*Brief Definitive Report*

# Lethal Exacerbation of *Pneumocystis carinii* Pneumonia in Severe Combined Immunodeficiency Mice after Infection by Pneumonia Virus of Mice

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## Summary

Mice homozygous for the mutant allele *scid* (severe combined immunodeficiency) have been described as excellent models for *Pneumocystis carinii* (*Pc*) pneumonia (PCP), a major health problem in patients with acquired immune deficiency syndrome (AIDS) and other immunodeficiency states. Other microorganisms have been shown to infect AIDS patients simultaneously with *Pc*, but whether one opportunist is able to directly influence the pathogenicity of another has not been determined previously. We have deliberately coinfected *scid* mice (with extant *Pc* infection) with a variety of primarily pneumotropic viruses and bacteria and have identified pneumonia virus of mice as causing a dramatic increase in the density of *Pc* organisms and the morbidity due to PCP in immunodeficient *scid* mice. This finding has clinical significance in the management of PCP, in that the identification and treatment of coinfecting pneumotropic pathogens may be as important as treatment targeted at *Pc*. A search for other synergistic (or antagonistic) microorganisms and determination of their mechanism(s) of action in altering the progression of PCP is indicated.

Patients with AIDS commonly develop and die from opportunistic infections (1). The spectrum of infectious diseases seen in AIDS patients includes those caused by viruses, bacteria, fungi, and protozoa (2). Chief among these pathogens is the fungus (3, 4) *Pneumocystis carinii* (*Pc*), with an incidence of ~80% in AIDS patients (5). Mixed infections involving *Pc* have been described in both AIDS (*Pc* plus CMV, *Mycobacterium spp.* or *Legionella pneumophila*) [2, 6]) and in malnourished children (where coinfecting organisms include *Toxoplasma gondii*, *Cryptosporidium*, *Candida sp.*, *Cryptococcus neoformans*, *Mycobacterium sp.*, *Chlamydia trachomatis*, CMV, herpes simplex, herpes zoster, EBV, and respiratory syncytial virus [RSV]) (7). Whether the presence of a specific coinfectious organism is neutral, antagonistic, or synergistic in interaction with *Pc* has been largely unaddressed. However, one retrospective study of patients with HIV-related pneumonia concluded that coinfection by CMV (the most commonly isolated viral copathogen in AIDS) did not adversely affect the prognosis of patients with *Pneumocystis carinii* pneumonia (PCP) (6).

In our original studies on PCP in *scid* mice (8), the longevity of *scid* mice reared in a barrier facility was greater than that of mice reared in a conventional room, although PCP was the principle cause of morbidity in both environments. Among 14 species of bacteria, mycoplasma, and viruses checked, the only identified difference in pneumotropic microorganisms between the two rooms was the persistent finding of *Pasteurella pneumotropica* (*Pp*) in the conventional room, although pneumotropic viruses such as pneumonia virus of mice (PVM) had historically also been found on rare occasions. We thus initiated experiments involving the deliberate challenge of *Pc*-infected *scid* mice with various microorganisms with tropisms primarily or secondarily directed toward tissues of the respiratory tract to determine whether such coinfection would exacerbate PCP.

## Materials and Methods

*Mice.* Immunodeficient mutant *scid/scid* (hereafter termed *scid*) mice were obtained originally from the Institute for Cancer Research (Philadelphia, PA) on inbred strain background C.B-17/Icr. The *scid* mutation was transferred by us to several standard inbred strains by greater that 10 backcrosses, and the congenic strains BALB/cBy-*scid* and C3H/HeSn-*scid* were then propagated by brother × sister matings. All *scid* mice maintained in our colony develop spontaneous PCP and die at median ages of 20 wk (BALB-*scid*) and 24 wk (C3H-*scid*). Additional details on the genetics, natural history, and pathology of PCP in *scid* mice have been described (8).

