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Defective Interfering Influenza Virus Inhibits Immunopathological Effects of Infectious Virus in the Mouse

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Mice inoculated intranasally with a lethal dose of standard influenza virus die with an immune-mediated pneumonia but are protected by coinoculation with defective interfering (DI) virus. Here we show that recruitment of immune cells into the infected lung is halved by treatment with DI virus although the $CD4^+/CD8^+$ cell ratio is not affected. Responsiveness of lung T and B cells to lectins is inhibited by standard virus, but coinoculation of mice with DI virus causes a 13-fold increase in T-cell proliferation and up to a 100-fold increase in immunoglobulin production. This effect appears to be due to lymphocyte-specific DI virus-mediated interference, since there is no inhibition of virus multiplication in the lungs. The net result is a shift from a lethal to a beneficial immune response.

Type A influenza virus infection of both mice and human beings causes a transient immunomodulation. This was first demonstrated during the great pandemic of 1918, when patients with influenza showed a decrease in the T-cell activity manifesting cutaneous sensitivity to tuberculin (4), and confirmed by later studies (21, 38). A wide range of human leukocyte functions is also suppressed or modulated by infection in vitro (reviewed in reference 22). Now with increased understanding and improved methodology, these findings are being reinvestigated and mechanisms of action are being sought. Inoculation of cultures of human leukocytes with influenza depresses the proliferative response to mitogen, but this was an indirect effect resulting from infection of macrophages (39). In vivo infection causes a slight polymorphonuclear leukocytosis and impairs phagocytosis and chemotaxis (32); it also impairs chemotaxis of monocytes and reduces their migration into inflammatory sites (23). Influenza virus infection frequently causes a lymphocytopenia, and differentiation of lymphocytes may be impaired (41). Infected lymphocytes also fail to proliferate in response to mitogen stimulation in vitro (6). In infected mice, aberrations in thymic activity were also detected (10). Influenza virus infection of B cells in vitro inhibited endogenous synthesis of antibody (9) and the antibody produced in response to mitogen stimulation (6). How influenza virus brings about these effects is not clear. In unstimulated lymphocytes, there is no virus multiplication or protein synthesis. After mitogen stimulation, viral proteins can be detected but the infection is abortive (5). In another study, infections of lymphocytes were also abortive and noncytolytic (6).

In mice, the net effect of immunomodulation by influenza virus is a lethal immunopathology centered on consolidation of the lungs, which become hemorrhagic, edematous, and heavily inflamed. Microscopically, there is a profound cellular infiltration into the alveolar walls and spaces (14). Severity of pathology is partly attributable to the level of virus multiplication (31). Cate and Mold (7) suggested that effector T cells were the principal mediator of pathology; adoptive transfer of activated Lyt 1^+2^- ($CD4^+$) cells, with delayed-type hypersensitivity reactivity, reduced survival of infected mice (24–26, 27), whereas Lyt 1^-2^+ ($CD8^+$) cells with cytotoxic activity were beneficial (1, 28–30, 44, 45).

Defective interfering (DI) viruses have a defective genome which is derived by deletion of part of the genome of the standard (infectious) virus; they are thus noninfectious, and they rely on the standard virus to provide missing functions necessary for their multiplication. This interaction can result in interference, that is, inhibition of infectious virus multiplication (15, 40). In vivo, DI viruses can protect against disease (3). A DI influenza virus population has one or more additional RNA segments (35).

Our current work is based on the protection of mice from lethal influenza by DI influenza virus. Previously we have described how protection was apparently not due to classical interference with gross virus multiplication (18), since there was no reduction in infectious virus or viral antigen in the lungs (11). However, protection required an active DI genome, since the course of infection in mice treated with DI virus inactivated by β-propiolactone (BPL) was unaltered and they all died. Since influenza in the mouse is an immune-mediated disease, we hypothesized that DI virus manifested protection by modulating the lethal immune response, and indeed we found qualitative differences in local antibody. Lungs of infected mice given inactivated or live DI virus both contained antineuraminidase immunoglobulin G (IgG) but antihemagglutinin (anti-HA) IgG was present only in lungs of the latter group (11, 33). Here we report on other immunological parameters which lead us to postulate that influenza virus modulates immune responses by infection of immune cells and that these responses are inhibited in a cell-specific manner by coinfection with DI virus.

