INTRODUCTION

The type 1 interferons (IFN-α and -β) are key components of antiviral host defence and modulators of the immune system. Produced within hours of viral infection, IFN-α/β induces an antiviral state in uninfected cells (Isaacs & Lindenmann, 1957) that helps to contain spread of the virus. IFN-α/β induction by horse serum-sensitive, but not -resistant, strains of influenza virus was inhibited in the presence of horse serum, indicating that binding of the virus to sialylated cell receptors is a necessary step in the induction process. Furthermore, influenza viruses A/PR/8/34 (H1N1) and A/WS/33 (H1N1), which were poor inducers of IFN-α/β in spleen cells, were shown to have a more active neuraminidase than strains that induced higher IFN levels, and IFN-α/β induction by A/PR/8/34 (H1N1) and A/WS/33 (H1N1) was restored in the presence of a neuraminidase inhibitor. Growth of virus in different cell types altered the level of IFN-α/β induced in spleen cells by particular virus strains, suggesting that the nature of the carbohydrate moieties on the viral glycoproteins may also influence IFN-α/β induction in this system. Consistent with this notion, treatment of egg-grown virus with periodate to oxidize viral carbohydrate greatly reduced its capacity for IFN-α/β induction. Furthermore, induction of IFN-α/β was inhibited in the presence of the saccharides yeast mannan and laminarin. Together these findings indicate: (i) a requirement for interaction of the virus with sialylated receptors on the IFN-producing cell; (ii) an influence of viral carbohydrate on the response; and (iii) possible involvement of a lectin-like receptor on the IFN-producing cell in the induction of IFN-α/β or in regulation of this response.

Virus–cell interactions in the induction of type 1 interferon by influenza virus in mouse spleen cells

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Inactivated influenza A virus and fixed, virus-infected cells induce type 1 interferon (IFN-α/β) production in murine splenocytes. In this study, we have explored the nature of the virus–spleen cell interaction that leads to IFN-α/β induction and the reason for the poor response to some virus strains. IFN-α/β induction by horse serum-sensitive, but not -resistant, strains of influenza virus was inhibited in the presence of horse serum, indicating that binding of the virus to sialylated cell receptors is a necessary step in the induction process. Furthermore, influenza viruses A/PR/8/34 (H1N1) and A/WS/33 (H1N1), which were poor inducers of IFN-α/β in spleen cells, were shown to have a more active neuraminidase than strains that induced higher IFN levels, and IFN-α/β induction by A/PR/8/34 (H1N1) and A/WS/33 (H1N1) was restored in the presence of a neuraminidase inhibitor. Growth of virus in different cell types altered the level of IFN-α/β induced in spleen cells by particular virus strains, suggesting that the nature of the carbohydrate moieties on the viral glycoproteins may also influence IFN-α/β induction in this system. Consistent with this notion, treatment of egg-grown virus with periodate to oxidize viral carbohydrate greatly reduced its capacity for IFN-α/β induction. Furthermore, induction of IFN-α/β was inhibited in the presence of the saccharides yeast mannan and laminarin. Together these findings indicate: (i) a requirement for interaction of the virus with sialylated receptors on the IFN-producing cell; (ii) an influence of viral carbohydrate on the response; and (iii) possible involvement of a lectin-like receptor on the IFN-producing cell in the induction of IFN-α/β or in regulation of this response.

