Mixed Viral Infections: Detection and Management

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INTRODUCTION

The occurrence of multiple virus infections in the same patient is, intuitively, not surprising and is not an unusual finding. Multiple virus infections associated with primary or secondary immunodeficiencies are relatively common. A latent infection, such as that following a primary infection with a human herpesvirus, in conjunction with other latent infections or with an acute infection with another virus also defines categories of mixed viral infections. More perplexing is the meaning of mixed, acute viral infections that occur at the same site in immunocompetent patients and involve viruses with similar expressions of disease. The incidence of such mixed infections, their clinical importance, and whether they warrant particular diagnostic or therapeutic considerations are unresolved questions. To focus on these questions, viruses of the respiratory tract were emphasized. Although multiple infections of the gastrointestinal tract are well recognized (3, 4, 72, 113), the methods of diagnosis and knowledge of pathogenesis are limited compared with those for the common respiratory viruses. The ability of viruses to be immunosuppressive and thereby affect the host relative to other viral infections is also well recognized (90) but was not considered in this review. Respiratory virus infections are the most numerous of acute viral infections. Several viruses that cause respiratory diseases circulate concurrently in the autumn, winter, and early spring (7, 14, 29–31). There is a tendency, however, for epidemic outbreaks of respiratory viruses not to occur simultaneously in a given population (28, 30, 31). Other viruses, such as herpes simplex virus (HSV) and cytomegalovirus (CMV), are generally not considered as respiratory agents but may occur in the respiratory tract. Additionally, enteroviruses enter the host by way of the mouth and may replicate initially in associated tissues. They are often overlooked, however, as agents of respiratory disease.

MIXED ACUTE VIRAL INFECTIONS IN IMMUNOCOMPETENT PATIENTS

Clinical and Epidemiologic Studies

Multiple virus infections occurring at the same site in immunocompetent patients are rarely searched for and are usually detected by serendipity, such as the simultaneous recovery of strains of influenza A and C from a patient in 1951 (66). Most of the early virus studies were oriented to associating clinical findings with particular viruses with the goal of establishing etiologies for the diseases (23, 39, 41, 46, 73, 75). The majority of the investigations employed serology as well as virus isolation. For example, Parrott et al. reported that 30% of patients with respiratory syncytial virus (RSV) also had serological evidence of another simultaneous viral infection (73). A similar study of 667 cases of respiratory disease noted a 5% dual infection rate as discerned serologically by rises in antibody titer; of 34 patients studied by virus isolation, 4 (11%) yielded two different viruses (39). In looking for an association of rhinovirus infection with lower respiratory disease, one report presented serological evidence showing that 38% of the study population had a concomitant viral infection (76); the authors noted the difficulty of interpreting such data when assigning an etiologic role for rhinoviruses. Portnoy et al. commented subsequently that concomitant infections in patients with respiratory disease may be relatively frequent and may thus create uncertainty regarding the assigning of an etiologic role to a particular virus (75). That study specifically addressed the question of multiple infections but failed to isolate more than one virus from any patient. As in previous reports, however, serological evidence suggested multiple infections. Serodiagnostic rises in titer to two viruses occurred in 14% of patients with lower respiratory disease and in 13% of patients with upper respiratory disease (75). In the absence of...
virus isolations, however, there was no direct evidence of simultaneous infection. Technical difficulties also compromised the diagnostic efforts. Experience with cell cultures and the ability to identify virus isolates differed between laboratories. Different cell cultures were often used, which affected the sensitivity of isolating different viruses. Additionally, rapidly growing viruses such as HSV or certain enteroviruses would prevent detection of more slowly replicating viruses, such as parainfluenza or influenza viruses. Wolontis et al. noted reports of mixed viral infections in other studies (28, 91). They reported a mixed infection rate of 35% in what appeared to be simultaneous epidemics of parainfluenza type 1 and influenza (112). Importantly, they commented that the use of a battery of cell cultures for isolation and holding the cultures for 3 weeks were important considerations for identifying mixed infections; from the same nasopharyngeal swab of one patient, they isolated adenovirus type 2, mumps virus, and parainfluenza viruses type 1 and 3 in three different cell lines.