C3H/He-mg ($H\text{-}2^k$) mice (originally obtained from Banting and Kingman Ltd., Grimston, Aldbrough, Hull, United Kingdom, and bred at the Warwick University Animal Facility) were used according to the guidelines laid down by the UK Coordinating Committee for Cancer Research. At 5 weeks of age, mice of either sex were inoculated intranasally under light ether anesthesia with 10 50% lethal doses of A/WSN (1,600 PFU) and 400 hemagglutinating units of UV-irradiated DI or UV-irradiated BPL-inactivated DI virus. A/WSN (H1N1) was inoculated at 10^3 PFU per egg, grown in the allantoic cavity of hen eggs, and quantified by plaque assay on chicken embryo fibroblast monolayers and

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TABLE 1. Cellular infiltration into lungs during influenza virus infection of C3H/He-mg mice

Mouse group	Total cells/ mouse[a] (10^6)	$CD4^+$:$CD8^+$: $CD4^-$ + $CD8^-$
Mock infected	1.7 ± 0.15	4.8:1:6.2[b]
WSN	4.6 ± 0.18	3.8:1:4.4
WSN + BPL-inactivated DI WSN	4.94 ± 0.53	3.6:1:3.9
WSN + DI WSN	2.76 ± 0.31	3.4:1:3.5

[a] Cells were prepared at 5 days p.i. as described in the text.
[b] Assayed by using a fluorescence-activated cell sorter with appropriate monoclonal antibodies.

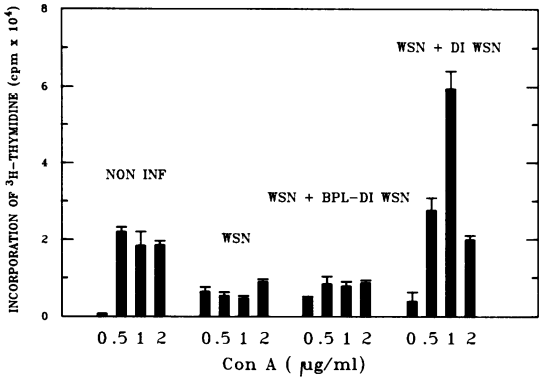

FIG. 1. ConA-induced proliferation of lymphocytes taken from the lungs of mice 5 days after intranasal inoculation with A/WSN virus alone, virus plus BPL-inactivated DI virus, and virus plus active DI virus, as shown. Noninfected mice (NON INF) received PBS. Cells were incubated for 30 h with ConA at the concentrations shown and then radiolabeled with [^{3}H]thymidine for 18 h. The incorporation of trichloroacetic acid-precipitable radioactivity is shown. The error bar represents 1 standard deviation.

by HA assay. DI WSN was prepared by three sequential passages of standard virus in ovo, starting at 10^9 PFU per egg. This preparation was then carefully UV irradiated to remove residual infectivity but not interfering activity (12). Control noninterfering preparations of DI virus were prepared by treatment with BPL (11). At 5 days postinfection (p.i.), mice were killed by cervical dislocation and their lungs were removed into RPMI 1640 medium supplemented as described above. Lung tissue was teased apart with forceps and digested with 0.5 U of collagenase (BCL; Boehringer Mannheim, Sussex, United Kingdom) per ml and 0.025% (wt/vol) DNase (Sigma, Poole, Dorset, United Kingdom) for 1 h at 37°C. Cells were filtered through stainless steel mesh and collected by centrifugation at 800 × *g* for 5 min. These cells were washed three times with medium at 37°C, and contaminating erythrocytes were lysed with distilled water at 4°C. Lymphocytes were then separated by centrifugation through Lymphoprep (Nygaard, Oslo, Norway) at 500 × *g* for 30 min. The number of viable cells was estimated by trypan blue exclusion. About 56% were $CD4^+$ or $CD8^+$ (Table 1). Cells were incubated with horse serum for 30 min at room temperature to minimize nonspecific attachment of antibody and then incubated with anti-CD4 and anti-CD8 monoclonal antibodies conjugated with phycoerythrin and fluorescein isothiocyanate, respectively [Becton Dickinson (UK) Ltd., Oxford, United Kingdom] for 30 min at 4°C. After washing, cells were examined by using a fluorescence-activated cell sorter (FACSTAR; Becton Dickinson) and fluorescence microscopy (Nikon UFX-IIA fluorescence microscope).

