

## **Original articles**

# **Interferon alfa, infectious virus, and virus antigen secretion in respiratory syncytial virus infections of graded severity**

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**SUMMARY** Interferon alfa was measured by an immunoradiometric assay in the nasopharyngeal secretions of a group of infants admitted to hospital with respiratory syncytial virus infection. Virus replication in the upper respiratory tract was assessed by infectivity assay and by an enzyme linked immunoadsorbent assay for the viral fusion protein on the same nasopharyngeal secretions. All infants were examined daily while in hospital and allocated a score based upon a subjective assessment of the severity of their illness. There was no significant correlation between interferon, virus, or fusion (F) protein secretion and severity of illness or age of infant. It is concluded that poor interferon alfa secretion does not underly the susceptibility of infants to severe infections with this virus.

A defect in the production of interferon has been associated with exacerbation of viral respiratory disease in children<sup>1 2</sup> and in animal model systems.<sup>3</sup> Respiratory syncytial virus is a major cause of respiratory disease, particularly bronchiolitis, in infants and children. This virus, although sensitive to the action of exogenously applied interferon in tissue culture, is a poor inducer of interferon in human cells.<sup>4 5</sup> Interferon concentrations in the nasal secretions of infants, children, and adults with respiratory syncytial virus infection are low when compared with those in the secretions of patients of similar age infected with influenza viruses.<sup>6–9</sup> This allows the suggestion that poor *in vivo* interferon production may contribute to the susceptibility of infants already compromised by the immaturity of their immune response during a primary respiratory syncytial virus infection. It is noteworthy that parainfluenza virus type 3, which is also associated with bronchiolitis in infants of this age, is also a poor inducer of interferon *in vivo*.<sup>9</sup>

In order to test this hypothesis we measured interferon alfa concentrations in the nasopharyngeal secretions of a group of infants with respiratory syncytial virus infection. The association between interferon secretion, the severity of illness, and the

degree of virus replication in the upper respiratory tract has been explored.

## **Patients and methods**

Fifty seven infants were studied during admission to hospital in Nottingham between January and March 1984. All were diagnosed as having acute viral bronchiolitis.<sup>10</sup> Respiratory syncytial virus antigens were shown in nasopharyngeal secretions at admission by immunofluorescence staining of cells in 54 and in all by an enzyme linked immunoadsorbent assay (ELISA) for viral fusion (F) protein (see below). There were 30 boys and 27 girls with a mean (SD) age of 15.3 (9.4) weeks, range 3–40 weeks. Prodromal symptoms had begun from a mean of 4.2 (2.5) days, range 1–14 days, before admission. One child was still being exclusively breast fed at 12 weeks, and 20 others had been breast fed for a mean of 5.3 (5.1) weeks, range 0.5 and 20 weeks. There was a first degree family history of asthma and eczema or hay fever, or both in 21 infants; 38 mothers and 34 fathers were smokers and one or other parent smoked in 46 cases.

While in hospital each patient was examined daily by one of us (MSCW); the clinical variables cough,

rhonchi, crepitations, subcostal recession, respiratory distress, and an empirical 'end of bed assessment' were allocated scores of 1-4 for minimal, mild, moderate, or severe respectively and the worst daily score (highest) for each variable noted at the time of discharge. Each child was reviewed three weeks after discharge and the number of days to full recovery recorded. To the above worst daily scores were added scores of 1-4 for hospital stay (1-2, 3-4, 5-6, 7 or more days) and total length of illness (prodrome+hospital+days to recovery 1-4, 5-8, 9-12, 13 or more days) and the resultant total score (range 8-32) was divided by the number of variables (8) to give a final illness severity score (range 1-4). These final severity scores were used for statistical analysis against virological variables described below.

Nasopharyngeal secretions were collected daily from all infants and flushed into a standard volume of virus transport medium containing 0.3 µmol/l phenolsulphonphthalein (phenol red), snap frozen in liquid nitrogen and stored at -70°C. The volume of secretion was calculated from the degree of dilution of the phenolsulphonphthalein in transport medium after the sample had been added as described previously.<sup>9</sup> Samples of nasal secretion below 30 µl could not be measured accurately and were excluded from the analysis.