**Clinical Implications**

Portnoy et al. did not observe differences in the duration of hospitalization between patients with dual infections and those with a single virus (75). Nor did Maletzky et al. find clinical differences between patients infected with one and those infected with two viruses (55). Nichol and Cherry, however, noted that patients with bronchiolitis or pneumonia more frequently grew multiple organisms than did patients with infections of the upper respiratory tract; dual viral infections were prominent in patients: nosocomial while mixed viral-bacterial infections predominated in patients with pneumonia (68). A total of 14 mixed viral infections were identified out of 69 respiratory cases (20%) by serology and viral isolation; only 4% of a comparable control group without respiratory illness had evidence of mixed viral infections. The relative contributions of serology and virus isolation to the study were not indicated (68). Miller et al. commented that they did not observe as many dual infections as Nichol and Cherry, possibly because their patients had milder illnesses (61).

Foy et al. noted the incidence of dual infections by serology and virus isolation in 10% of pneumonia cases, 17% of bronchiolitis cases, and 5% of group cases; the data did not discern between mixed viral infections and a viral-Mycoplasma pneumoniae coinfection; nor was there an attempt to compare degrees of illnesses (23). Similarly, Hall et al. reported dual infections with viruses in 4% of RSV-infected patients but emphasized the significance of coinfections with bacteria (35).

A single mixed infection of RSV and influenza A was reported in a concurrent outbreak of RSV and influenza in a nursing facility for the elderly; the patient developed pneumonia as did five patients with influenza only (56). A report of nine dual isolations of RSV and influenza A virus noted that there was no unusual severity of disease seen in the patients with dual infections; the same degree of illness could be seen in infections with either virus alone (33). Valenti et al. noted similar clinical findings in patients infected with rhinovirus or RSV (102).

The use of nonculture techniques for diagnosis of viral infections did not add to the knowledge base regarding the role of mixed infections in disease. Respiratory viruses were isolated and/or identified by immunofluorescence (IF) in necropsy material of 24 children who died of respiratory disease; only 1 case (4%) was a dual infection, parainfluenza and influenza (15). Meissner et al. employed viral isolation and direct immunofluorescence (DIF) of cells obtained from nasal wash specimens to study a simultaneous outbreak of RSV and parainfluenza virus type 3 (PIV 3) in a newborn nursery (59). Of 14 patients yielding virus, 2 had dual infections with RSV and PIV 3. These infants could not be distinguished clinically, however, from infants infected with RSV or PIV 3 alone.

Tristram et al. used similar methods and identified four RSV patients coinfected with adenoviruses and one RSV patient coinfected with CMV (101). Three of the four RSV-adenovirus patients died of respiratory failure. Importantly, all four of the patients had serious underlying problems that may have enhanced the likelihood of severe complications from the dual infections. In comparison, adenovirus was associated with 6 of 20 fatal cases reported in a 3-year survey of acute lower respiratory disease in 805 children. Three of the cases involved a mixed adenovirus-bacterial infection, and three were cases of adenovirus alone; both categories of infection occurred in patients with underlying conditions (6). Considering these reports together, the relative threat of an adenovirus-mixed viral infection in an immunocompetent subject can not be discerned.

A retrospective study reported that 7.6% of 666 specimens with RSV (detected by isolation and/or DIF) also contained another virus; six additional dual infections not involving RSV were also noted. No significant difference in the severity of disease was seen, however, in a case control study of patients with RSV alone versus patients with RSV and another virus (93). A prospective study involving 1,246 healthy infants also found no difference in the severity of disease in cases of mixed infection versus cases of RSV infection alone (82). The latter study concluded that when RSV is detected in previously healthy infants, there is no clinical reason to search for other concomitant viral infections.

Studies of respiratory viral infections in developing countries also reported mixed viral infections (13, 26, 43, 70, 77, 79). The incidence (<10%) was comparable to that reported in studies from industrialized countries (discussed below). Nor was there any indication of increased severity of disease. Rather, the role of mixed viral and bacterial infections was emphasized.

In summary, evidence for an association of mixed infections with increased severity of disease was not forthcoming. The primary intent of the majority of the studies was to associate agents with clinical syndromes relative to age and sex of the patients. In most instances, little more than anecdotal experiences with mixed infections were provided. Much of the data were derived from serological studies with few dual organism isolations reported. There were few instances of observations of clinical severity, and where noted, the reports did not indicate any increased severity that might be attributed to mixed infections.

