Detection of respiratory syncytial virus infection in nasal aspirate samples by flow cytometry

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Abstract

Hypotheses: (i) Flow cytometry has the potential for rapid detection of respiratory viral antigens. (ii) This technique can be applied to viral diagnosis in clinical samples.

Objectives and study design: (i) To study the identification of six common respiratory viral pathogens by flow cytometry, in virus infected and uninfected cultured cells, as models of positive and negative clinical samples. (ii) To compare flow cytometry with the established techniques of viral isolation and immunofluorescent microscopy in the diagnosis of respiratory syncytial virus infection in 68 naso-pharyngeal aspirates taken from children and sent to the virology laboratory for routine virological diagnosis.

Results: (i) For each virus analysed, populations of infected and non-infected cells were clearly discernable, confirming potential for this method in rapid viral diagnosis in clinical samples. (ii) Two definitions were employed for a sample to be positive by flow cytometry, these were compared with the combined established techniques. The sensitivity, specificity, positive and negative predictive values of flow cytometry were 41%, 98%, 92% and 71% for the first definition and 74%, 88%, 80% and 84% for the second definition respectively.

Conclusions: As tested in this study, flow cytometry is less sensitive than established techniques as well as recently developed rapid diagnostic techniques for the diagnosis of respiratory syncytial virus infection. Further evaluation of the potential of flow cytometry in rapid viral diagnosis is warranted.

Key words: Flow cytometry; Rapid viral diagnosis; Respiratory syncytial virus

1. Introduction

Acute respiratory tract infections are among the most common causes of illness in infants and young children, and respiratory syncytial (RS) virus is a major cause of severe illness requiring hospitalisation, causing 50% of bronchiolitis cases, and 25% of community acquired pneumonias in the first months of life (McIntosh and Chanock, 1985). Standard procedures for diagnosing viral respiratory infections, particularly in infants and young children, include the examination of exfoliated epithelial cells in respiratory secretions by immunofluorescence microscopy with virus-specific antibodies (IF), and viral isolation in tissue cultures (TC). Immunofluorescence microscopy can be relatively time-consuming with results frequently not available until the following day, is subjective rather than objective, with the diagnosis frequently being made on the presence of only a few fluorescent cells, and requires highly trained operators. Viral isolation is extremely time-consuming, can take up to 3 weeks for results to be available, and is inferior to IF in the diagnosis of respiratory viral infections (Grover et al., 1990). The availability of effective therapy for RS virus infections, together with the need for early preventive measures to reduce nosocomial infections, has prompted the development of techniques for rapid diagnosis of RS virus infection. These include several enzyme immunoassays (EIA) for detection of viral antigens in clinical samples (Halstead et al., 1990; Subbarao et al., 1989; Johnston and Siegel, 1990; Kok et al., 1990; Freymuth et al., 1991), and a shell vial technique (Smith et al., 1991). When compared to TC, these methods have sensitivities of 71–95% and specificities of 80–100% in several recent studies. The polymerase chain reaction is also being increasingly used for viral diagnosis in clinical samples with encouraging results, but is expensive, and requires elaborate precautions to avoid contamination leading to false positive results. (Johnston et al., 1993; Paton et al., 1992). These new methods of rapid diagnosis for RS virus infection are promising, but require further development before their use can be recommended in routine clinical practice.

Flow cytometry is a technique allowing very rapid and objective assessment of cell-associated fluorescence. This technique therefore has the potential to provide a rapid and objective means to identify respiratory viral infections in a variety of clinical samples such as nasal aspirate, sputum or bronchoalveolar lavage. For example it has been used for in vitro viral detection of Pseudorabies virus in porcine leukocytes (Wang et al., 1988) and cytomegalovirus (CMV) in cultured lymphoblasts (Link et al., 1992), and in vivo for the detection of herpes simplex virus type 2 in cervical epithelial cells (Aurelian, 1982), rubella viruses in human leukocytes (O'Shea et al., 1988), and CMV in bronchoalveolar lavage (Elmendorf et al., 1988) and peripheral blood mononuclear cells (Link et al., 1992). A flow-cytometric assay with immunoreactive beads has been used to detect antibodies to human immunodeficiency virus in human blood, and proved superior to enzyme immunoassay (Scillian et al., 1989). Leukocyte populations in nasal aspirates taken during rhinovirus upper respiratory tract infections have been studied using flow cytometry (Levandowski

et al., 1988), confirming that this technique is capable of identifying cell populations in nasal aspirate samples.