ASSAY OF INFECTIOUS VIRUS AND INTERFERON IN SECRETIONS

Thawed nasal secretions were diluted in twofold steps in cell maintenance medium and assayed for the presence of infectious virus on microtray monolayers of HeLa cells. At three days postinoculation the monolayers were stained with a pool of subgroup cross reactive antirespiratory syncytial virus monoclonal antibodies<sup>11</sup> and foci of infection were visualised by the immunofluorescence method described by Routledge *et al.*<sup>12</sup> In parallel an aliquot of secretion was sonicated to disperse mucus and assayed for interferon alfa using an immunoradiometric kit as described by Taylor *et al.*<sup>9</sup>

ASSAY OF F PROTEIN IN SECRETIONS

Aliquots of secretion were diluted in 1% bovine plasma albumin, homogenised by sonication and dialysed against distilled water. Each specimen was concentrated approximately fivefold by freeze drying and resuspension in phosphate buffered saline. Respiratory syncytial virus F glycoprotein in the concentrated specimens was assayed by a double antibody sandwich ELISA.<sup>13</sup> The primary solid phase antibody layer was a pool of two monoclonal antibodies, 1A12 and 4E5, specific for separate epitopes on the F protein of respiratory syncytial virus

and cross reactive with both subgroups of virus.<sup>14</sup> The detecting antibody combination was a polyclonal rabbit antiserum raised against the Long strain of respiratory syncytial virus as described by Gardner and McQuillin<sup>15</sup> followed by peroxidase conjugated swine antirabbit IgG (Dako Ltd). Specimens were tested in parallel with a standard pool of known positive secretions and appropriate corrections made for plate to plate variation. Titres were read from a standard curve constructed from seven replicate titrations of this standard pool.

STATISTICAL ANALYSIS

Infectivity, interferon, and antigen titres in original secretions were calculated after determination of secretion volume by the phenolsulphonphthalein dilution method. The sensitivity of recovery for each variable depended upon the sensitivity of the assay and the quantity of secretion recovered, which varied from patient to patient. The sensitivity of infectivity, interferon, and F antigen detection was taken as 2.6 log<sub>10</sub> plaque forming units/ml, 1.28 log<sub>10</sub> units/ml, and 1.5 log<sub>10</sub> units/ml of secretion respectively. For statistical analysis, where assays were negative or titres/ml fell below these values, the titre/ml for the specimen was taken as equivalent to the sensitivity of detection.

Summated yields of infectivity, interferon, and F antigen over the first three days after admission were calculated from the formula:

$$\text{Summated titre} = \frac{x_1/\bar{x}_1 + x_2/\bar{x}_2 + \dots + x_n/\bar{x}_n}{n}$$

where  $x_1$  to  $x_n$  = titre on day 1 to day n and  $\bar{x}_1$  to  $\bar{x}_n$  = mean titre for specimens for all patients tested on that day. Expression of each value as a fraction of the mean in this way allowed summation where samples from one of the three days was missing. Pairs of coordinates were analysed by product moment and Spearman's rank correlation.

Results

Severity scores were normally distributed around a mean (SD) of 2.55 (0.62). There was no correlation between the age of the infants and the severity of infection.

Measurable quantities of secretion were obtained on at least one of the first three days after admission for 53 of the 54 cases studied. Virus was demonstrable from 30 (57%) of these patients. Mean infectivity titres were highest for the first sample tested falling thereafter so that after three days only two of 32 tested were positive (figure A). Infectivity in the secretions of five infants was followed throughout