**MIXED VIRAL INFECTIONS IN IMMUNODEFICIENT PATIENTS**

The virus-host relationship changes in the immunodeficient patient. Viruses that are normally latent in the immunocompetent host may reactivate and cause acute disease in the immunodeficient host; viral infections that are normally suppressed by the immune system may become chronic infections; and generally, the severity of disease resulting from a viral infection is likely to be greater in immunodeficient patients.

In a study of children with acute lymphoblastic leukemia, 20 of 99 (20%) patients had multiple viruses isolated from the same specimen (5); significantly, only 20 of 796 (2.5%) control patients (no malignant disease or immunosuppressive therapy) had similar mixed infections (5, 12). When HSV was excluded, only three mixed infections were identified in healthy children. However, 17 mixed infections were identified in the leukemic
group. CMV was a coisolate in six of the leukemic children but in none of the control children. The multiple isolations from leukemic patients accounted for a significantly larger proportion of mixed infections than those from normal patients (P = < 0.001), suggesting that the leukemic children were more susceptible to mixed viral infections. Similarly, Hall et al. reported that 6 of 47 (12.8%) immunocompromised children with RSV had a mixed infection (34). Jarvis et al. noted that 3 of 12 patients with severe combined immunodeficiency had dual respiratory virus infections (47).

There was an indication of increased incidence of mixed infections in immunocompromised patients. The severity of respiratory disease in the patients with mixed infections, however, appeared comparable to that of single infections in patients with similar underlying diseases. The increased incidence may be attributable to the underlying condition of the patients. Immunocompromised patients excrete virus longer than immunocompetent patients (12, 21, 34, 47). Latent viruses, such as HSVs and CMV, are commonly reactivated and may produce chronic infections (40, 108, 109). Adenovirus infections also become prolonged and may result in serious disease (54, 116). The immunocompromised patient, therefore, represents a diagnostic dilemma for correlating laboratory findings with clinical presentation.

The above analysis indicates the need for an increased awareness of mixed infections in immunocompromised patients. Although there is no evidence that mixed infections result in enhanced disease, any single virus is likely to cause increased morbidity and/or mortality over that caused by the same virus in immunocompetent patients. Rapid diagnosis to identify all of the viruses present, therefore, becomes more critical for timely treatment with antiviral agents. Acyclovir is available for HSVs and varicella-zoster virus, ganciclovir is available for CMV; amantadine is available for influenza A; ribavirin is available for RSV; and foscarin is available for CMV retinitis and, in some circumstances, for disseminated CMV infection. Use of immune modulating agents, such as interferon and lymphokines, also requires a specific and rapid diagnosis. Additionally, there should be an awareness of the impact of diagnosis of mixed viral infections on the pharmacology of antiviral agents. Vogt et al., for example, reported antagonism between ribavirin and azidothymidine (104).

THE INCIDENCE AND NATURE OF MIXED VIRAL INFECTIONS

Rapid Methods of Viral Diagnosis

The advent of nonculture methods for the rapid diagnosis of viral infections provided opportunities for assessing the occurrence and importance of mixed viral infections. Analogous to earlier investigations that were intended to establish etiologies for clinical syndromes, however, the studies of rapid methods for diagnosis were not intended to identify mixed viral infections. The fact that the technique under evaluation required isolation in cell culture or the use of other established techniques for confirmation of the rapid methods resulted in data that contained incidences of mixed infections. The two prominent rapid methodologies studied were DIF (58) and enzyme immunoassay (EIA) (8, 16, 85, 86, 114). More recently, the shell vial technique, which was originally described for the rapid identification of CMV (27), has been used for the diagnosis of respiratory viruses (18, 49, 57, 69, 78, 89).

Data reporting only on immunocompetent patients were examined for the occurrence of mixed viral infections and for the identity of the viruses in the mixtures (1, 5, 8, 9, 13, 15, 23, 24, 28, 35, 38, 39, 41, 43, 45, 46, 50, 52, 56, 57, 59, 63, 70, 71, 74, 77-79, 81, 92-95, 97, 99, 105-107, 112). Pediatric and adult patients constituted the populations studied. No effort was made to distinguish outpatients from hospitalized patients. Mixed infections were categorized as two or more viruses identified in the same specimen by isolation in cell culture and/or antigen detection.