Homozygous *scid* and congenic +/+ mice for these experiments were inoculated with specific microorganisms and thereafter maintained in polycarbonate cages with filter tops until necropsy. All

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cage changes and experimental procedures were performed in a biological safety cabinet, and all cages, bedding, feed, and water were autoclaved. The number of mice studied in each experiment is indicated as the denominator (number at risk) in the morbidity data presented (see Fig. 1 and Table 1).

*Histopathology.* At necropsy, lungs were removed, fixed in Bouin's solution, embedded in paraffin, sectioned at 5 $\mu$M, and stained with hematoxylin and eosin (H&E) or Grocott's methenamine silver (GMS) and light green (LG). Tissue sections were evaluated for the presence and intensity of PCP-related histopathology and cyst levels as described previously (8).

*Immunohistochemistry.* PVM and *Pc* were identified on serial sections of infected *scid* lung by capillary gap technology using a robotic immunohistochemistry workstation (9) (Code-On; Fisher Scientific, Pittsburgh, PA). Paraffin sections to be stained for PVM were predigested for 30 min in a solution of 0.25% crude pancreatic type II trypsin (Sigma Immunochemicals, St. Louis, MO) in 0.02% $CaCl_2$, followed by overnight incubation with polyclonal rat anti-PVM antiserum. Sections were then incubated for 30 min with biotinylated goat anti-rat Ig, then streptavidin-horseradish peroxidase (SA-HPO; Southern Biotechnology Associates, Birmingham, AL), and finally with chromagen 3-amino, 9-ethylcarbazole (AEC; Biomeda, Foster City, CA). *Pc* was revealed (without trypsinization) on adjacent serial sections by 30-min incubations with polyclonal mouse anti-*Pc* (10), biotinylated goat anti-mouse Ig $\kappa$, and finally SA-HPO and AEC as above. Stained slides were viewed and representative fields were digitized using an Olympus research microscope equipped with a Sony DXC-930 CCD color video camera and a Macintosh IIci computer with a Scion LG-3 8-bit grayscale frame grabber (Scion Corp., Frederick, MD). Acquisition and initial manipulation of the images were done using the public domain National Institutes of Health (NIH) Image 1.47 program written by W. Rasband (available by anonymous Internet FTP from zippy.nimh.nih.gov). The superimposed images of PVM- and *Pc*-stained tissue (from adjacent serial sections of a dually infected *scid* lung; see Fig. 2) were produced by merging the NIH Image-produced TIFF files using Adobe Photoshop followed by printing on a ColorTone dye-sublimation printer (GCC Technologies, Bedford, MA).

*Microorganisms and Inoculation Procedures.* The following microorganisms were investigated to determine their ability to alter the course of PCP in *scid* mice. Recipient mice were 6–10-wk-old at time of inoculation. Unless otherwise stated, C3H mice were used in these experiments because of the relative hardiness and breeding success of C3H-*scid* mice among various congenic *scid* stocks in our colony.

*PVM.* 200 fluorescent focus units (FFU, see below) of PVM were inoculated intranasally (11) into C3H-*scid* and +/+ mice. The PVM inoculum was a pooled lung homogenate from naturally infected athymic mice (12) that was isolated and quantified in BHK 21 cell cultures as previously described (11).

*Mouse Hepatitis Virus-Y (MHV-Y).* The isolation and characterization of strain MHV-Y was described by Barthold et al. (13). Isolate No. 78318 was passaged in mycoplasma-free NCTC1469 cell cultures (American Type Culture Collection [ATCC], Rockville, MD). 100 median tissue culture infectious doses ($TCID_{50}$) of this isolate were inoculated intranasally into C3H-*scid* and +/+ mice.

*RSV.* RSV (VR-26) was grown on HEp-2 (CCL-23) cells (both obtained from ATCC). $10^5$ $TCID_{50}$ of RSV were inoculated intranasally into BALB/c-*scid* and +/+ mice. Mice of the BALB/c background were used in this experiment because of the known susceptibility of BALB/c and resistance of C3H mice to RSV (14).