In this study, a preliminary study using cell cultures alone, and cell cultures inoculated with RS virus, influenza type A, parainfluenza virus type 3, coronavirus type 229E, adenovirus and CMV as models of negative and positive clinical samples respectively, was carried out to investigate the potential of this technique in rapid viral diagnosis. We then carried out a prospective comparison between conventional virological techniques (IF and TC) and flow cytometry in the diagnosis of RS virus infection in naso-pharyngeal aspirate (NPA) samples sent for routine viral diagnosis over a 4-month period.

2. Materials and methods

Tissue culture cells

The following tissue culture cell lines and virus inocula were used: HEp2 cells for RS virus and adenovirus, MDCK cells for influenza type A and parainfluenza virus type 3, MRC5 cells for CMV, and Clone16 cells for coronavirus 229E. Infection of the inoculated cells was confirmed by typical cytopathic effect (CPE) and for parainfluenza virus type 3 by immunofluorescence microscopy of the cultured cells. Details of the monoclonal antibodies (mAbs) used in the pilot study are as follows: RS virus: IgG1, specific to F protein, used as supplied from Genetic Systems Corp. WA 98121; coronavirus 229E: IgG2a, specific to group-specific 110/120 kD peplomere glycoprotein, used at a dilution of 1 in 40, supplied from Chemicon, CA 92390; influenza type A: IgG2, specific to types H_1N_1, H_2N_2 and H_3N_2, used as supplied; parainfluenza virus type 3: IgG1, specific to parainfluenza virus type 3, used as supplied; adenovirus: IgG1, specific to the group-specific pentonbase (65–70 kDa) protein, used as supplied, all from CIS UK Ltd.; CMV: IgG1, specific to CMV early antigen, used at a dilution of 1 in 40, supplied from Dakopatts a/s, Denmark, and goat anti mouse-FITC, used at a dilution of 1 in 40, from Southern Biotechnology Associates Inc., AL 35226. For each mAb, both infected and the appropriate uninfected cells were processed and analysed to test the ability of flow cytometry to differentiate infected from non-infected cells.

Cells were harvested by gentle scraping of a single culture tube (approx. 2×10^5 cells), spun at 500 $\times g$ for 5 min and resuspended in 2 mls phosphate-buffered saline (PBS) (Oxoid Ltd., Basingstoke UK). Those for incubation with anti-adenovirus mAb were fixed in an equal volume of ice-cold 100% ethanol for 10 min, while the remainder were left unfixed (preliminary studies had demonstrated that adenovirus-infected cells only stained satisfactorily when fixed, while the other virus-infected cells stained equally well fixed or unfixed). All cells were then washed twice in PBS and spun finally. Cell pellets were incubated at 4°C for 40 min with 10 µl FITC-conjugated mAbs for RS virus, adenovirus, influenza type A, and parainfluenza virus type 3, and for 20 min with 10 µl murine mAb for coronavirus 229E and CMV, followed by a wash with PBS and a further 20-min incubation with 10 µl

goat anti mouse-FITC mAb. Labelled cells received a further wash in PBS and then were resuspended in 0.5 ml PBS for analysis.

Flow cytometry was performed using a Facscan analyser (Becton-Dickinson) with 5 W argon ion laser excitation at 488 nm. Green fluorescence, wavelength 530 nm, was measured on a logarithmic scale, 5000 cells from each tube were counted and histograms constructed of cell numbers versus fluoresence.

Clinical samples

All NPA samples sent to the Department of Virology at Southampton General Hospital for routine viral diagnosis between the period 1st January 1991–30th April 1991 were included in the prospective comparison between flow cytometry and the combined results of IF and TC together, in the diagnosis of RS virus infection.