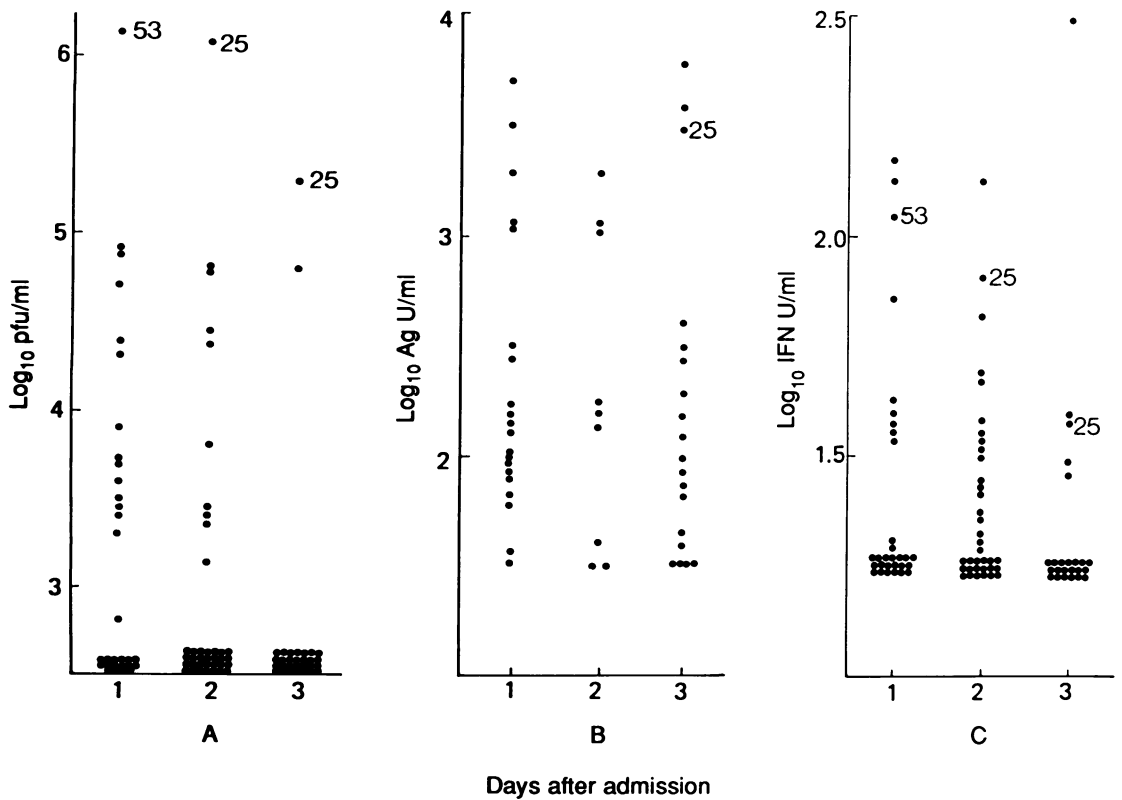


Figure A: infectious virus ( $\log_{10}$  plaque forming units (pfu)/ml), B: viral F protein ( $\log_{10}$  Ag U/ml), and C: interferon alfa ( $\log_{10}$  IFN U/ml) recovered from the nasopharyngeal secretions of infants admitted to hospital with respiratory syncytial virus infection. Each point represents one patient and the points representing patients 25 and 53 are singled out.

their stay in hospital. None showed a rise in virus titre after the third day.

In a preliminary experiment F protein was assayed in daily nasopharyngeal secretions from six patients throughout their stay in hospital. Mean levels recovered, uncorrected for volume, were similar for three days postinfection but then declined, although four of the six patients still secreted detectable F protein when tested one week after admission. Thirty six patients were tested by the same technique during the first three days after admission. All but two were positive on at least one day. Mean concentrations of F protein/ml of secretion remained essentially constant over three days (figure B). There was a highly significant positive correlation between summated F protein and infectivity titres (table).

Although interferon was detectable in all but eight of the 54 infants tested only 26 secreted more

Table Correlations of infectious virus, interferon, and F protein concentrations in nasopharyngeal secretions with age and severity of infection

	Severity	Infectivity*	Interferon*	F protein*
Age	-0.06	-0.26	-0.11	-0.15
Severity		+0.18	+0.1	+0.22
Infectivity			(+0.27)	(+0.47)**
Interferon				(+0.23)

\*Summated values for the first three days after admission.