*Sendai Virus (SV).* SV isolate No. 771076 was derived at Yale University (15) and used in the form of an infected lung homogenate from specific pathogen-free, random-bred weanling mice. 100 FFU were inoculated intranasally into C3H-*scid* and +/+ mice.

*Pp.* The *Pp* inoculum was obtained from Dr. H. Bedigian at The Jackson Laboratory (Bar Harbor, ME), and was cultured and frozen at −70°C until needed. C3H-*scid* and +/+ mice received an intranasal inoculum of $1.1 \times 10^7$ CFU per mouse.

*Postinoculation Assessment of Infection.* Since *scid* mice lack the capability of producing normal antibody responses and normal +/+ mice can clear infectious microorganisms, both serology and microorganism cultures were used to confirm successful inoculation.

*Serology.* Antibody was detected in 1:10 diluted serum by an indirect fluorescent antibody assay (IFA) as previously described (16, 17) using infected BHK21 cells for PVM and SV, NCTC1469 cells for MHV-Y, and Hep2 cells for RSV. In all cases, the secondary antibody was FITC-conjugated goat anti-mouse Ig (Antibodies, Inc., Davis, CA).

*Virus Isolation and Titration.* Analyses for PVM and SV were performed by inoculating BHK21 cells grown on chamber slides (Lab-Tek; Miles Scientific Division, Naperville, IL) with supernatants of test lung homogenates. After 6 d of incubation, these culture slides were processed for IFA. Virus titers were expressed as $\log_{10}$ FFU per gram of lung.

*Pp Cultures.* Groups of mice were necropsied at 1, 2, and 3 mo after intranasal inoculation. Nasopharyngeal washes were obtained and plated on blood agar media, and growth was documented after 24 h of culture.

*Statistics.* Results were expressed as the arithmetic mean ± SE of the mean. Two-tailed Student's *t* tests (for comparison of unpaired samples) were performed, and *p* values $\leq 0.05$ were considered to be significant.

## Results

*Synergistic Effect of PVM on PCP.* All 26 C3H-*scid* mice inoculated with PVM died or were necropsied when morbidly ill 25–28 d after infection (dpi) (Fig. 1 *A*). No morbidity was exhibited by any of the noninoculated *scid* mice or by treated or untreated C3H-+/+ mice. Histological examination revealed that morbidity was associated with pulmonary pathology typical of PCP (8). Characteristic alveoli-filling foamy matrix comprised primarily of *Pc* organisms, activated macrophages, remnants of phagocytic cells, and proteinaceous exudate was found to be extensive in distribution and density. Thickening of alveolar interstitium was also pronounced. The extent of pulmonary pathology (Fig. 1 *B*) was significantly greater in PVM-treated *scid* mice than in their noninoculated counterparts. Neither PVM-treated nor untreated C3H-+/+ mice had significant pulmonary pathology, although some of the PVM-treated +/+ mice did exhibit occasional intraalveolar inflammatory cells, minor focal hyperemia, and hemorrhage characteristic of PVM infection (12, 17). Mutant *scid* mice inoculated with PVM had nearly five times greater density of *Pc* cysts than noninoculated control *scid* mice (Fig. 1 *C*). As expected, no *Pc* cysts were observed in either PVM-inoculated or noninoculated C3H-+/+ mice.

To test the efficacy of viral inoculation, lungs were removed at necropsy (25–28 dpi) and PVM virus was titrated. Virus