Many cell types can produce IFN-α/β in response to virus infection. Double-stranded RNA (dsRNA) synthesized during the virus replicative cycle (Jacobs & Langland, 1996) is considered a likely trigger, since synthetic dsRNA, e.g. polyinosinic–polycytidylic acid [poly(I)·poly(C)] is known to be a potent inducer of type 1 IFN (De Clercq, 1981). A second pathway of IFN-α/β induction that is independent of virus replication or gene expression is seen in the response of certain cells of haemopoietic origin to enveloped viruses. These so-called ‘natural’ IFN-producing cells (IPC), found in human and porcine peripheral blood and mouse spleen, produce type 1 IFN in response to physically and chemically inactivated virus, to fixed virus-infected cells, or to cells transfected with particular viral glycoproteins (Fitzgerald-Bocarsly, 1993; Ito, 1994). This IFN-inducing activity has been described for a range of enveloped viruses including herpes-, lenti-, rhabdo-, corona-, orthomyxo- and paramyxoviruses and appears to result from a direct interaction of viral glycoproteins with the surface of the IPC (Charley & Laude, 1988; Fitzgerald-Bocarsly, 1993; Feldman et al., 1994). The principal IPC in human blood has been identified as a particular subset of immature DCs, the type 2 DC precursor or plasmacytoid DC (Cella et al., 1999; Siegal et al., 1999). Recently, several groups have reported the isolation of a CD11c+ B220+ Gr-1+ cell population from mouse spleen that produces large amounts of IFN-α on stimulation with live (Nakano et al., 2001) or inactivated (Asselin-Paturel et al., 2001) influenza virus or with herpes simplex virus (HSV) (Bjorck, 2001) and appears to be the murine counterpart of the human plasmacytoid DC. The response of this cell to inactivated virus and its failure to respond to poly(I)·poly(C) (O’Keeffe et al., 2002) is consistent with previous studies indicating that the response to virus is mediated by the viral glycoproteins, rather than by dsRNA.
In examining the response of murine spleen cells to inactivated influenza A virus, we observed a markedly lower response to certain virus strains. In the present study, we have attempted to explore further the nature of the virus–cell interaction between influenza virus and the murine IPC and the basis for the difference between virus strains in their IFN-α/β-inducing ability.

METHODS

Viruses. Influenza A viruses used were the Mt Sinai strain of A/PR/8/34 (H1N1) (PR/8/34); HKx31 (H3N2), B/jx109 (H3N2) and Phil/82 (H3N2), which are laboratory-derived, high-yielding reassortants of PR/8/34 with A/Beijing/353/89 (H3N2) and A/Philippines/2/82 (H3N2), respectively; A/Memphis/1/71 (H3N1), which is a reassortant of A/Memphis/1/71 (H3N2) with A/Bel/42 (H1N1); A/Victoria/75 (H3N2) (Victoria/75); A/Guangdong/25/93 (H3N2) (Guangdong/93); A/NSW/33 (H1N1) (WS/33); A/NWS/33 (H1N1) (NWS/33); and A/Brazil/1178 (H1N1) (Brazil/78). Also used were receptor mutants of Mem/71H1-Bel2 (H3N1), which is a reassortant of A/Memphis/1/71 (H3N2) with A/Bel/42 (H1N1); A/Victoria/75 (H3N2) (Victoria/75); A/Guangdong/25/93 (H3N2) (Guangdong/93); A/NSW/33 (H1N1) (WS/33); and A/Brazil/1178 (H1N1) (Brazil/78). Also used were receptor mutants of Mem/71H1-Bel2 and Victoria/75 viruses (Mem/71H1-Bel2/HS and Victoria/75/HS, respectively). These viruses, which were selected by growth of the parent virus in the presence of horse serum, displayed specificity for sialic acid linked α2,3 to galactose (SAα2,3Gal) in contrast to the SAα2,6Gal specificity of the parent viruses (Anders et al., 1986) and were resistant to inhibition by the γ-inhibitor (equine α2-macroglobulin) in horse serum (Rogers et al., 1983).

Influenza viruses were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs (Anders et al., 1990). For some experiments, viruses were also grown in Madin–Darby canine kidney (MDCK) cells or vero cells in the presence of trypsin (Kaverin & Webster, 1995). Mouse-lung-grown stocks of B/jx109 influenza virus were prepared as described previously (Reading et al., 1997). Infectivity titres were determined by plaque assay on MDCK cells (Anders et al., 1994). For UV-inactivation of virus, virus was placed in a 60 mm Petri dish and irradiated for 30 min at a distance of 15 cm from a 20 W germicidal lamp. β-Propiolactone-inactivated Guangdong/93 virus (BPL Guangdong/93) was provided by Michael Hocart (Influenza Process Development, CSL Ltd, Melbourne, Australia).

For treatment of virus with periodate, one vol. of virus in allantoic fluid was incubated with 3 vols 0-011 M KIO4 for 15 min at room temperature; the KIO4 was then inactivated by the addition of 6 vols 0-22 % (w/v) glycerol in water. For mock treatment, the periodate and glycerol were mixed 15 min before addition of virus.

