOI) of 1 for 1 h at 37 °C in serum-free RPMI 1640 supplemented with 2 mM glutamine, 100 units penicillin G and 100 μg/ml streptomycin (37). For sham-exposures, cells were exposed to a volume of chicken allantoic fluid equal to that used for virus infections. After 1 h of exposure to virus or sham-exposure, the cells were washed in warm medium, centrifuged, and reincubated at 37 °C in medium supplemented with 10% heat-inactivated defined fetal calf serum (DFCS) (Hyclone).

2.3. Flow cytometric analysis of cell phenotype

Acquisition, cell sorting and analysis were done using a FACSort (Becton Dickinson). Acquisition and analysis was performed using Cellquest software (Becton Dickinson). Phenotypes of cells were determined using monoclonal antibodies to identify monocyte-macrophages ($CD14^+$) or $CD3^+$ lymphocytes (Becton Dickinson or BD Pharmingen) and corresponding immunoglobulin (IgG) matched isotype control antibodies were used to set baseline values for analysis of markers (Nichols et al., 2001; Roberts, 1988; Nichols et al., 1993; Roberts and Nichols, 1989). Immunofluorescent staining for influenza hemagglutinin (HA) or neuraminidase (NA) expression was done as previously described (Roberts and Horan, 1985; Nichols et al., 1993).

2.4. Assays of viral protein synthesis and expression

Cells were suspended in methionine-free medium and pulse labeled with 100 μCi of ^{35}S-methionine for 2 h. Cells were then washed and lysed by sodium dodecyl sulfate (SDS)-containing detergent buffer with subsequent analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Laemmli, 1970; Mock et al., 1987). Immunoprecipitation was performed using NIAID Reference Reagent antibodies specific for the H1 (Reagent #V-314-511-157) or H3 (#V-314-591-157) viral hemagglutinin (HA), the N1 (#V-308-513-157) or N2 (#V-308-541-157) neuraminidase (NA), and influenza A matrix (M) protein (#V-306-501-157) (Roberts, Jr. and Horan, 1985; Van Wyck et al., 1981; Nichols et al., 1993). In brief, 5 μL of a mixture of the above 4 antibodies or 5 μL of *anti*-N1 or *anti*-N2 alone were incubated for 1 h at 37 °C with 40 μL of cell lysate. These samples were subsequently incubated with staphylococcal protein A sepharose beads at 25 °C for 1 h, washed and boiled with 20 μL of Laemmli buffer before SDS-PAGE.

Cells were stained for surface expression of CD3 prior to staining for viral protein expression. After fixation in 2% PAF, the cells were permeabilized for 10 min in 0.6% n-octyl β-D-glucopyranoside (Sigma Chemical Co.). Expression of influenza virus proteins was determined by indirect immunofluorescent staining using goat *anti*-H1 or *anti*-H3 hemagglutinin, *anti*-N1 or *anti*-N2 neuraminidase, and *anti*-M polyclonal antisera (NIH reference reagents, described above). Cells were incubated in 20 μl of a 1:500 dilution of goat anti-influenza antibodies for 1 h. After this step cells were washed and stained with 20 μl of a 1:500 dilution of FITC conjugated rat anti-goat IgG antibody (Organon Teknika) for 1 h.

2.5. Analysis of cell apoptosis

Early stages of apoptosis induction were evaluated using Annexin V binding, an indicator of early apoptosis (Huppertz et al., 1999). Annexin V-PE or -FITC (Pharmingen, San Diego, CA) staining was combined with staining for cell phenotype. Measurements of late-stage apoptosis were determined by quantitation of DNA strand breaks (Nichols et al., 2001) using the TUNEL Assay (In Situ Cell Death Kit, Boehringer Mannheim, Mannheim, Germany). In a subset of experiments, the apoptosis was confirmed after cell sorting by demonstration of DNA fragmentation (Gavrieli et al., 1992).

Caspase-3 level was evaluated by staining 10^6 sham-exposed or virus-exposed PBMC with 20 μl of PerCP conjugated anti-CD3 antibody (44). After 30 min the cells were washed and fixed in 2% PAF for 2 h. The $CD3^+$ stained cells were permeabilized for 10 min using 0.6% n-

octyl glucopyranoside (Sigma Chemical Co.) and then stained for caspase-3 expression with 20 μl of FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody (clone C-92-605) (BD Pharmingen). After 30 min cells were washed and analyzed immediately.