Incidence. Percent mixed infection was calculated by dividing the number of specimens containing two or more viruses by the total number of specimens yielding viruses. The analysis revealed that 63.1% of the reports identified between 1 and 5 mixed viral infections (average, 3.4%), 18.4% reported mixed infections in 5 to 10% of cases, and 13.1% reported mixed infections in 10 to 15% of cases (Table 1). One study reported mixed infections in 19% of cases (94), and one study observed greater than 35% mixed infections in patients with viral infections (112); the total number of positive specimens in these studies was small, 21 and 17, respectively. The latter study was conducted during a simultaneous outbreak of paramyxovirus and influenza.

Composition of mixed infections. Two hundred fifty-one mixed infections were identified in the literature reviewed (Table 2). RSV, influenza viruses, adenoviruses, and parainfluenza viruses occurred in 82.4, 26.3, 25.1, and 23.5%, respectively, of the mixed infections. The combination of RSV and influenza was most common, occurring in 25.6% of the mixed infections identified.

Differential diagnosis. The articles on rapid testing were further examined for the number of viruses identified other than the one targeted by the rapid procedure. Data were retrieved from 23 reports (1, 8, 9, 24, 36-38, 42, 45, 48-50, 60, 62, 74, 77, 78, 94, 97, 99, 105-107). In 13 studies, 15 to 41% of

| Table 1. Analysis of published reports of the occurrence of mixed virus infections |
|-----------------|-----------------|-----------------|
| No. (%) of reports (n = 38) | % Specimens with >1 virus |
| Range | Avg |
| 24 (63.1) | 1-5 | 3.4 |
| 7 (18.4) | 5-10 | 7.2 |
| 5 (13.1) | 10-15 | 12.8 |
| 1 (2.6) | 15-20 | 20.0 |
| 1 (2.6) | >20 | 35.0 |

| Table 2. Analysis of 251 mixed virus infections |
|-----------------|-----------------|-----------------|
| Virus(es) identified in mixed infections | No. (%) of occurrences in mixed infections | No. (%) of occurrences with: |
| RSV | Influenza viruses | Adenoviruses | Parainfluenza viruses | Enteroviruses | CMV | Rhinoviruses | HSV | Mumps |
| 207 (82.4) | 66 (26.3) | 63 (25.1) | 59 (23.5) | 34 (13.5) | 34 (13.5) | 22 (8.8) | 13 (5.2) | 2 (0.8) |
| 207 | 53 (25.6) | 39 (18.9) | 40 (19.3) | 27 (13.0) | 29 (14.0) | 16 (7.7) | 3 (1.4) | 0 (0) |

a Includes six adenoviruses.
b Includes six influenza viruses, seven parainfluenza viruses, and six HSVs.
c Includes seven adenoviruses and four enteroviruses.
d Includes six adenoviruses.
the specimens that were not reactive for the targeted virus yielded other viruses (Table 3).

The identification of other viruses was possible because multiple diagnostic methods were used in the studies, primarily isolation in cell culture or DIF. If the additional methods were not employed, the other viruses would not have been detected. This analysis demonstrated the need for a comprehensive strategy to identify both the mixed infections and, more importantly, the viruses present in specimens that would be considered negative if only one virus was targeted for diagnosis.

**DIAGNOSTIC CONSIDERATIONS**

This review has focused on multiple viruses that are identified in the same clinical specimen. Simultaneous viral infections, however, often occur in different body sites in the immunocompromised patient. Both categories of mixed infections require the necessary laboratory resources to effect a comprehensive diagnosis. Virus isolation in cell cultures, identification of antigen directly in specimens, and centrifugation culture (shell vial technique) are the common methods that are most applicable to the problem.