On receipt, samples were divided into 3 equal portions for processing by TC, IF and flow cytometry. The first aliquot was inoculated onto HEp2 cells and observed for the development of CPE through 3 passages. Samples demonstrating CPE were confirmed as RS virus-infected by immunofluorescent microscopy of a cytospin preparation of tissue culture cells after incubation with FITC conjugated anti-RS virus mAb (a cocktail of two IgG1 mAbs specific to RS virus N and F proteins and one IgG2b mAb specific to RS virus F protein) used as supplied (NovoBiochem, Nottingham, UK), as used routinely for RS virus diagnosis. The second aliquot was washed for 10 min in 10 ml 0.2% N acetyl cysteine at room temperature, cytospun onto glass slides, fixed in acetone and air-dried, stained with FITC conjugated mAb to RS virus using identical methodology, and examined by immunofluorescent microscopy. The sample for flow cytometry was processed as for cultured cells described above. The mAb to RS virus for flow cytometry in clinical samples was also obtained from NovoBiochem, and used as detailed above, to permit a valid comparison between the methods to be made.

Flow cytometry in clinical samples

Flow cytometry was performed using the methods described above for the cultured cells. Initial studies in known positive and negative samples suggested that a gate could be set on the histograms such that less than 1% of cells fell within the gate in negative samples, while more than 1% of cells fell within the gate for positive samples. This gate was used for the prospective analysis of all samples in the study. Comparison of flow cytometry with the combined results from TC and IF was also made using a definition of >0.5% of cells within the gate to denote a positive sample, to determine whether this would improve sensitivity without loss of specificity. The sensitivity, specificity and positive and negative predictive values of flow cytometry compared with TC and IF together, and IF alone, were calculated for each definition.

The study was approved by the Southampton Hospitals Joint Ethical Subcommittee.

3. Results

Tissue culture cells

There was a clear differentiation between infected and uninfected samples for each of the viruses studied (Fig. 1). In the parainfluenza virus type 3 infected sample there appeared to be two distinct cell populations: half with identical staining intensity to the uninfected sample, and half with increased staining, suggesting that only half the cells in the infected sample were infected. In the other infected samples, however, there was one population of cells almost entirely separate from the uninfected cells, suggesting that the majority of cells were infected. Gates could be set
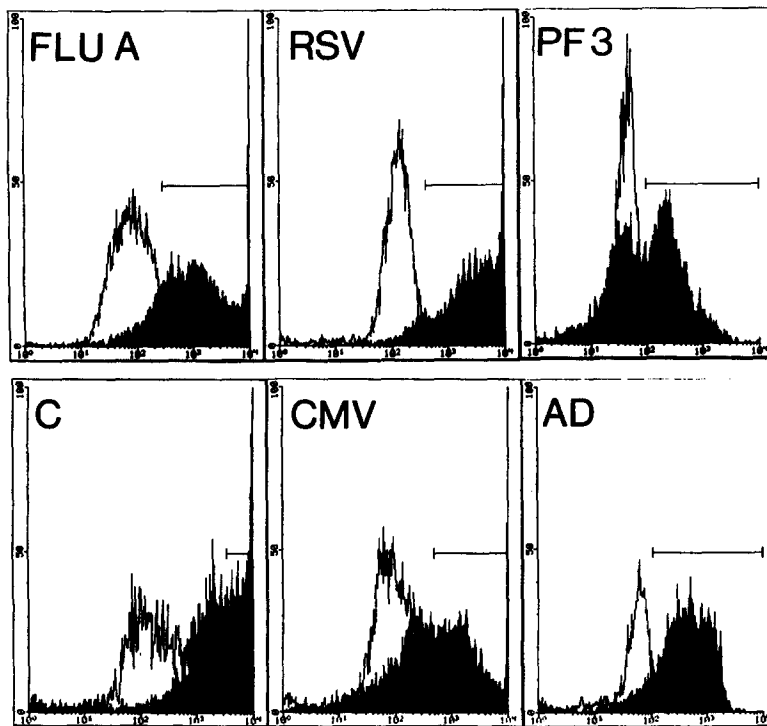