Media and reagents. Serum-free (SF) medium was RPMI 1640 (Gibco BRL) supplemented with 2 mM glutamine, 2 mM pyruvate, 22 % (w/v) glycerol in water. For mock treatment, the periodate and 14 M NH4Cl in 17 mM Tris, pH 7 (IU) by comparison with a laboratory IFN-α standard that had been calibrated against the NIH muIFN-α standard Ga02-901-511.

IFN bioassay. IFN was assayed by inhibition of the cytopathic effect (CPE) of Semliki Forest virus (SFV) on murine L929 cells. L929 cell monolayers in 96-well culture plates were incubated with serial twofold dilutions of samples overnight and then challenged with 2-5 × 103 TCID50 SFV. Controls included L929 cells exposed to SF medium throughout or to a further 7 h in SF medium followed by SFV (virus control). After 2 days, the degree of CPE in individual wells was determined by a colorimetric assay based on that of Mosmann (1983): 10 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml−1 in PBS; Sigma M-2128) was added directly to each well, the plates were incubated at 37 °C for 8 h and the cells were subsequently solubilized by the addition of 100 µl 10 % (w/v) SDS, 0-1 % HCl in water. The following day, absorbance was read using a test wavelength of 540 nm and a reference wavelength of 690 nm, and the titre of IFN was expressed as the reciprocal of the sample dilution causing 50 % reduction in CPE, corresponding to an absorbance midway between that of cell and virus controls. The data were converted to International Units (IU) by comparison with a laboratory IFN-α/β standard that had been calibrated against the NIH muIFN-α standard Ga02-901-511.
**Elution of virus from spleen cells.** Influenza virus (50–100 HAU) was incubated with 5 × 10^7 spleen cells in 1 ml SF medium supplemented with either 50 μM zanamivir containing 187 μM lactose, or 187 μM lactose alone, on ice. After 20 min, a sample (140 μl) was taken, centrifuged at 10 000 g for 5 min, and the supernatant was taken and stored on ice. Following transfer of the cell mixtures to a 37 °C water bath, further samples were taken at various intervals and treated similarly. The haemagglutinating titre in each supernatant was determined by standard microassay and expressed as a percentage of the haemagglutinating titre of the original virus used.

**Statistics.** Statistical analysis of results was performed using the two-tailed Student’s t-test.

## RESULTS

**Distinct mechanisms of IFN-α/β induction in peritoneal macrophages and spleen cells in response to influenza virus**

Incubation of murine peritoneal macrophages or spleen cells with influenza virus resulted in the production of IFN that was identified as type 1 (IFN-α/β) by its stability at pH 2 and neutralization by antiserum to IFN-α/β (data not shown). The two cell populations differed, however, in their requirement for virus infectivity. Macrophages responded only to live virus (Fig. 1B), whereas spleen cells also responded to virus that had been inactivated by UV irradiation for 30 min, or by treatment with β-propiolactone, and to glutaraldehyde-fixed, virus-infected cells (Fig. 1A). These findings confirmed the earlier observations of Ito et al. (1978), and suggested that the major IPC in the spleen is stimulated by a cell-surface interaction with influenza viral glycoproteins. In subsequent experiments to examine the nature of this interaction, the viruses were all inactivated by β-propiolactone or by 30 min UV irradiation before use.

**Requirement for virus binding to sialic acid for IFN induction**

Sialic acid binds to a pocket at the distal tip of the haemagglutinin (HA) molecule of influenza virus and represents the primary receptor for virus attachment to cell surfaces (Wilson et al., 1981; Weis et al., 1988). Sialic acid-independent infection of MDCK cells by influenza virus has recently been reported (Stray et al., 2000), however, indicating that other modes of interaction of the virus with cell-surface molecules are also possible.

To determine whether induction of IFN-α/β required the sialic acid-binding function of the viral HA, the effect of horse serum on the response was examined. Horse serum contains a sialylated glycoprotein, α2-macroglobulin, that inhibits the infectivity and haemagglutinating activity of H2 and H3 subtype human influenza viruses by competing with sialylated cell receptors for binding to the receptor-binding pocket of HA (Rogers et al., 1983). As shown in Fig. 2, induction of IFN-α/β in spleen cells by Mem/71H-BelN (H3N1) and Victoria/75 (H3N2) viruses was inhibited in the presence of 2.5 % (v/v) horse serum. In contrast, IFN-α/β induction by receptor mutants of these two viruses that are resistant to the horse serum inhibitor (Mem/71H-BelN/HS and Victoria/75/HS; see Methods) was unaffected by horse serum, ruling out a non-specific inhibitory effect of the serum on the IPCs. These data indicate that binding of the viral HA to sialylated cell-surface molecules is a necessary step in the induction of IFN-α/β in splenic IPCs.