Anti-NA antibodies (N1 or N3 - NIAID Reference Reagents), or the NA inhibitors 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (5 mM) or N-methyldeoxynojirimycin (5 mM) were added to cells in a subset of experiments to inhibit NA activity.

2.6. Data analysis

Results are presented as the mean ± SD for six or more experiments unless indicated otherwise in the figure legend. Two-way analysis of variance was performed to evaluate the virus strain-specificity of apparent differences. These analyses and determinations of SD, variance, and significant differences by paired *t*-test were performed using the commercially available SYSTAT (Systat, Inc., Evanston, IL) software program.

3. Results

3.1. Cell infection and apoptosis induction in CD3$^+$ lymphocytes

Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues that have redistributed to the outer leaflet of the plasma membrane in early stages of apoptosis prior to the loss of membrane integrity or DNA fragmentation, and can be used for the detection of apoptosis, commonly via flow cytometric analysis (Huppertz et al., 1999; Elmore, 2007). Caspase 3 is an important effector or executioner caspase in the apoptosis pathway (Cohen, 1997; Elmore, 2007).

PBMC were sham-exposed or exposed to IAV. After 24 h cells were collected and stained for production of viral proteins (within the exposed cell population, using a mixture of anti-HA, anti-NA and *anti*-M antibodies) and for annexin V binding or expression of active caspase-3 (Fig. 1). Analyses of the virus-exposed cell preparations showed that the percentage of CD3$^+$ cells that were infected was less than the percent of apoptotic cells, suggesting that direct effects of cell infection by the virus cannot account fully for the high level of cell death.

3.1.1. Role of monocytes/macrophages in apoptosis induction

Our earlier studies demonstrated a potentially important role for monocytes/macrophages in induction of CD3$^+$ lymphocyte apoptosis after exposure to IAV (Nichols et al., 2001), although the inducer of the process was unknown. We therefore further determined the effect of monocyte-macrophage depletion on IAV-induced lymphocyte apoptosis. The percent of CD3$^+$ cells that were TUNEL-positive was reduced in virus-exposed, monocyte-macrophage-depleted cultures at 24 (P = 0.069) hours, and was significantly reduced at 48 (P = 0.017) and at 72 h (P = 0.004), whether by TUNEL analysis (Fig. 2,A) or by assay for DNA fragmentation (Fig. 2,B). If monocytes/macrophages were removed 1 h after exposure to virus and then added back 24 h later, levels of apoptosis seen in CD3$^+$ lymphocytes increased from levels that were seen for depleted cultures but did not reach the level seen in undepleted PBMC cultures (Fig. 3).

3.1.2. Role of NA in the induction of Caspase-3 in T lymphocytes

The role of monocytes/macrophages or NA-expressing cells in the induction of active caspase-3 in CD3$^+$ lymphocytes was examined further. PBMC were sham-exposed or exposed to IAV and, after 1 h, cultures were depleted of monocytes/macrophages or NA-positive cells. In a subset of experiments, the depleted CD14$^+$ or NA-positive cells were added back 2 h later. The percent of CD3$^+$ cells that were apoptotic was significantly reduced in virus-exposed, monocyte/macrophage-depleted cultures at 24 h (P = 0.00302) and in NA-expressing cell-depleted cultures 24 h (P = 0.00022) after exposure (Fig. 3). The