Fig. 1. Detection of respiratory viral infection by flow cytometry of cultured cells. Histograms of fluorescence intensity (horizontal axis) against number of cells on an arbitrary scale (vertical axis), for each of 6 monoclonal antibodies to common respiratory viruses. Filled tracings are infected tissue cultures, empty tracings are control (uninfected) tissue cultures. The horizontal bar represents the gate used to define positive and negative samples (see Table 1). FLU A: influenza type A infected, and uninfected MDCK cells incubated with influenza mAb. RSV: respiratory syncytial virus infected and uninfected HEp 2 cells incubated with respiratory syncytial virus mAb. PF 3: parainfluenza type 3 infected and uninfected MDCK cells incubated with parainfluenza type 3 mAb. C: coronavirus type 229E infected and uninfected Clone 16 cells incubated with coronavirus 229E mAb. CMV: cytomegalovirus infected and uninfected MRC5 cells incubated with cytomegalovirus mAb. AD: adenovirus infected and uninfected HEp 2 cells incubated with adenovirus mAb.

Table 1
Detection of respiratory viruses isolated in tissue culture cells, by flow cytometry (see Fig 1)

Virus type	Percentage of cells within 'positive' gate	
	Infected culture	Uninfected culture
Influenza virus type A	80.6	9.1
Respiratory Syncytial Virus	94.6	4.8
Parainfluenza virus type 3	53.1	4.2
Coronavirus 229E	53.4	9.8
Cytomegalovirus	52.6	4.1
Adenovirus	81.1	7.1

(Fig. 1) such that $<10\%$ of cells from the uninfected cultures fell within the gate, while $>50\%$ of cells from the infected cultures fell within the gate (Table 1).

Clinical samples

A total of 68 NPA samples were processed for flow cytometry during the study period: all 68 were also inoculated onto cell cultures and 65 were examined by IF, 3 samples were unsuitable due to insufficient cells on the cytospin preparation. Of the 68 samples 19 (28%) were RS virus-positive by IF and 25 (37%) by TC, resulting in a combined total of 27 (40%) for both methods together.

Flow cytometry, with the definition for a positive result being $>1\%$ of cells within the gate, yielded 12 (18%) RS virus-positive samples (Fig. 2). When compared with TC and IF combined, flow cytometry had a sensitivity of 41%, a specificity of 98%, and positive and negative predictive values of 92% and 71% (Table 2).

With the definition for a positive result being $>0.5\%$ of cells within the gate, flow cytometry yielded 25 (37%) RS virus-positive samples. When compared with TC and IF combined, flow cytometry had a sensitivity of 74%, a specificity of 88%, and positive and negative predictive values of 80% and 84% (Table 3).

4. Discussion

This study has demonstrated that flow cytometry can be used for the diagnosis of RS virus infection in clinical samples. Although less sensitive than the current methods in widespread clinical use, flow cytometry was specific, samples take 40 to 80 min to process, depending on whether the staining is direct or indirect, and large numbers can be analysed very rapidly. However, considerable further development would be required before flow cytometry could be considered to have a place in rapid viral diagnosis.