**Influence of viral glycosylation on induction of IFN-α/β**

Influenza virus stocks that had been prepared in different cell types were found to differ in their ability to induce IFN-α/β (Table 1). For each of three viruses tested, virus grown in eggs induced higher levels of IFN-α/β than virus grown in MDCK cells, which in turn induced higher levels than vero cell-grown virus. Since the oligosaccharide moieties on influenza viral glycoproteins are known to differ depending on the host cell in which the virus is grown (Nakamura & Compans, 1979; Deom & Schulze, 1985), these observations suggested a possible influence of viral carbohydrate on the IFN response. Stimulation of the splenic IPCs was not restricted to influenza viruses that had been propagated in cells of a different species; BJx109 influenza virus that had been grown in mouse lung, thus bearing glycans derived from the homologous host, was also able to induce IFN-α/β in this system (Table 1).
The effect of treating influenza virus with periodate to oxidize viral oligosaccharides was also examined. Since any effect of periodate on the sialic acid-binding capacity of the virus might also be expected to affect IFN induction, the haemagglutinating activity of the virus following treatment was also monitored. Treatment of UV-irradiated egg-grown Guangdong/93 virus with 0.011 M KIO₄ for 15 min led to a fourfold reduction in haemagglutinating titre (data not shown), but to a 32-fold (i.e. a further eightfold) reduction in IFN-inducing ability (Fig. 3); similar results were obtained with a second strain of virus, Mem71H-BelN (data not shown). Together these results suggest that differences in structure of viral glycans can influence the efficiency of IFN-α/β induction in spleen cells by influenza virus.

Effect of saccharides on IFN-α/β induction by influenza virus

If triggering of the IFN-α/β response in spleen cells involved interaction of viral glycans with a lectin-like receptor, the response might be inhibited by the appropriate sugar ligand. A range of monosaccharides (D-mannose, L-fucose, L-rhamnose and N-acetyl-D-glucosamine) were tested for their ability to inhibit IFN induction by influenza virus BJx109. No significant effect was detected at concentrations of 25–50 mM (data not shown). IFN induction was inhibited, however, by yeast mannan, a polymer of mannose residues, and by laminarin, a β-glucan polymer, in a dose-dependent manner (Fig. 4). The inhibition is unlikely to reflect inhibition of virus binding to sialic acid, since neither saccharide is sialylated. Furthermore, we have shown previously that mannan does not inhibit binding of radiolabelled influenza viral HA and neuraminidase (NA)

**Table 1.** Induction of IFN-α/β in spleen cells by influenza virus strains grown in different cell types

Spleen cells (1 × 10⁷) were incubated with 1 × 10⁶ p.f.u. UV-irradiated virus stocks that had been grown in hens’ eggs, MDCK or Vero cells, or with 1 × 10⁵ p.f.u. UV-irradiated virus that had been propagated in mouse lungs. NT, Not tested.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Eggs</th>
<th>MDCK cells</th>
<th>Vero cells</th>
<th>Mouse lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phil/82</td>
<td>722 ± 239</td>
<td>214 ± 121</td>
<td>10 ± 0.6</td>
<td>NT</td>
</tr>
<tr>
<td>BJx109</td>
<td>664 ± 69</td>
<td>154 ± 13</td>
<td>27 ± 1</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>HKx31</td>
<td>307 ± 5</td>
<td>26 ± 0</td>
<td>6 ± 0.6</td>
<td>NT</td>
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</table>

**Fig. 2.** IFN-α/β induction by receptor variants of H3 subtype influenza A viruses and the effect of horse serum on the response. Spleen cells (1 × 10⁷) were exposed to 1 × 10⁶ p.f.u. UV-inactivated Mem/71+BelN, Mem/71+BelN/HS, Victoria/75, or Victoria/75/HS virus in the absence (solid bars) or presence (hatched bars) of 2·5 % (v/v) heat-inactivated (56 °C, 30 min) horse serum. Horse serum was omitted from subsequent washings and overnight culture of cells. Means and standard deviations of triplicate samples are shown and the data are representative of two similar experiments.