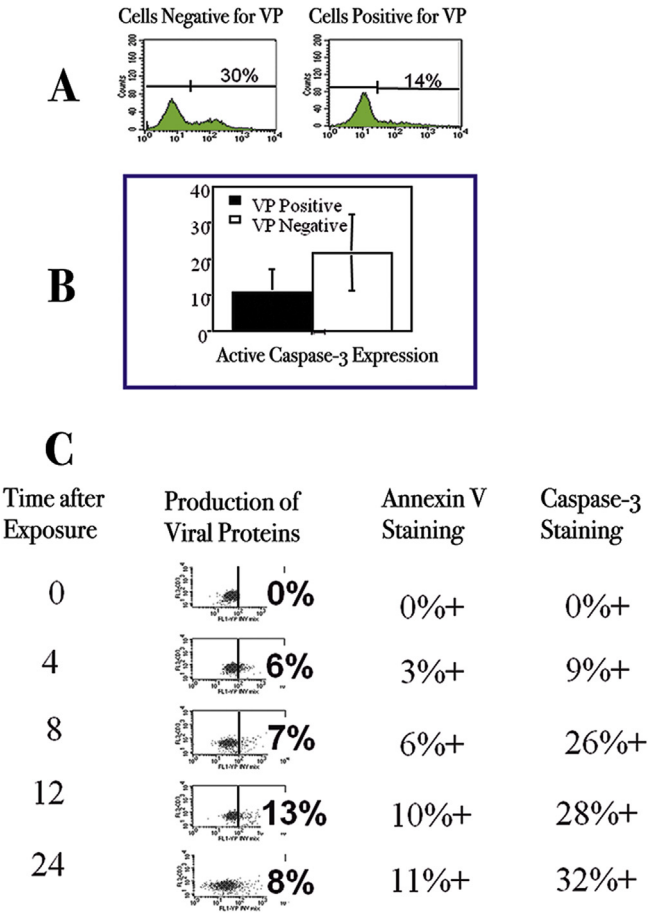


Fig. 1. Cell infection and the induction of CD3$^+$ lymphocyte apoptosis. PBMC were sham-exposed or exposed to IAV. After 24 h cells were collected and stained for production of viral proteins (VP, identified using a mixture of anti-HA, -NA and -M antibodies) and for expression of active caspase-3. (A). VP-negative and VP-positive CD3$^+$ cells within the IAV-exposed cell population examined for expression of caspase-3. Data from one representative experiment of six are shown. (B). Results from six experiments are presented as the mean percentage of caspase-3 positive CD3$^+$ cells ± SD. For each sample, data from 10,000 CD3$^+$ cells were collected. (C). In a subset of experiments, production of viral proteins as described in (A) and annexin V binding or active caspase-3 production was determined. Data are presented from one representative experiment out of three showing the percent of CD3$^+$ cells positive for production of viral HA, NA and M, annexin V or active caspase-3.

data suggested that monocyte/macrophage depletion might be closely related to elimination of the cell population expressing NA.

3.1.3. The influence of Anti-NA antibody or NA inhibitor treatment on the production of active Caspase-3

In PBMC cultures in which virus-exposed monocyte/macrophages were treated with anti-NA antibody prior to addition of lymphocytes to the cultures, production of the active form of caspase-3 was reduced in CD3$^+$ lymphocytes. Anti-NA antibody treatment (Fig. 4) or treatment with a NA inhibitor reduced the level of caspase-3 production in CD3$^+$ lymphocytes from virus-exposed PBMC cultures. NA inhibitors 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid and N-methyldeoxynojirimycin reduced the levels of caspase-3 production (mean 7% ± 1.6 [P = 0.0002] and 38.1% ± 5.3 [P = 0.07], respectively for three experiments).

3.1.4. Monocyte/macrophage production and surface expression of NA

Synthesis of influenza NA by infected monocytes/macrophages varied markedly depending upon the strain of virus to which the cells