Increase in cellular infiltration into lungs during infection. Lungs taken from infected mice showed an increase in cellular infiltration of nearly threefold at 5 days p.i., but infected mice protected by DI virus contained about half the number of lymphocytes obtained from lethally infected mice given killed DI virus (Table 1). This finding corresponds very well with the level of gross consolidation seen in the lungs of these mice at this time. Analysis by cell sorting showed that $CD4^+$ and $CD8^+$ cells comprise about half of this cell population. The ratios of $CD4^+$ to $CD8^+$ cells are approximately equal in the lethally infected and the infected, DI-protected groups (Table 1). In this system, the $CD4^+$ and $CD8^+$ cell responses are independently lethal, as mice die if one subset is depleted by immunosurgery with the appropriate monoclonal antibody, but when both subsets are depleted, the majority of mice survive (34a).

Proliferation of T lymphocytes in response to ConA stimulation. Lung lymphocytes taken from infected and noninfected groups of mice were seeded into 96-well round-bottom tissue culture plates at 5 × 10^5 cells per well in medium containing 0, 2, 5 and 10 μg of concanavalin A (ConA; Sigma) per ml. After 30 h, cells were pulsed with 2 μCi of [3-*methyl*-^{3}H]thymidine (Amersham) per well for 18 h. Cells were then collected with a cell harvester (Inotech Biosystems International, Lansing, Mich.), and incorporation of trichloroacetic acid-precipitable radioactivity was measured. Figure 1 shows the ConA-induced proliferation of T lymphocytes taken from the lungs of lethally infected and DI virus-protected mice at 5 days p.i. There was negligible spontaneous proliferation of T cells from noninfected mice, but this was increased about 10-fold by the addition of ConA and was usually maximal at 1 μg/ml. T cells from mice infected with virus alone showed a marked decrease of responsiveness (fourfold at 1 μg of ConA per ml) which was not overcome by increasing concentrations of ConA to 2 μg/ml. A similar suppression of proliferation was seen when infected mice were coinoculated with BPL-inactivated DI virus. However, T cells from infected mice treated with DI virus showed an increased responsiveness of 13-fold compared with the value for infected mice, which peaked at 1 μg of ConA per ml. This was a reproducible finding in three experiments. The data demonstrate that T cells from lethally infected mice were less responsive than those from naive animals and suggest that the virus is immunosuppressive. T cells from infected mice treated with DI virus, on the other hand, responded even better than those from noninfected mice. We conclude that standard virus and DI virus are affecting T-cell activities antagonistically.

Synthesis of antibody by B cells in response to stimulation with LPS. Lung lymphocytes were plated as described above in medium containing 0, 0.5, 1.0, and 2.0 μg of lipopolysaccharide (LPS; *Escherichia coli* O55/B:5; Sigma) per ml. Cells were incubated for 5 days, and then tissue culture fluids were analyzed for the presence of either IgM, IgG, or IgA by enzyme-linked immunosorbent assay. Ninety-six-well flat-bottom tissue culture plates were coated with rabbit antiserum to mouse IgG, IgM, and IgA (Dako Ltd., High Wycombe, United Kingdom) at 2 μg per well overnight at 4°C. Nonspecific binding sites of the plates were blocked with phosphate-buffered saline (PBS) containing 1% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween 20 (Sigma). Dilutions of tissue culture fluids were then added to the wells and incubated overnight at 4°C. The plates were

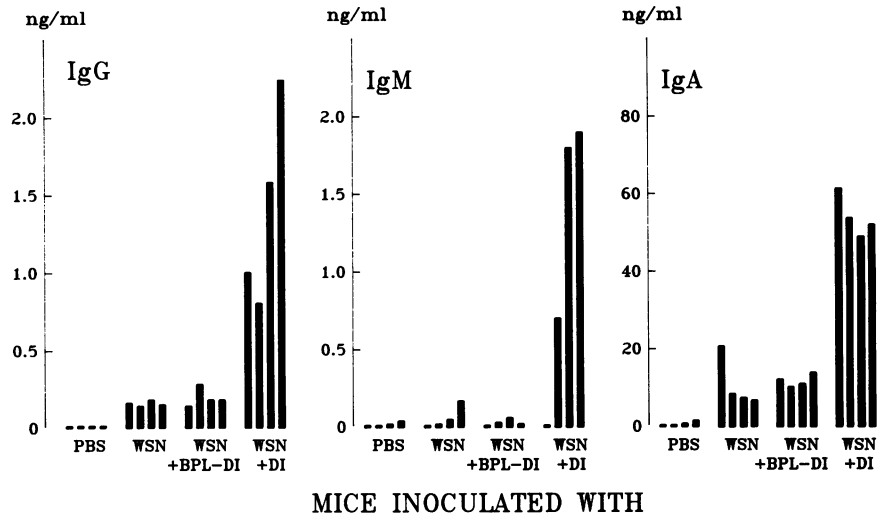

FIG. 2. LPS-induced synthesis of immunoglobulins from lymphocytes taken from the lungs of mice as described for Fig. 1. Columns represent LPS concentrations in the culture medium of, from the left, 0, 0.5, 1.0, and 2.0 μg/ml. Cells were incubated for 5 days before assay for released immunoglobulin.