**Fig. 3.** Periodate treatment of influenza virus inhibits IFN-α/β induction in splenocytes. Guangdong/93 virus (960 HAU) was UV-irradiated, then treated (Δ) or mock-treated (○) with periodate as described in Methods, or diluted 1 : 10 in Tris-buffered saline, pH 7·2 (□, not treated). Periodate treatment resulted in a fourfold reduction in haemagglutinating titre compared with the untreated and mock-treated virus (not shown). The IFN-α/β response of 1 × 10⁷ spleen cells to serial twofold dilutions of these virus preparations was examined and the results are presented in terms of the experimentally determined number of HAU of each virus preparation used at each point on the curve.
glycoproteins to the surface of murine macrophages (Reading et al., 2000). The findings thus suggest the possible involvement of a lectin-like receptor on the IPC, either in the induction of IFN-α/β or in regulation of this response.

Induction of IFN-α/β by PR/8/34 and other H1N1 influenza virus strains

PR/8/34 (H1N1) influenza virus was observed to be a weak inducer of IFN-α/β in murine spleen cells. PR/8/34 carries less glycosylation than the other strains of virus tested and is known to bind poorly to endogenous lectins such as serum mannose-binding lectin, lung surfactant protein D and the macrophage mannose receptor (Reading et al., 1997, 2000). To explore whether poor induction of IFN-α/β by PR/8/34 virus was related to its lower level of glycosylation, additional H1N1 influenza virus strains were tested that differed in the level of glycosylation of their HA molecules. These included NWS/33, which is identical to PR/8/34 Mt Sinai in its potential N-linked glycosylation sites on HA1 (four sites on the stalk and none on the head of HA; Caton et al., 1982; Ward & de Koning-Ward, 1995); WS/33, which carries an additional site on the head of HA (Ward & de Koning-Ward, 1995); and Brazil/78, which is heavily glycosylated, with four potential glycosylation sites on the head of HA in addition to four on the stalk (Raymond et al., 1983). No direct relationship between glycosylation of the head of HA and induction of IFN was observed. WS/33 was found, like PR/8/34, to be a poor inducer of IFN, whereas NWS/33 induced substantially higher levels, similar to levels induced by Brazil/78 (Fig. 5). These findings were not consistent with low glycosylation being the explanation for low IFN-α/β induction by A/PR/8/34.

Role of viral NA activity in IFN-α/β induction by influenza virus

NWS/33 virus has been reported to possess low NA activity (Sawyer, 1969; Sedmak & Grossberg, 1973). Since we had shown an interaction with sialylated receptors is necessary

### Potential glycosylation sites on HA1 at amino acid residues:

<table>
<thead>
<tr>
<th></th>
<th>stalk</th>
<th>head</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil/78</td>
<td>[20, 21], 33, 271, 289</td>
<td>94a, 131, 158, 163</td>
<td>(Raymond et al., 1983)</td>
</tr>
<tr>
<td>NWS/33</td>
<td>[20, 21], 33, 271, 289</td>
<td>-</td>
<td>(Ward &amp; de Koning-Ward, 1995)</td>
</tr>
<tr>
<td>WS/33</td>
<td>[20, 21], 33, 271, 289</td>
<td>165</td>
<td>(Ward &amp; de Koning-Ward, 1995)</td>
</tr>
<tr>
<td>PR/8/34</td>
<td>[20, 21], 33, 271, 289</td>
<td>-</td>
<td>(Caton et al., 1982)</td>
</tr>
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</table>

Fig. 4. Inhibition of the splenic IFN-α/β response to influenza virus by saccharides. Spleen cells (1 × 10^7) were stimulated with BJx109 (m.o.i. 0.05) in the absence or presence of 1, 2 or 5 mg mannan (□) or laminarin (■) ml⁻¹. The two saccharides were tested in separate experiments. The saccharide was present both at the time of virus adsorption and during subsequent overnight culture of the splenocytes. The presence of mannan or laminarin in test samples did not affect the detection of IFN in the bioassay (data not shown).

Fig. 5. Induction of IFN-α/β in spleen cells by different H1N1 strains of influenza virus. The responses shown were obtained from 1 × 10⁷ spleen cells incubated with 1 × 10⁶ p.f.u. UV-irradiated influenza virus. Results shown are representative of three similar experiments. Location of potential glycosylation sites on HA1 of each virus is also shown using the H3 numbering system.
for IFN-α/β induction in splenic IPCs, we investigated whether a difference in strength of the viral NA activity may account for the difference between high- and low-inducing strains of virus.