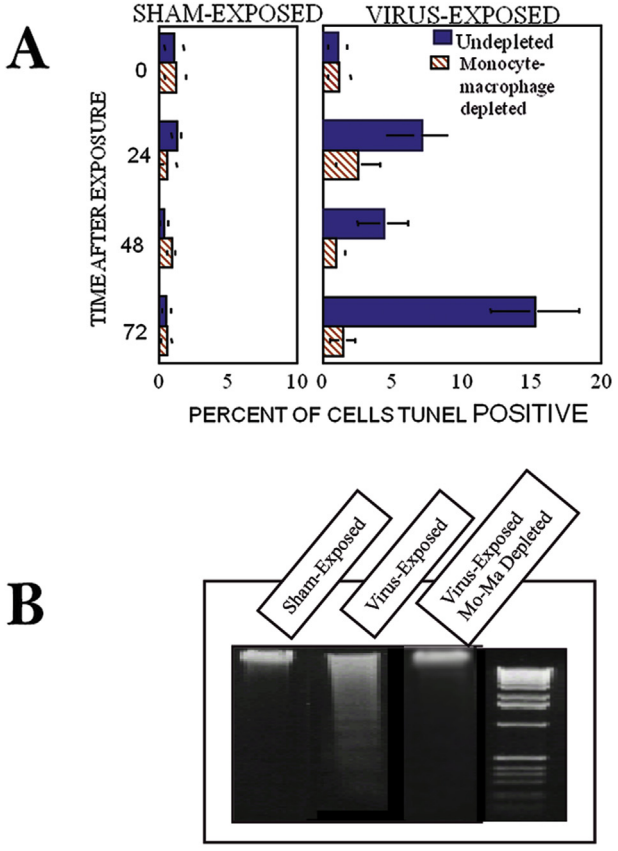


Fig. 2. Role of monocytes/macrophages in apoptosis induction. (A) TUNEL analysis for apoptosis of sham-exposed or IAV-exposed $CD3^+$ lymphocytes with or without monocyte/macrophage depletion at 72 h after exposure. (B) Assay for DNA fragmentation of apoptosis in sham-exposed and IAV-exposed $CD3^+$ lymphocytes and in lymphocytes with monocyte/macrophage depletion at 72 h after exposure.

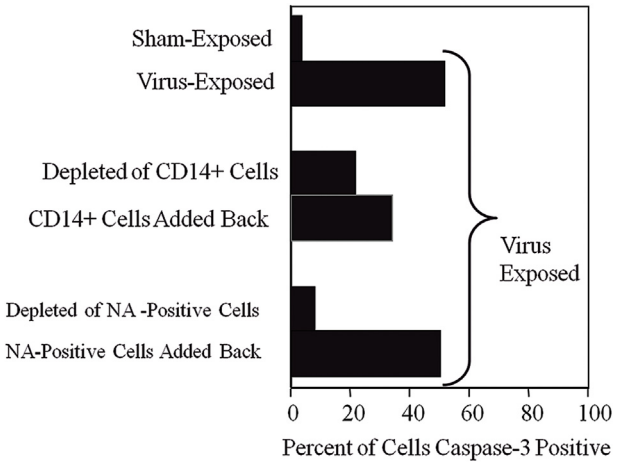


Fig. 3. The role of monocytes/macrophages or NA-expressing cells in the induction of active caspase-3 in $CD3^+$ lymphocytes. PBMC were sham-exposed or exposed to IAV and, after 1 h, cultures were depleted of monocytes/macrophages or NA-positive cells. In a subset of experiments, the depleted $CD14^+$ or NA-positive cells were added back 2 h later. Results from five experiments are presented as the mean percentage of caspase-3 positive $CD3^+$ cells. For each sample, data from 10,000 $CD3^+$ cells were collected.

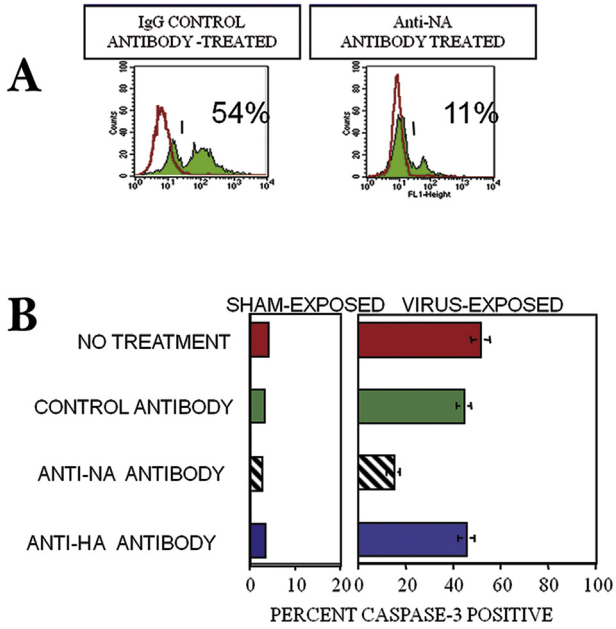


Fig. 4. The influence of anti-NA antibody treatment on the production of active caspase-3. (A) Data from one representative experiment out of five showing the percent of $CD3^+$ cells positive for production of active caspase-3 for unexposed cells with no treatment (in red), or after exposure to virus and treatment with an IgG matched isotype control antibody or with an anti-NA antibody (in green) as specified in figure. (B) Active caspase-3 production in $CD3^+$ lymphocytes in untreated (red bars), control antibody-treated (green bars), anti-NA antibody-treated (hatched bars) or anti-HA antibody-treated (blue bars) cells. Results represent the mean percentage of caspase-3 positive cells ± SD for six experiments. For each sample, data from 10,000 $CD3^+$ cells were collected.