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<th>No. of reports (n = 23)</th>
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**TABLE 3.** Analysis of published reports in which specimens were positive for a virus other than the virus targeted by a specific rapid technique

**Diagnostic Strategies**

Strategies for the rapid diagnosis of multiple viral infections, whether from the same specimen or from specimens originating from different anatomic sites, must take into account both feasibility and cost; a major component of cost is hands-on time. The problem of diagnosis of multiple virus infections with cell cultures was addressed by Hsuing over 25 years ago (44). A single cell culture is rarely useful for the identification of multiple viruses (Fig. 1); even if it is permissive for several viruses, one agent, such as HSV, may overgrow another. Wolontis et al. advocated the use of multiple cell cultures and the prolonged maintenance of them after inoculation (112). Recently, Fong and Landry presented data obtained prospectively to support the proposition that multiple cell cultures should be employed with other techniques to maximize the recovery of viruses from one specimen (22); the strategy is equally amenable to diagnosis of multiple viruses from different anatomic sites. Conventional isolation procedures, however, are time-consuming. Use of hemadsorption (65) or IF (92) methods before the appearance of a cytopathic effect has been advocated as an adaptation to routine cell culture to speed the rapid identification of isolates in cell cultures.

The shell vial technique combines the specificity of isolation in cell culture with the rapid diagnostic capabilities of IF. The method is particularly useful when good specimens for DIF can not be obtained. Like conventional isolation techniques, however, multiple cell lines are required to permit an adequate differential diagnosis. The use of multiple cell cultures and conventional handling of specimens for inoculation and IF staining makes the procedure labor intensive.

In the studies reporting >5% mixed infections, viruses were detected by isolation in cell culture, cell culture and DIF, or cell culture and EIA (Table 1). Mixed infections were found in only two of seven studies in which the shell vial technique was used for identification of respiratory viruses. In these studies, the mixed infection rate was 1% (57) and 3% (78). Additional experience is needed to fully evaluate the shell vial technique and its potential for detecting multiple viruses.

**FIG. 1.** Cytopathic effects of adenovirus (A) and CMV (C) in a culture of MRC-5 cells inoculated with a urine specimen.
DIF permits rapid diagnosis of multiple viruses in the same specimen. Cells obtained from a nasopharyngeal wash or aspirate, bronchial lavage, or lesion may be aspirated to numerous circumscribed wells on glass slides and stained by IF for as many viruses as the availability of cells and reagents permits (81, 106). The viruses present are thereby identified in their natural host cell(s). Preventing the interaction of two or more viruses in a cell culture eliminates the possibility of interference or interferon production, either of which could compromise a diagnosis. Currently, DIF provides the optimum requirements for rapid and cost-effective analysis of multiple infections. Good monoclonal antibodies for the common viral pathogens and accompanying anti-immunoglobulin conjugates are available commercially, and the initial investment in a fluorescence microscope is modest.

The available EIA tests are targeted for a few important agents but are not available commercially as panels for differential diagnosis. EIA tests for most viruses, however, can be constructed in the laboratory, and comprehensive panels may be assembled. The general experience suggests that to account for their variable sensitivities and specificities EIA tests should be supported by virus isolation in cell cultures and/or DIF (36, 37, 49, 74, 94, 95, 97, 107).

Future Prospects

An expanded use of cell cultures for diagnosis will require the identification of novel cell lines with enhanced abilities to support the replication of common viral pathogens and non-cultivable viruses. Genetically engineered cell lines, such as 293 cells for adenoviruses 40 and 41 (96), could be developed for use as centrifugation cultures (shell vials) to provide comprehensive and rapid diagnosis of the common and fastidious agents.

Recent presentations have reported a rapid immunofluorescence adaptation to DIF that permits a diagnosis in 20 min, a time comparable to that of the rapid EIA tests (64, 100). Otherwise, DIF seems to have reached its limit of performance and remains very dependent on the quality of specimen collected and the acquired skill of the laboratory staff.

The newest adaptations to EIA have resulted in tests that produce results in 20 min (95, 105, 107). As discussed above, commercial EIA tests are focused on individual agents. Unit panels of EIA tests for the common viruses (such as a respiratory virus panel) with sensitivities and specificities comparable to those of DIF are necessary for comprehensive diagnostic screening.

Detection of viral nucleic acids provides the specificity of a genomic sequence and permits identification of a latent virus (111). The relative merits of nucleic acid detection versus antigen detection, however, are controversial (115). The procedural aspects of either hybridization on filters or hybridization in solution are not readily amenable to routine diagnosis. The sensitivity of the reaction is also dependent on the amount of viral nucleic acid present in the specimen. Additionally, radioisotope-labeled probes in hybridization formats are not practical for routine use, and nonisotope probes often do not provide the desired sensitivity. Molecular strategies for detection of multiple viruses in a single specimen have not been pursued.