To compare the relative NA activity of PR/8/34 and NWS/33 viruses, virus was adsorbed to spleen cells at 0 °C and the rate of elution following transfer of the cells to 37 °C was measured by assaying haemagglutinating activity in the supernatant sampled at different times. After 1 min at 37 °C, PR/8/34 had completely eluted from splenocytes, whereas 75 % of NWS/33 remained bound to the cells after 30 min (Fig. 6A). As expected, inclusion of the NA inhibitor zanamivir blocked the elution of both viruses. The effect of zanamivir on the ability of PR/8/34 and NWS/33 to induce IFN-α/β from spleen cells was then examined. No effect was seen on the level of IFN induced by NWS/33, but induction by PR/8/34 was substantially increased by the inclusion of zanamivir, to a level similar to that induced by NWS/33 (Fig. 6B).

When tested with other virus strains, zanamivir significantly enhanced IFN-α/β induction by WS/33 but had no effect on the response to Guangdong/93 or BJx109 viruses (Table 2). Consistent with this observation, WS/33 was shown, like PR/8/34, to elute rapidly from spleen cells in the absence of zanamivir, whereas BJx109 and Guangdong/93 showed little or no detectable elution by 30 min (data not shown). Taken together, the data indicate that the low IFN-inducing ability of PR/8/34 and WS/33 viruses in mouse spleen is due to their more rapid elution from the IPCs through the activity of the viral NA.

**NA activity of influenza viruses grown in different cell types or following periodate oxidation**

We had found previously that egg-grown influenza virus induced higher levels of IFN-α/β than MDCK-grown virus (Table 1). Since, as shown above, NA activity could clearly influence the level of IFN induction, we compared egg-grown and MDCK-grown stocks of BJx109 virus for possible differences in their NA activity. No differences were observed in their binding or pattern of elution from spleen cells: both viruses were completely adsorbed by the cells and neither showed any detectable elution after 30 min at 37 °C (data not shown). Furthermore, periodate-treated egg-grown Guangdong/93 virus, which was a poor inducer of IFN-α/β (Fig. 3), showed the same low level of elution from spleen cells as the untreated virus (data not shown). The difference in IFN-α/β induction by virus grown in the two cell types, or following periodate oxidation, is thus not related to their NA activity and, as originally proposed, may reflect structural differences in the carbohydrate moieties present on the viral glycoproteins.

**DISCUSSION**

In this study we have explored the nature of the virus–cell interaction involved in the induction of IFN-α/β in murine spleen cells by inactivated influenza A virus. Influenza viruses were found to differ in their ability to induce IFN-α/β depending on the strength of their NA enzymic activity and the host cell in which they were grown. These and other observations indicate: (i) a requirement for interaction of the virus with sialylated receptors on the IPC; and (ii)
an influence of viral carbohydrate on the response. Furthermore, IFN-α/β induction was inhibited in the presence of mannan or laminarin. Since these saccharides lack sialic acid, and mannan has been shown previously not to affect overall binding of influenza virus HA and NA glycoproteins to the cell surface (Reading et al., 2000), the inhibition of IFN-α/β induction by these saccharides suggests the possible involvement of a lectin-like receptor on the IPC, either in the induction of the IFN-α/β response, or in its regulation.

Given the established role of sialic acid as the primary receptor for influenza virus, the requirement for binding of the virus to sialic acid for IFN-α/β induction was not unexpected. This point needed to be established, however, since IFN-α/β is induced by viruses such as HSV, human immunodeficiency virus type 1 (HIV-1) and vesicular stomatitis virus (Feldman et al., 1994), which do not bind sialic acid. Furthermore, influenza virus may also interact with cells in other ways. Stray et al. (2000) have recently reported influenza virus infection of MDCK cells by a sialic acid-independent mechanism that is not yet understood. Binding of enveloped viruses through their glycans to cell membrane C-type lectins also occurs, for example binding of Sendai virus to the asialoglycoprotein receptor on hepatocytes (Markwell et al., 1985), HIV-1 to DC-SIGN on DCS (Geijtenbeek et al., 2000) and influenza virus to the mannose receptor on macrophages (Reading et al., 2000). For IFN-α/β induction, the requirement for sialic acid binding was indicated by two observations: (i) horse serum inhibited IFN-α/β induction by strains of influenza virus known to bind the sialylated γ-receptor, equine α2-macroglobulin, but had no effect on the response to inhibitor-resistant strains of virus; and (ii) viruses that eluted rapidly from spleen cells due to a very active NA were poor inducers of IFN-α/β in this system but induction was restored in the presence of the NA inhibitor, zanamivir. The latter finding suggests that the signal for IFN-α/β induction is not generated immediately on binding of virus to the cell surface; rather, time at the surface may be required for the virus to encounter and cross-link several molecules of a critical receptor for a signal to be transmitted.