Flow cytometry has certain theoretical advantages over current methods in addition to the speed of processing. The assessment of the number of cells expressing a given degree of fluoresence is analysed objectively according to consistent predetermined criteria, and is performed on a larger number of cells than in routine IF
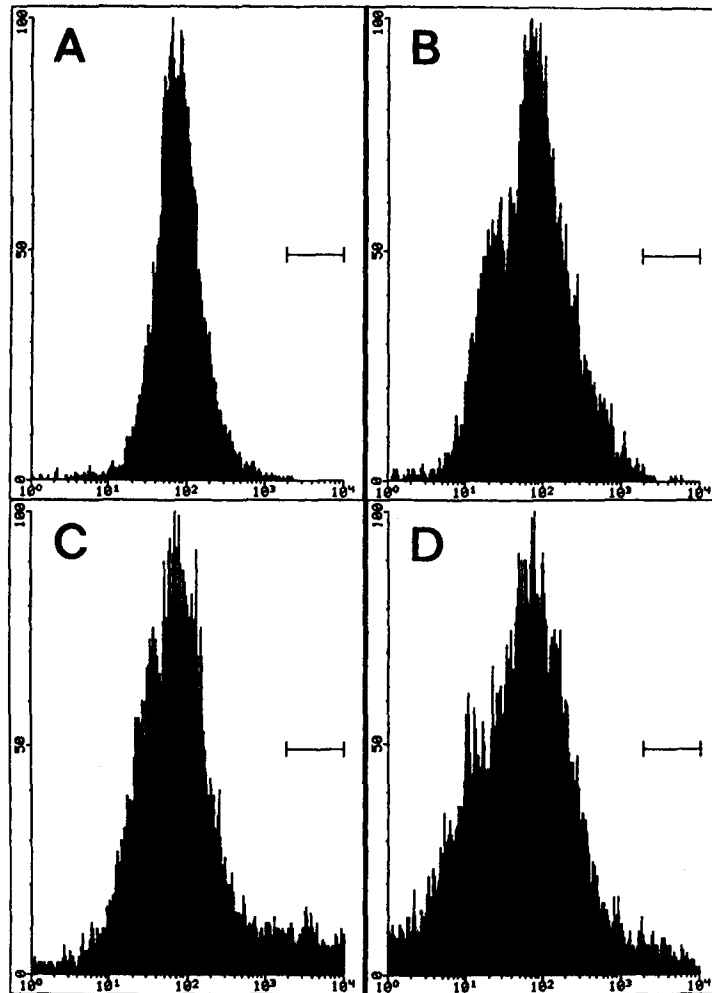
Fig. 2. Detection of respiratory syncytial viral infection by flow cytometry of naso-pharyngeal aspirate samples. Graphs of fluorescence intensity (horizontal axis) against number of cells on an arbitrary scale (vertical axis) in naso-pharyngeal aspirate samples stained with monoclonal antibody to respiratory syncytial virus. The horizontal bar represents the gate used to define positive and negative samples. Samples A and B are negative, and samples O and D are positive.

testing. In this study, the minimum number of cells within the positive gate was 25 cells for the 0.5% cut-off and 50 cells for the 1% cut-off, and the maximum was 350 cells.

Flow cytometry could be used as an alternative to immunofluorescent microscopy of cytospins of tissue culture cells in the confirmation of virus isolation by cell culture. This study has not directly compared flow cytometry with immunofluorescence of cultured cells, but the pilot study carried out in cultured cells demonstrated

Table 2
Comparison of flow cytometry with tissue culture and immunofluorescent microscopy, and immunofluorescent microscopy alone, using 1% of cells within the gate as the definition of a positive sample by flow cytometry

| | | Tissue culture and immunofluorescent microscopy | | |
		+ve	−ve	total
Flow	+ve	11	1	12
Cytometry	−ve	16	40	56
	total	27	41	68
Sensitivity		−11/27 =41%		
Specificity		−40/41 =98%		
Positive predictive value		−11/12 =92%		
Negative predictive value		−40/56 =71%		

| | | Immunofluorescent microscopy alone | | |
		+ve	−ve	total
Flow	+ve	9	3	12
Cytometry	−ve	10	43	53
	total	19	46	65
Sensitivity		− 9/19 =47%		
Specificity		−43/46 =93%		
Positive predictive value		− 9/12 =75%		
Negative predictive value		−43/53 =81%		

very clear differentiation between positive and negative samples for RS virus, influenza type A, adenovirus and parainfluenza virus type 3, while CMV and coronavirus 229E were slightly less clear-cut with a broad range of intensity of fluorescence, and relatively high fluorescence in the negative samples (Fig. 1 and Table 1).