were exposed (Fig. 5,A). Both NA production and the intensity of cell surface expression varied with the virus strain. The mean fluorescence intensity for NA expression was highest for strains that induced high levels of apoptosis. This was evident despite relatively equivalent synthesis of the viral hemagglutinin and matrix proteins by cells exposed to the different strains of virus (Fig. 5,B), which suggested relatively equivalent infectivity of the different strains for the cells. Results of viral protein synthesis using wild type A/Kawasaki and derived vaccine strains are shown in Fig. 5,B to indicate that differences in NA synthesis are not limited to the H3N2 viruses which showed the greatest variation in NA synthesis. It is important to note that the wild type and vaccine strains for both the H3N2 and H1N1 viruses contained the same (wild type) NA.

3.1.5. Induction of apoptosis by different strains of IAV

All of the infectious influenza viruses induced significant levels of apoptosis in $CD3^+$ cells (Fig. 6). The wild-type A/Bethesda virus induced substantially higher levels of apoptosis than the cold-adapted Bethesda or A/Marton strains, suggesting that synthesis and expression of neuraminidase and consequent induction of apoptosis may be associated with strain virulence.

4. Discussion

Cell death plays an important role in the host response to viral infection, and in vivo studies demonstrate that apoptosis plays a crucial role in that regard (Orzalli and Kagan, 2017). Apoptosis of IAV-infected cells makes them susceptible to phagocytosis (Hashimoto et al., 2007), and this mechanism for elimination of the virus is conserved among multicellular organisms (Nainu et al., 2017). Such a defense could be expected to be of greatest benefit if the infected apoptotic cells would have supported a productive infection by the virus, and perhaps less so

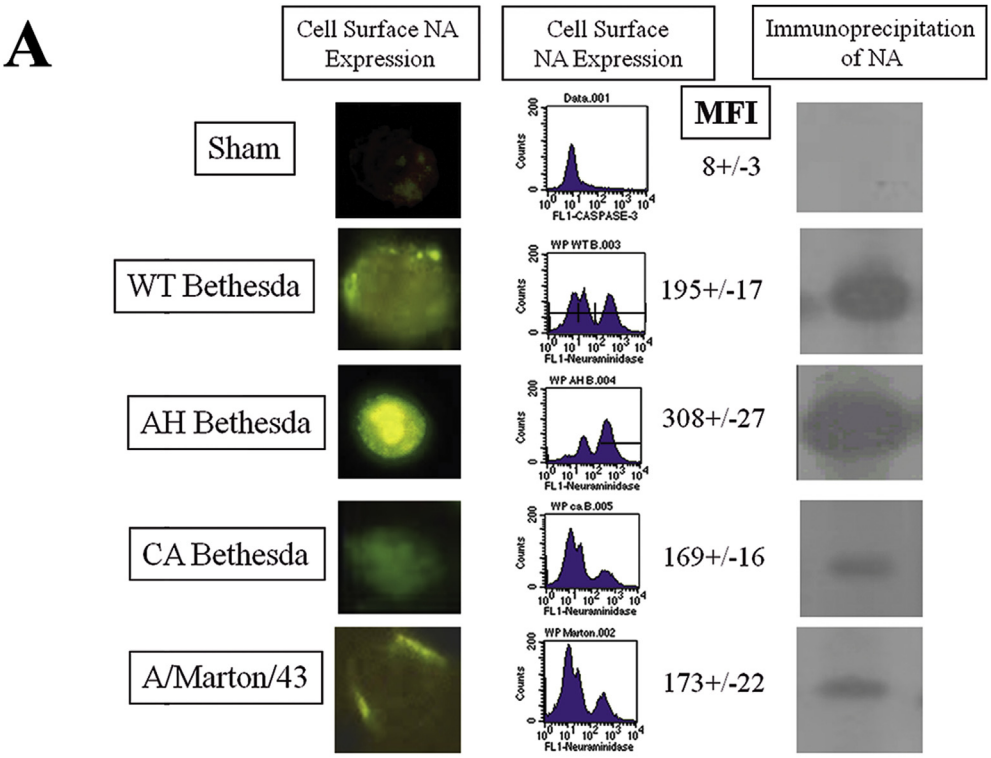
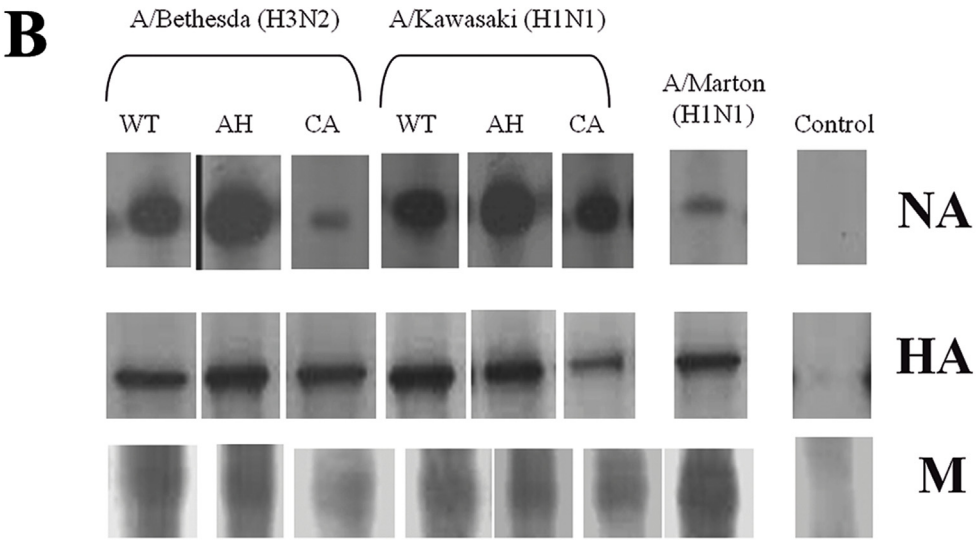