Preferential specificity of the HA molecule for sialic acid linked α2,6 or α2,3 to galactose did not appear to have a marked effect on efficiency of induction of IFN-α/β, since comparable levels of IFN-α/β were induced by the horse serum-sensitive (SAα2,6Gal-specific) and -resistant (SAα2,3Gal-specific) variants of both Mem/711-Bel5 (H3N1) and Victoria/75 (H3N2) viruses. This result contrasts with our earlier studies of the mitogenic activity of H3 subtype influenza A viruses for murine B cells (Anders et al., 1986). In that case, mitogenic activity was restricted to SAα2,6Gal-specific viruses and was proposed to result from interaction with a critical receptor bearing α2,6-linked sialic acid only. In the induction of IFN-α/β, binding to sialic acid may serve simply to concentrate the virus on the cell surface, facilitating its subsequent binding to a second, triggering receptor through a sialic acid-independent interaction. Alternatively, if binding to sialic acid on a specific triggering molecule is necessary, the molecule in question may bear sialic acid linkages of both types.

IFN-α/β induction by influenza virus was substantially reduced following oxidation of viral carbohydrate with periodate, to an extent greatly exceeding the reduction in haemagglutinating activity. The efficiency of IFN-α/β induction was also found to be dependent on the host cell type in which the virus was grown, egg-grown virus being a stronger inducer than virus grown in MDCK cells or Vero cells. Here, the difference in IFN-α/β induction was not mediated through a difference in NA activity, since egg-grown and MDCK-grown Bx109 viruses, and periodate-treated and -untreated Guangdong/93 viruses, all displayed a very low rate of elution from spleen cells. Differences in the glycosylation of influenza viruses grown in different host

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**Table 2. Effect of zanamivir on IFN-α/β induction in spleen cells by different influenza virus strains**

IFN induction was examined in $1 \times 10^7$ murine splenocytes incubated with $1 \times 10^7$ p.f.u. UV-irradiated influenza virus in SF medium, either unsupplemented or containing 187 μM lactose monohydrate, or 187 μM lactose monohydrate and 50 μM zanamivir. The respective supplements were also present during subsequent washing and overnight culture of the splenocytes. The experiment was performed in triplicate. Means ± SD are shown.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>IFN-α/β titre (IU ml$^{-1}$)</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Medium + lactose</td>
</tr>
<tr>
<td>Guangdong/93</td>
<td>914 ± 165</td>
<td>1098†</td>
</tr>
<tr>
<td>Bx109</td>
<td>806 ± 73</td>
<td>687 ± 14</td>
</tr>
<tr>
<td>NWS/33</td>
<td>959 ± 47</td>
<td>1120 ± 0</td>
</tr>
<tr>
<td>WS/33</td>
<td>251 ± 31</td>
<td>248 ± 29</td>
</tr>
<tr>
<td>PR/8/34</td>
<td>143 ± 24</td>
<td>182 ± 21</td>
</tr>
</tbody>
</table>

*Ratio of IFN level induced by virus in the presence of zanamivir + lactose to that induced by virus in the presence of lactose alone.
†Due to insufficient cells, these samples were only assayed in duplicate. Average titre is shown.
‡Significantly different from IFN titre induced in the absence of zanamivir ($P<0.001$).