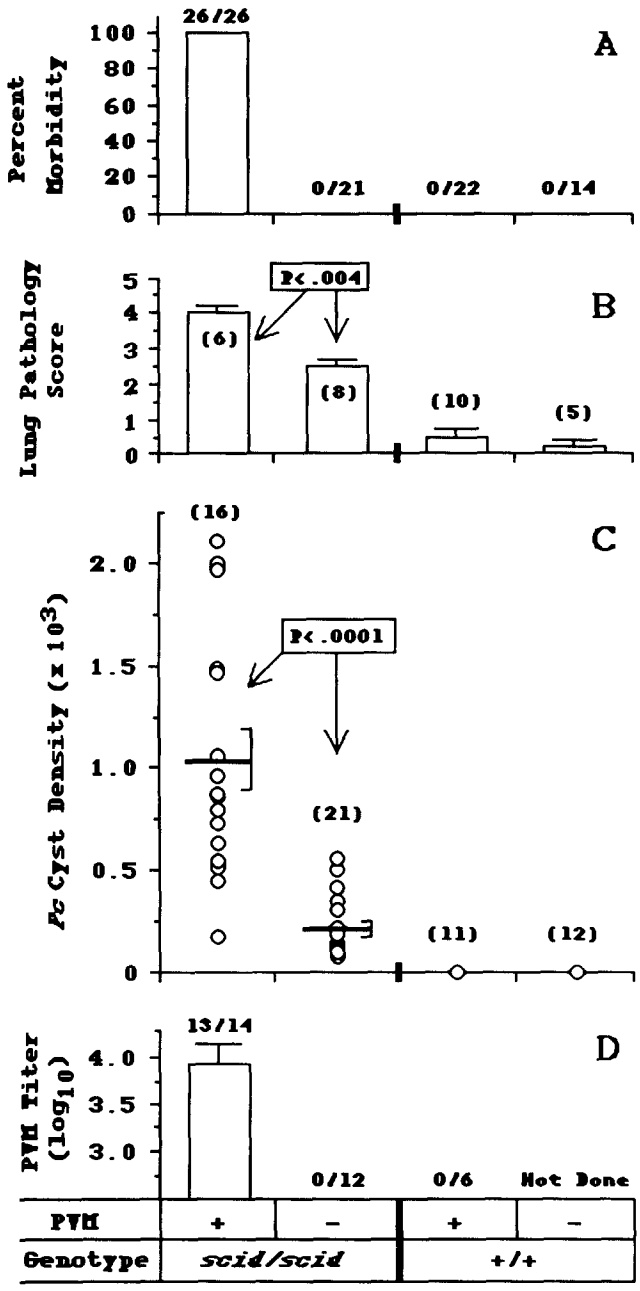


**Figure 1.** Analysis of *Pc*-related pathology and morbidity in C3H-*scid* mice after inoculation with PVM. (*A*) Percent morbidity (No. dead or moribund/total No. at risk). (*B*) Lung pathology. (*c*) *Pc* density (cysts/mm$^2$). (*D*) PVM virus recovery ($log_{10}$ FFU/gram of tissue). No. of positive > 2.5 $log_{10}$ FFU) mice/No. tested.

recoveries <2.5 $log_{10}$ FFU per gram of lung were considered negative. 13 of 14 (93%) inoculated *scid* mice were positive (Fig. 1 *D*), whereas none of the noninoculated *scid* mice or the inoculated +/+ mice was positive, indicating that: (*a*) *scid* mice were free of PVM before experimental infection; and (*b*) wild-type mice, but not the immunodeficient *scid* mice, were able to clear PVM. Viral serology was also conducted. All of 22 inoculated +/+ mice were seropositive whereas none of 14 noninoculated +/+ mice and none of 14 inoculated *scid* mice was positive. These data document lack of prior infection and successful intranasal inoculation. Overall, these findings demonstrate that PVM directly increases infection and morbidity by *Pc* organisms.

As demonstrated by microscopy and image analysis, PVM and *Pc* in dually infected *scid* lungs were distributed independently of each other at the microanatomical level. PMV was detected primarily within the cytoplasm of alveolar and bronchiolar epithelial cells, whereas *Pc* was found extracellularly but in close proximity to the alveolar epithelial lining. Clusters of *Pc* and PVM-containing cells were diffusely scattered throughout the pulmonary parenchyma but in general did not colocalize (Fig. 2). PVM could not be detected in the lungs of *scid* mice that were not deliberately infected.