Fig. 5. Monocyte-macrophage production and surface expression of NA. (A) Analysis of NA cell surface expression or production by sham-exposed and virus-exposed monocytes/macrophages. Staining and immunoprecipitation data are from one representative experiment out of six showing photomicrographs of the most commonly occurring cell for each exposure, the pattern of fluorescence staining using flow cytometry, and the production of NA by immunoprecipitation. Measurements of mean fluorescence intensity (MFI) are presented as the mean MFI ± SD for six experiments. For each experiment the data from 10, 000 $CD14^{+}$ cells were collected. (B) Monocyte-macrophage synthesis of IAV proteins. Autoradiograms of immunoprecipitated lysates, using *anti*-N1, *anti*-N2, *anti*-H1, *anti*-H3 or *anti*-M antibodies, from monocyte-macrophages that were sham-exposed or exposed to influenza A strains A/Marton/43, wild type Bethesda/85 (wild type, or WT), A/Mallard/NY6750/78 x Bethesda/85 (avian-human reassortant, or AH), A/Ann Arbor/6/60 x Bethesda/85 (cold-adapted, or CA), A/Kawasaki/87 (WT), A/Mallard/NY6750/78 x Kawasaki/87 (AH), A/Ann Arbor/6/60 x Kawasaki/87 (CA). Data presented are all from the same gel from the same representative experiment shown in Fig. 5,A to indicate that the different strains of virus showed essentially equivalent infectivity for the cells, illustrated most clearly by production of the HA.



in the case of human PBMC which demonstrate abortive infection (Roberts, Jr. and Domurat, 1989). Furthermore, in the current as well as in previous (Nichols et al., 2001) studies, a significant portion of uninfected lymphocytes became apoptotic after exposure of PBMC to IAV. It is notable that, despite IAV-induced human lymphocyte apoptosis (Nichols et al., 2001), the infection most commonly resolves without major sequelae and with development of immunity to the challenging virus. In fact, therapeutic targeting of apoptotic cell death pathways is being investigated as a new approach to cure viral infections, especially for those viruses that persist in the host (Cooney et al., 2018; Naderer and Fulcher, 2018). Thus, further examination of the process of IAV-induced apoptosis of human lymphocytes has been warranted.