washed in PBS–0.05% Tween 20, and bound antibody was detected by using alkaline phosphatase-conjugated goat antiserum specific for mouse IgM, IgG, or IgA (Sigma) and 100 μg of *p*-nitrophenyl disodium salts (Sigma) per well. The amount of antibody present was quantified by reference to IgM, IgG, and IgA standard concentration curves. Figure 2 shows the production of immunoglobulins (IgM, IgG, and IgA) by B cells in response to stimulation by LPS. Lymphocytes were prepared from lungs taken at 5 days p.i. Cells from the lungs of mock-infected mice showed little if any spontaneous production of immunoglobulin and barely responded to LPS. IgM production by infected mice and infected mice given BPL-inactivated DI virus showed a slight rise from background upon LPS stimulation by over 12-fold. In contrast to IgM and IgG, IgA synthesis was highest in nonstimulated cultures in all infected groups and LPS was inhibitory even at the lowest concentration. Nonetheless, the same trend is seen with the other immunoglobulins: similar lower responses in infected mice or infected mice given killed DI and higher production at all doses of LPS in infected mice treated with DI virus. (Note also that the endogenous level of IgA synthesis was over 2 orders of magnitude higher than that of IgG.)

The major pathological effect of a lethal respiratory tract infection in the mouse by influenza virus is consolidation of the lungs (14), and this investigation was undertaken to determine how DI virus protects mice from an otherwise lethal influenza virus infection without inhibiting the gross multiplication of virus in the main target organ, the lung (11). We have shown how influenza virus modulates functions of lymphocytes in vivo, giving rise to the immune pathology and death with which this infection is associated. Others have shown that infection of lymphocytes in vitro with influenza virus modulates their functions in a similar way (6). We now hypothesize that during infection in vivo, DI virus also enters the lymphocyte and there inhibits the immunomodulatory effects of standard virus (Fig. 3). All available data show that a functional DI genome is required, as DI virus activated by BPL had no effect on any of the parameters tested. DI virus alone is ineffective in protecting mice from lethal infection or stimulating HA-specific lung antibody (11, 34). The cell-specific nature of the generation, propagation, and interference by DI viruses is well known: different DI genomes are generated in cells of different species (8, 19, 20), and some cells appear incapable of generating DI virus (16, 17, 37, 42). Furthermore, some cells will not replicate DI genomes with which they have been inoculated (9a, 16, 43). Finally the degree of interference can be affected by the cell (2, 13, 36). These data are entirely consistent with our findings in vivo that cells of the mouse lung which support the productive infection of WSN do not permit interference by inoculated DI WSN (11) and with our hypothesis that there is classical DI-mediated interference in the cells affecting immune responses. This conclusion is in accord with our earlier finding that anti-HA antibody is only found in the lungs of infected mice treated with DI virus. However, the immunomodulatory activity of DI virus is probably more subtle than the terms in which we have phrased it, since both groups of mice produced identical amounts of antineuraminidase IgG (11, 33). We do not yet know whether the type of change indicated by the alteration in mitogen responsiveness is also responsible for the decrease in number of lymphocytes in the lung, and we have yet to ascertain the relative importance of lymphocyte

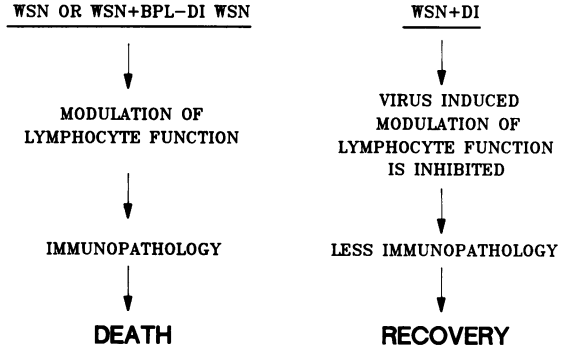

FIG. 3. Hypothesis to explain protection of mice given a lethal dose of influenza virus by DI influenza virus.

number versus changes in lymphocyte physiology to the lethal pneumonia.

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