*Microorganisms Not Producing a Synergistic Effect on PCP.* Four other pneumotropic microorganisms besides PVM were evaluated for possible synergism with *Pc*. There was no evidence of exacerbation of PCP in any of the following dually infected *scid* mice (Table 1).

*MHV-Y.* Morbidity signs were exhibited by all 25 inoculated C3H-*scid* mice allowed to age and survival ranged from 31 to 59 dpi. This morbidity was not related to heightened presence of *Pc* organisms as both cyst density (Table 1) and histopathology associated with PCP (data not shown) were similar among infected and noninfected *scid* mice. Only one of four noninfected *scid* mice became moribund (at 34 dpi); the remaining thrifty mice were necropsied at 45 dpi. Serological tests of C3H-+/+ mice confirmed the success of intranasal MHV-Y infection. As expected, *scid* mice failed to generate detectable antibody.

*RSV.* RSV did not significantly exacerbate *Pc* cyst density, pathogenicity, or morbidity from *Pc* in BALB/c-*scid* mice. 6 out of 16 RSV-inoculated and 2 out of 4 noninoculated *scid* mice became ill and were necropsied between 33 and 55 dpi. Serological tests of BALB/c-+/+ mice confirmed the success of the RSV inoculation.

*SV.* All 20 *scid* and eight +/+ mice inoculated with SV became morbidly ill by 12 dpi; all noninoculated control mice remained thrifty and were necropsied in parallel. The 19 *scid* mice tested had recoverable SV 7–12 dpi with titers 5.1–5.5 $log_{10}$ FFU. Two of the inoculated +/+ mice were virus positive (4.8 ± 0.8 $log_{10}$ FFU) at 7 dpi; one +/+ assayed at 12 dpi was virus negative. There was no significant difference in the density of *Pc* cysts (Table 1) or in PCP-associated pulmonary histopathology (data not shown) between SV-infected and uninfected *scid* mice. Because of the rapidity of disease development after this dose of SV, there may have been insufficient time to alter the pathogenesis of PCP.

*Pp.* By 30 dpi, 4 of 24 *Pp*-inoculated *scid* mice, but none of 11 noninoculated *scid* and none of 8 inoculated +/+ mice, had died. This slightly enhanced morbidity was not associated with increased presence of *Pc* (Table 1) or characteristic PCP histopathology (data not shown). *Pp* was cultivated from nasopharyngeal washes from one of nine uninoculated and approximately three fourths of inoculated *scid* mice ($\chi^2 = 6.69$; $p < .01$). Although not exacerbating PCP, the increased morbidity found in *Pp*-inoculated *scid* mice may par-