Murine models have demonstrated substantial recruitment of PBMC, both monocytes/macrophages and lymphocytes, to the lung after IAV challenge (Wyde et al., 1978; Cerwenka et al., 1999). These recruited cells play important roles in defense against and recovery from the virus infection (Wyde et al., 1982; Ennis et al., 1978; MacKenzie et al., 1989; Wells et al., 1981). Notably, recruited human PBMC may themselves become infected by IAV in the context of developing the immune response in the respiratory tract (Roberts, Jr. and Domurat, 1989).

Monocytes/macrophages specifically play an important role in

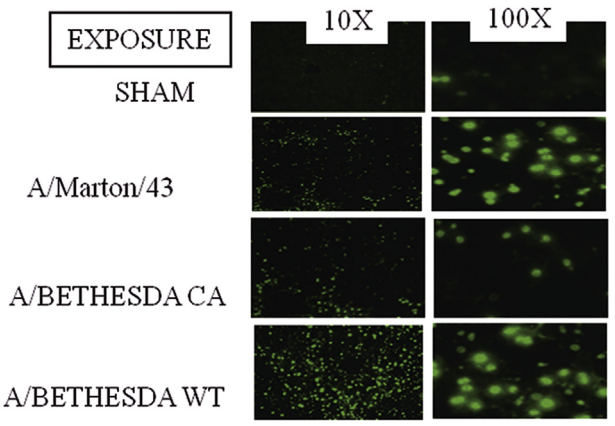


Fig. 6. Induction of apoptosis by strains of IAV. Representative immunofluoresence assays for TUNEL-positive $CD3^+$ cells after sham exposure or exposure to different strains of IAV are shown.

antiviral defense (Mogensen, 1979; Ettensohn and Roberts, Jr., 1984). They produce IFN and IL-1 and other cytokines after exposure to IAV (Roberts et al., 1979; Roberts et al., 1986; Roberts, Jr. and Nichols, 1989; Houde and Arora, 1989) and support the proliferative and non-proliferative responses of virus-specific lymphocytes (Roberts, Jr. and Nichols, 1989; Salkind et al., 1991). In the process of initiating such antiviral responses, the macrophages become infected, express viral proteins, and even facilitate the infection of lymphocytes (Roberts, Jr. and Horan, 1985; Mock et al., 1987). Therefore, experiments regarding infection of macrophages and their subsequent interactions with lymphocytes are relevant to the pathogenesis of IAV infections.

Numerous studies, including our own (Ettensohn et al., 2016), which have been reviewed elsewhere (Roberts, Jr. and Domurat, 1989), have suggested that the infection of macrophages by IAV is abortive. That is, no infectious progeny virus was released into the culture medium by the cells, and the virus titer steadily decreased from the inoculum level after addition to the cell cultures. Nonetheless, several IAV-specific proteins are produced, as shown also in the current studies, and expressed on the surface of the infected cells (Mock et al., 1987; Roberts, Jr. and Horan, 1985). Earlier studies, using immunofluorescent staining, suggested that approximately 75% of the macrophages could be infected even when a high MOI (MOI = 10) was used to assure that virtually all cells were exposed to the virus (Roberts, Jr. and Horan, 1985). In contrast, only a small percent of lymphocytes are infected, requiring monocytes/macrophages in co-culture for their infection (Mock et al., 1987). The current studies were performed using an MOI of one, which should assure exposure of approximately 65% of the macrophages. Such an MOI would allow detection of enhanced infection, that is, more cells infected and/or greater synthesis of viral proteins by infected cells, resulting in greater evidence of infection by Northern blots or SDS-PAGE of cell lysates and by staining for surface expression by the cells. It is notable that the different strains of virus appeared to have similar infectivity for the cells as judged by synthesis of the viral hemagglutinin, especially, and the matrix protein, even though they differed substantially in synthesis of the neuraminidase. It is also important to note that all of the vaccine strains with differing synthesis of NA contained the wild type NA, suggesting that other IAV gene products influenced NA synthesis. It is surprising and important to note that different strains of influenza virus could have quite different magnitudes of neuraminidase synthesis and expression by hunan monocytes/macrophages despite essentially equivalent synthesis of other viral proteins such as the HA and M protein, as illustrated in Fig. 5. The strains of virus that induced greater expression of neuraminidase by infected cells induced more apoptosis in lymphocytes, shown in Fig. 6.