\*\* $p < 0.01$ .

Figures in parentheses represent Spearman rank coefficients of correlation.

than or equal to 1.28  $\log_{10}$  units/ml (19 units/ml), the baseline for inclusion as positive in statistical analyses. Mean interferon concentrations were similar on the first two days after admission. On the third day, however, there was a sharp decline in the number of

infants with detectable interferon in their secretions (figure C). Four infants were followed for four to nine days. Although interferon was detectable in secretions of three of the four beyond three days and one remained positive until day eight, concentrations were low or unquantifiable because of the small volumes of secretion recoverable. There was a positive correlation between recovery of interferon with infectious virus and F protein but neither was significant (table).

Recovery of infectious virus, F protein, and interferon were negatively correlated with the age of the infants but positively correlated with the clinical severity of infection. In no case, however, did correlations approach significance. It is noteworthy that two infants, 53 and 25 (figure), secreted very high titres of infectious virus together with high concentrations of F protein and interferon. Both were less than 7 weeks old and were among the most severely ill in this study (severity scores 4.0 and 3.38 respectively).

## Discussion

It is generally recognised that infants under 1 year of age are most vulnerable to respiratory syncytial virus, that the incidence of bronchiolitis peaks in the second or third month of life and that infants under 1 month of age generally are less severely affected.<sup>16</sup> Despite this appreciable age related variability in the pathogenesis of infection, in the group of infants studied here there was no correlation between age and clinical severity score. This bears out a previous study on a smaller group of patients<sup>17</sup> and probably relates to the relative uniformity of the group studied. All of the patients were between 3 and 40 weeks of age and all had been admitted to hospital with respiratory disease later diagnosed as respiratory syncytial virus infection.

The uniformity of the group may also explain why no significant correlation was observed between virus secretion and the age of the infants. Hall *et al.*,<sup>18 19</sup> while finding lower secretion of virus in infants under 1 month of age, found no such correlation in a group ranging from 3 weeks to 21 months. Interferon secretion has also been shown previously to be independent of age in such patients.<sup>7</sup> Within this relatively homogeneous group we have sought evidence that poor interferon production correlates with defective limitation of virus replication.

Virus replication was monitored in secretions after storage at  $-70^{\circ}\text{C}$ . As the virus is highly labile to freezing and thawing, storage in this way reduces virus titres but patterns of secretion remain discernible as all the samples were treated in the same way. As expected, recoveries of virus were relatively poor

with only half of the infants yielding detectable infectious virus, although all were positive for virus antigen. Infectivity titres fell considerably over the first three days after admission. In a study by Hall *et al* mean virus titres in nasal secretions did not fall significantly for five days.<sup>18</sup> In that study, however, the authors indicated that titres from samples taken early after admission may have been underestimated. For two young, severely ill individuals virus secretion was exceptionally high. Other infants who scored highly in the clinical assessment of severity, however, secreted only modest concentrations of virus and overall there was no correlation of virus secretion and severity of disease.

Secretions were also titrated for viral F protein by ELISA. The F protein antigen is relatively stable and is unaffected by prolonged periods of storage (data not shown). Although F protein secretion was strongly correlated with yields of infectious virus, secretion patterns differed. All of the infants tested were positive for F protein and levels of secretion were much more stable over the period of study. Furthermore, log summated values for F protein secretion were normally distributed.

Interferon secretion showed no significant correlation with clinical assessment of the severity of infection and was positively correlated with both virus infectivity and F protein antigen secretion whereas our hypothesis predicted a negative correlation. This pattern is similar to that found in other primary virus infections of the respiratory tract,<sup>20 21</sup> and this suggests that limitations in the secretion of alfa interferons, at least of those species detectable by the YOK-1 monoclonal antibody, do not underly the development of bronchiolitis. That neither interferon, infectivity, nor F protein secretion were significantly associated with clinical severity assessment also diminishes hope that such easily measured variables might assist in prognosis.

We wish to thank Dr David Appleton for advice on the handling of the data presented here.

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Accepted 29 June 1989

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