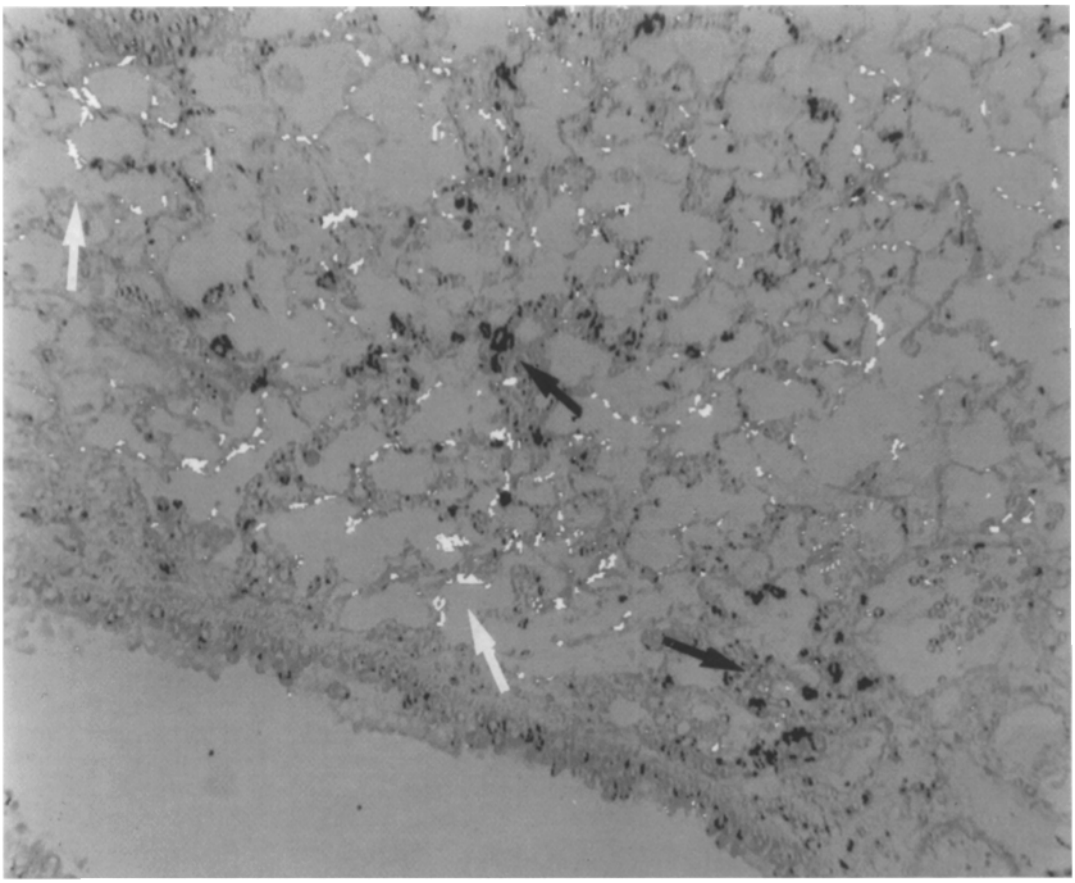

**Figure 2.** Composite computer image of adjacent serial sections of the lung of a dually infected *scid* mouse immunostained with anti-PVM (*black clusters* and *arrows*) or anti-*Pc* (*white clusters* and *arrows*) (×260).

tially explain the reduced longevity of *scid* mice in the conventional versus barrier facilities described in the introduction.

## Discussion

The findings presented here constitute the first evidence of a fatal synergism between a coinfective pneumotropic microorganism and *Pc*. PVM, alone among the microorganisms tested, caused an increase in the density of *Pc* organisms, an increase in the severity of histopathologically defined PCP, and earlier morbidity than those of *scid* mice not coinfected. The uniqueness of PVM's influence is suggested by the observations that MHV-Y, SV, and *Pp* were infective and caused morbidity but did not increase the density of *Pc* or augment the severity of PCP. Coinfection with human RSV was not detrimental to *scid* mice. That *scid* mice (deficient in both cell-mediated and humoral immunity) were readily infected with PVM (considered an apathogenic virus) is not surprising based on earlier studies of the respiratory disease produced in T cell–deficient nude (*nu*) mice singularly challenged with PVM (12). In contrast with this fatal synergism of PVM and *Pc*, Pesanti (18) described a protective effect of preexisting *Pseudomonas* pneumonitis on the development of glucocorticoid-induced PCP.

Some histopathological hallmarks of both PVM and *Pc* infection are similar (interstitial pneumonitis with a preponderance of macrophages), yet each has unique characteristics. PVM infection of an immunocompromised host has marked intraalveolar hemorrhage and substantial fibrin deposition, whereas PCP typically has an extensive intraalveolar accumulation of trophozoite-rich foamy matrix. It should be emphasized that all dually infected *scid* mice necropsied had typical PCP pathology. The hallmarks of PVM infection were seen only in diminished form in less than a quarter of the cases.