Application of PCR to the detection of nucleic acids circumvents the problems of sensitivity and source of specimen (2, 84, 87). Unique segments of the target nucleic acid are amplified to obtain quantities that are easily detected by nonisotopic methods. Theoretically, multiple primer pairs directed against different viruses could be included in a single reaction mixture to amplify the various viral nucleic acids that may be present. In practice, however, different primer pairs often require specific optimum conditions that require individual reaction mixtures. A provisional diagnosis may be made in one working day, but confirmation usually requires an additional day. New variations of PCR permit rapid amplification that can be completed in minutes (110). A provocative suggestion has been to use PCR in a diagnostic panel by spotting any number of known nucleic acids onto a membrane strip to bind with amplified products (53).

MIXED VIRAL INFECTIONS IN SINGLE CELLS

Individual cells may also experience a mixed virus infection (Fig. 2) in which two infecting viruses simultaneously enact part or all of their replicative cycles (103). These infections may prove to be more clinically relevant than mixed infection of the same organ system or simultaneous infections of multiple organ systems. Findings concerning heterologous transactivation of viruses suggested that a dual infection of the same cell may have important implications for the host (98). For example, in a cell culture model of HSV infection, CMV induced the synthesis of infectious HSV (11). Subsequently, it was reported that the product of the major immediate-early gene of a porcine virus, pseudorabies, activated transcription of human adenovirus genes that are normally activated by the immediate-early gene products of adenovirus (20). Thus, it was recognized that a gene product from one virus may function analogously, in trans, as the like product of an unrelated virus.

Coinfection with some human herpesviruses can affect human immunodeficiency virus (HIV) gene expression (25, 32, 80); certain viral antigens also stimulate cytokine production which in turn can up-regulate expression of HIV type 1 (HIV-1) (10). These findings raise the question of whether such dual infections may be associated with reactivation of latent HIV. Transactivation of HIV genes requires a dual infection. Given the common association of several viruses and HIV with lymphoid tissue, the opportunity for coinfection appears considerable (17, 51, 83).

There is evidence that dual infections of single cells occur in patients. CMV and HIV-1 were shown to coexist in brain cells (67) and retinal cells in patients with AIDS (88). In the absence of CMV, the HIV-1-infected cells within the neural retina were not associated with inflammation or necrosis (88). The rapid diagnosis of mixed infections, therefore, may be important for rapid initiation of treatment to diminish the likelihood of dual infections of individual cells.

PERSPECTIVE

Publications on respiratory virus infections were reviewed to evaluate the significance of multiple viruses found in the same clinical specimen. The review focused on immunocompetent patients and certain immunocompromised patients. A salient feature was the lack of controlled studies involving patients with dual viral infections in comparison with patients with single virus infections. The majority of reports did not identify mixed infections or reported them in the context of the total data accumulated.

The literature survey showed that 63% of the articles with data on mixed infections identified them in <5% of the total number of viral infections. RSV occurred in 82.4% of all mixed infections, with RSV and influenza being the most common virus pair identified. Of importance was the coincident finding of the effect of focusing on only one virus for diagnosis. A total
of 87% of the studies with available data identified a virus in >10% of the specimens that were negative for the virus targeted by one technique; 56.5% of the reports recognized a virus in >15% of negative specimens.

An assessment of the available reports did not indicate that mixed infections, per se, served as a clinical disease promotion factor (19) by functioning additively or synergistically in causing disease. Dual infections by certain viruses of the same cell, however, may function as a clinical disease promotion factor at the molecular level to reactivate latent infections and/or to initiate or stimulate a disease process. The clinical impact of the latter findings has yet to be determined.

A comprehensive strategy of diagnosis is necessary for (i) the diagnosis of mixed infections, (ii) the diagnosis of viral infections occurring at different anatomical sites, and (iii) the achievement of a differential diagnosis if one virus is targeted for rapid diagnosis by a single method. This approach is also relevant for choosing the appropriate antiviral agent and for monitoring its efficacy. Future directions in methodology, whether based on antibody or nucleic acid probes, should consider these requirements.

ACKNOWLEDGMENT

I gratefully acknowledge Christine Robinson, The Children's Hospital, Denver, Colo., for providing the photograph of the dual infection of RSV and CMV (Fig. 2).

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