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cells are well documented and include differences in both size and composition of viral glycans (Nakamura & Comans, 1979; Deom & Schulze, 1985). Taken together, these findings suggested that the carbohydrate moieties decorating the viral glycoproteins might influence the triggering process in some way. Interestingly, an effect of viral glycosylation on IFN-α/β induction in porcine leukocytes by the coronavirus transmissible gastroenteritis virus has previously been reported (Charley et al., 1991; Laude et al., 1992). The particularly strong IFN-α/β response to egg-grown influenza virus may represent an effect of egg-derived glycans that is not physiologically relevant to natural infection, although it may be to vaccination. However, we also demonstrated a significant IFN-α/β response of spleen cells to a low dose of inactivated mouse-lung-grown influenza virus, confirming that stimulation also occurs with virus bearing glycans derived from the homologous host.

In one possible scenario for stimulation of the IPC by influenza virus, triggering may result from direct multivalent binding of HA, through sialic acid, to molecules of a particular critical receptor on the splenic IPC. It is known that changes in size or composition of viral glycans on the HA molecule that result from growth of the virus in different host cells can affect the receptor-binding properties of the virus (Crecelius et al., 1984; Deom & Schulze, 1985; Gambaryan et al., 1998). Such viruses might therefore differ in the repertoire of sialylated molecules on the IPC with which they interact. Differences in glycosylation may also affect the density of packing of the viral glycoproteins in the viral membrane (Rudd et al., 1999) and hence the avidity of interaction with critical cell receptors.

Alternatively, triggering of the IPC may require interaction of the virus with a putative second receptor following binding to sialic acid. This second receptor, a pattern recognition receptor (PRR) (Medzhitov & Janeway, 2000), would recognize some molecular pattern displayed by the viral glycoproteins or envelope that is brought into close apposition with the membrane of the IPC through multivalent binding of virus to siaylated receptors. Host-derived differences in carbohydrate moieties on the viral glycoproteins might affect the conformation of the viral epitope in question, or differentially impede its access to the PRR through steric hindrance.

In either model, the observed inhibition of IFN-α/β induction by mannan and laminarin may be mediated through delivery of a negative signal via a separate lectin-like receptor on the IPC. DC populations are known to express a number of different C-type lectins on their surface (Figdor et al., 2002) and precedents for negative regulation of cytokine responses by saccharides or by antibodies to C-type lectins exist in the literature (Dzienek et al., 2001; Nigou et al., 2001). Alternatively, the PRR may itself be lectin-like and interact with the viral glycans to trigger the IFN-α/β response, viral glycans derived from different cell types binding with differing avidity depending on their composition. In the latter case, inhibition by mannan and laminarin could conceivably be mediated through competition with the viral glycans for binding to the PRR, although these saccharides themselves do not induce an IFN-α/β response.

The IPC in mouse spleen has recently been identified by others as the plasmacytoid pre-DC precursor (Asselin-Paturel et al., 2001; Bjorck, 2001; Nakano et al., 2001). In a collaborative study with M. O’Keeffe, K. Shortman and others, we have confirmed responsiveness of purified splenic plasmacytoid pre-DCs to BPL-inactivated influenza virus and shown a lack of response to poly(I)-poly(C) (O’Keeffe et al., 2002), consistent with previous studies indicating that the viral stimulus for the splenic IPC is provided by the viral glycoproteins rather than by dsRNA (Fitzgerald-Bocarsly, 1993; Ito, 1994). The IFN produced was a mixture of IFN-α and IFN-β, and the yield of IFN-α/β from purified plasmacytoid pre-DCs was sufficient to account for all of the IFN-α/β produced by mouse spleen cells in response to BPL-inactivated influenza virus (J. L. Miller & M. O’Keeffe, unpublished results), indicating that the plasmacytoid pre-DC is the major IFN-α/β producing cell in the spleen responding to this stimulus.

The ability to work with defined populations of murine plasmacytoid pre-DCs should facilitate further study of the triggering receptor(s) and possible inhibitory receptor(s) involved in the IFN-α/β response to inactivated influenza virus and the existence of common or distinct pathways of stimulation by other enveloped viruses and microbial stimuli. The involvement of toll-like receptors (TLR) will be of particular interest, since this family of PRRs is known to play a critical role in the recognition of various microbial and viral components and signalling of innate immune responses (Takeda & Akira, 2001). Two non-viral inducers of type I IFN in human plasmacytoid pre-DCs – oligodeoxynucleotides containing unmethylated CpG motifs and small imidazoquinoline compounds – have been shown to signal through TLR9 and TLR7, respectively (Hemmi et al., 2000; Kadowaki et al., 2001; Krug et al., 2001; Hemmi et al., 2002; Ito et al., 2002).

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