At present, we can only speculate regarding the possible mechanisms underlying the synergism of PVM and *Pc*. We demonstrated that PVM and *Pc* were diffusely scattered throughout the lung parenchyma, but only infrequently colocalized. Thus, a contact-dependent effect of PVM on enhancing the proliferation of *Pc* organisms seems unlikely. The possibility that PVM could sufficiently alter the biochemical microenvironment (pH, metabolic factors, etc.) to the benefit of *Pc* should be considered. Alternatively, PVM may detrimentally affect the number or function of alveolar macrophages, the principle means of controlling *Pc* organisms in *scid* mice. Since macrophages are dominant in the inflammatory responses to both *Pc* and PVM, infection by PVM may saturate the capacity for production, immigration, or activation of these phagocytes, resulting in increased numbers of *Pc*. Also, as morbidity in PCP seems to be related to pulmonary

**Table 1.** *Morbidity and Pc Levels in scid Mice after Inoculation of Various Murine Pathogenic Microorganisms*

| Organism | Recipients | Inoculated | Infection | Morbidity | *Pc* Cysts | Probability |
|---|---|---|---|---|---|---|
| MHV-Y | *scid* | + | 0% (19) S* | 100% (25)‡ | 118 ± 37 (21)§ | >.5 (NS)‖ |
| | | − | 0% (3) | 25% (4) | 108 ± 15 (3) | |
| | +/+ | + | 86% (7) | 12.5% (8) | 0.9 ± .6 (7) | |
| | | − | 0% (3) | 0% (3) | 0.7 ± .7 (3) | |
| RSV | *scid* | + | 6% (16) S | 38% (16) | 339 ± 41 (16) | <.2 (NS) |
| | | − | 0% (4) | 50% (4) | 625 ± 334 (4) | |
| | +/+ | + | 83% (6) | 0% (6) | 0 | |
| | | − | 0% (2) | 0% (2) | 0 | |
| SV | *scid* | + | 100% (19) C | 100% (20) | 503 ± 84 (19) | <.4 (NS) |
| | | − | 0% (4) | 0% (22) | 409 ± 72 (22) | |
| | +/+ | + | 67% (3) | 100% (8) | 0.6 ± .6 (3) | |
| | | − | ND | 0% (9) | 0 (6) | |
| *Pp* | *scid* | + | 72% (18) C | 17% (24) | 212 ± 44 (19) | <.4 (NS) |
| | | − | 11% (9) | 0% (11) | 303 ± 86 (9) | |
| | +/+ | + | 42% (12) | 0% (8) | 1 ± 1 (12) | |
| | | − | 0% (8) | 0% (6) | 0.3 ± .3 (8) | |

* Percent infected (No. tested) based on serology (S) or culture (C).
‡ Percent mice dead (No. at risk).
§ *Pc* cysts/mm$^2$ (mean ± SEM [*n*]).
‖ Probability (Student's *t* test).

insufficiency associated with both thickening of the alveolar septa as well as dense alveolar obstruction (organisms, desquamated epithelia, and active and remnant phagocytic cells), any factor causing additional recruitment, proliferation, and activation of macrophages may be detrimental to the host. In support of this scenario, we have previously shown that *scid* mice have heightened morbidity due to the hyperresponsiveness of macrophages after the transfer of immunocompetent *Pc*-sensitized CD4$^+$ T cells (10). Finally, the nonspecific host response to PVM may lead to an altered balance of cytokines critical to the anti-*Pc* response. For example, TNF-$\alpha$ produced by alveolar macrophages has been reported to play a major role in the control of PCP in AIDS patients (19).

Human (and bovine) RSV (HRSV) and PVM are classified by morphological criteria as unique members of the genus *Pneumovirus* (20) but are reported to either share (21) or not share (22) antigenic surface determinants. Recent molecular studies have shown weak homology between proteins coded by genes 1 and 2 of PVM and analogous genes 1C and 1B of HRSV (23). Thus, the fatal synergism of PVM and *Pc* described here may or may not be predictive of similar consequences in HRSV-*Pc* dual infections in immunodeficient (e.g., AIDS) patients.

In summary, these results support the broad notion that coinfection by secondary microorganisms may have significant clinical relevance in terms of diagnosis, prognosis, and therapy of primary (e.g., *Pc*) opportunistic infection. Further, this experimental model may be exploitable for determining the fundamental mechanism(s) and effective therapies for such detrimental synergy.

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