Although IAV infection is well recognized to induce cellular caspases and consequent apoptosis, the consequences for the virus and the host can be debated (Ludwig et al., 2006). IAV NA has been reported to be important for the initiation of infection in human airway epithelium cultures, acting early after exposure to the virus (Matrosovich et al., 2004), and effects of the NA on apoptotic cellular responses are also important for the success of the viral infection. Thus, NF-κB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL has been shown to be crucial for efficient IAV propagation (Wurzer et al., 2004). A pro-apoptotic response to the virus appears to enhance the infection. As those studies noted, it has been commonly thought that both NF-κB activation and apoptosis induction are antiviral responses to IAV infections. The data did show that NF-κB regulates expression of antiviral cytokines such as IFN-β but that the effect that is finally dominant for the outcome of virus propagation is the regulation of proapoptotic, provirally acting factors such as TRAIL, Fas, and FasL. Also, IAV-induced apoptosis could be potentiated by α/β interferons themselves (Balachandran et al., 2000). Further studies showed that caspase 3 activation during the onset of apoptosis is essential for efficient IAV propagation, using an inhibitor of the enzyme as well as partial depression of levels by small interfering RNAs (Wurzer et al., 2003). The studies further showed that acetylsalicylic acid (ASA) blocks IAV propagation via its NF-κB-inhibiting activity, involving impaired expression of proapoptotic factors, and subsequent inhibition of caspase activation as well as blocking of caspase-mediated nuclear export of viral ribonucleoproteins (Mazur et al., 2007).

Immune responses to influenza NA are becoming increasingly recognized as an important component of the host's antiviral defense. For example, Halbherr and colleagues generated monospecific immune sera by vaccination with recombinant RNA replicon particles encoding NA. These immune sera inhibited hemagglutination in an NA subtype-specific and HA subtype-independent manner and interfered with infection of MDCK cells (Halbherr et al., 2015). In addition, they inhibited the sialidase activities of various influenza viruses of the same and even different NA subtypes. When chickens were immunized with the NA recombinant replicon particles and subsequently infected with low-pathogenic avian influenza virus, inflammatory serum markers were significantly reduced and virus shedding was limited or eliminated. The findings suggested that NA antibodies can inhibit virus dissemination by interfering with both virus attachment and egress. Furthermore, a recently reported healthy volunteer IAV challenge study showed that a NA inhibition (NAI) titer is more predictive of protection and reduced disease than the long-established hemagglutination inhibition (HAI) titer that has been used in vaccine trials (Memoli et al., 2016). The baseline NAI titer correlated more significantly with all disease severity metrics after challenge and had a stronger independent effect on outcome.

It is currently unclear why cellular expression of the influenza neuraminidase triggers lymphocyte apoptosis. We also do not know whether exogenous neuraminidase would have effects on lymphocytes similar to those of the cell-expressed neuraminidase. We further believe that studies regarding the potential effects of neuraminidase inhibitors on the infection and lymphocyte apoptosis are warranted. Nonetheless, studies reviewed above and the current studies suggest that the influenza neuraminidase should be considered an important virulence factor for the virus and further suggest that vaccine development with increased attention to measurement of anti-NA responses is warranted. The benefit, or cost, to the host of lymphocyte apoptosis also warrants continued investigation.

Author contributions

J.E.N. and N.J.R. conceived and designed the experiments; J.E.N., J.A.N. and E.H.F. performed the experiments; J.E.N., E.H.F. and N.J.R. analyzed the data; and J.E.N. and N.J.R. wrote the paper.

Conflicts of interest

The authors disclose no potential conflicts of interest.

Funding

This work was supported by an NIH Institutional Training Grant (T32-AI07526) and a Robert D. Watkins Minority Fellowship from the American Society for Microbiology (E.H.F.), and the Paul R. Stalnaker, M.D. Endowment Fund (N.J.R.). The funding sources had no role in the design and conduct of the studies; data collection and analysis; preparation or approval of the manuscript; and decision to submit the manuscript for publication.

Acknowledgments

We thank Molly Roth for help in preparing the manuscript.

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