Flow cytometry could also be used as an alternative to IF testing in the rapid diagnosis of clinical samples directly, where the benefit of both a rapid and objective diagnosis is attractive. Compared with IF alone and using the 1% cut off, the specificity of flow cytometry was 93%, but the sensitivity was only 47%, while using the 0.5% cut off, the sensitivity was 74%, but at the cost of reduced specificity of 80%. The clinical samples demonstrated quite variable degrees of non-specific fluorescence, such that a lower gate setting would have resulted in large numbers of false positive results. Attempts were made to block such fluorescence with bovine serum albumin, newborn goat serum, foetal calf serum and cows milk proteins without success (data not shown). This application clearly requires considerable further development: possible methods of improving the results include the use combined flow cytometry and nucleic acid hybridisation as recently reported for the

Table 3

Comparison of flow cytometry with tissue culture and immunofluorescent microscopy, and immunofluorescent microscopy alone, using 0.5% of cells within the gate as the definition of a positive sample by flow cytometry

		Tissue culture and immunofluorescent microscopy		
		+ve	−ve	Total
Flow	+ve	20	5	25
	−ve	7	36	43
Cytometry	total	27	41	68
Sensitivity		−20/27 =74%		
Specificity		−36/41 =88%		
Positive predictive value		−20/25 =80%		
Negative predictive value		−36/43 =84%		

		Immunofluorescent microscopy alone		
		+ve	−ve	total
Flow	+ve	14	9	23
	−ve	5	37	42
Cytometry	total	19	46	65
Sensitivity		−14/19 =74%		
Specificity		−37/46 =80%		
Positive predictive value		−14/23 =61%		
Negative predictive value		−37/42 =88%		

diagnosis of CMV infection (Link et al., 1992), or by employing double staining with mAbs to cytokeratin or desmosomal proteins to identify epithelial cells which could then be gated, and the virus-specific fluoresence assessed.

As in all such studies the quality of the test is very dependent on the quality of the mAb. All the mAbs used in this study were commercially available, and were not characterised by the authors. It is possible that the mAbs used for identification of CMV and coronavirus 229E were less specific in their binding than the other mAbs. Both CMV and coronavirus 229E are also notoriously difficult to diagnose using IF, and in addition, coronavirus 229E is extremely difficult to culture (McIntosh et al., 1978), while CMV may take up to 3 weeks to grow.

A difficulty with the interpretation of this study is the ability of the existing testing methods used as gold standards, to accurately identify negative samples. Reducing the number of cells required within the gate, or moving the position of the gate to a lower fluorescence level would have resulted in larger numbers of 'false positives' with flow cytometry. However,it is possible that such samples are in fact 'false negatives' with existing methods. We have recently demonstrated with the use of the

polymerase chain reaction (PCR) for the diagnosis of picornavirus infections, that there is a very high 'false negative' rate with cell culture methods for picornaviruses, PCR detecting more than 3 times as many positive samples in clinical specimens (Johnston et al., 1993). However a recent report using PCR for the diagnosis of RS virus infection in clinical samples showed PCR to be equivalent to cell culture (Paton et al., 1992), suggesting that for RS virus, the false negative rate with cell culture may be low. The shapes of the histograms (Fig. 2) suggest a large population of uninfected cells, with only small numbers of infected cells within the gate, also suggesting that there are unlikely to be large numbers of false negative samples.

Several recent studies have reported very good specificities and sensitivities for EIA in comparison to culture, for the rapid diagnosis of RS virus infection in clinical specimens (Halstead et al., 1990; Subbarao et al., 1989; Johnston and Siegel, 1990; Kok et al., 1990; Freymuth et al., 1991). Although flow cytometry, as used in this study, was not as sensitive as several reported EIA techniques, further develpoment of flow cytometry as outlined above could considerably improve its performance.

Flow cytometry may also have a role in the diagnosis of infections in other clinical samples such as bronchoalveolar lavage, which may be of value in the diagnosis of RS virus infection (Derish et al., 1991), and in the investigation of the pathogenesis of viral infections, in particular to determine which cell types are infected in samples taken from subjects with active viral infections.

Further studies are required to examine the potential of flow cytometry in the diagnosis of viral infections with viruses other than RS virus, to improve upon the methods employed in this study, and to carry out comparisons with other possible methods of rapid viral diagnosis.

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