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Spontaneous *Pneumocystis carinii* Pneumonia in Immunodeficient Mutant *scid* Mice

Natural History and Pathobiology

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The opportunistic pathogen **Pneumocystis carinii** ***(Pc) poses a major clinical health problem in individuals with immune deficiency, including those patients with human immunodeficiency (HIV)-associated acquired immune deficiency disease (AIDS). Heretofore,*** **in vivo** ***investigations of the biology of Pc and pathogenesis of pneumocystosis have generally employed steroid-induced immune suppression with antibiotic prophylaxis and protein deprivation. This approach has many drawbacks, chief among them being the widespread, multiple interacting effects caused by the inducing agents. Athymic (nude) mice and rats have been used, but are less than ideal, as the immune defect primarily affects T lymphocytes. This article describes the natural history, pathobiology, and environmental effects on Pc pneumonitis in nonaxenically housed mice homozygous for the autosomal recessive mutation 'severe combined immunodeficiency' (*** **scid** ***), which almost totally lack both cell-mediated and antibody-mediated immune functions. The predictability, unequivocal expression, high morbidity, and well-defined genetic basis make*** **scid/scid** ***mutant mice the model of choice for*** **in vivo** ***studies of spontaneous pneumocystosis. (Am J Pathol 1990, 136:1173–1186)***

In man, *Pneumocystis carinii* (Pc) represents a significant international health problem usually presenting clinically as pneumonia in immunologically compromised individuals.[1] Before 1980, the majority of cases of Pc pneumonitis were identified in children with protein-calorie malnutrition[2]; patients with primary diseases such as leukemia or other malignancies[3]; genetically caused immune deficiency disorders[4]; and organ transplant recipients, in whom immunosuppressive radiation or drug therapy are thought to be key determinants in development of Pc pneumonitis.[5] Since 1980 the majority of Pc pneumonitis cases have been reported in patients with human immunodeficiency virus (HIV)-associated acquired immune deficiency syndrome (AIDS).[1] Moreover, Pc is the most common opportunistic infection and is the major diagnosed cause of morbidity in patients with AIDS.[6]

Pneumocytis carinii, a eukaryotic obligate parasitic microorganism, has been classified as either a protozoan or a fungus based on morphology, life cycle, and drug sensitivity characteristics.[1] A recent phylogenetic study based on 16S ribosomal RNA sequences provided strong evidence that Pc is a member of the fungi.[7] Based on findings of serum anti-Pc antibody, it has been estimated that nearly all healthy individuals have had latent Pc infection during the first 2 years of life.[8] It is still unknown whether overt Pc pneumonia is a result of activation of latent infection, primary infection, or reinfection. The host is thought to acquire Pc from the environment by an airborne route, yet the infective stage has not been identified.[9] *Pneumocystis carinii* has been found in other mammalian species. Studies using rat monoclonal anti-*Pneumocystis* antibodies have indicated, based on antigenic differences, that there are host-specific species of Pc.[10] Based on findings that oligonucleotides prepared to rat Pc 16S rRNA recognize human Pc, Edman et al[7] suggested that human and rat Pc are closely related.

In vitro cultivation of Pc, most commonly using embryonic chick epithelial lung (CEL) cells, is difficult, and Pc can not be separated from host cell fragments.[1,11] Most studies of Pc and its relationship with the host have focused on cortisone-treated rats.[12,13] Spontaneous pneumocystosis has been identified in athymic rats (mutation:

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Rowett nude, *rnu*)[14] and athymic mice (mutation: nude, *nu*).[15] Homozygosity at the *nu* locus causes a failure of normal thymic development, and consequently a deficiency in T-cell–dependent immune functions. Walzer et al[16] recently described outbreaks of *pneumocystis* pneumonia in both *nu/nu* and *scid/scid* mice, mice homozygous for the autosomal recessive mutation 'severe combined immunodeficiency' (*scid*), at several institutions.

Mice homozygous for the autosomal recessive mutation *scid* are deficient for both B- and T-lymphocyte function. Homozygous *scid/scid* mice (hereafter referred to as *scid* mice) have small, sparsely populated thymuses, yet some large thymocytes express high levels of the Thy-1 surface antigen,[17] and spontaneous Thy-1^+ lymphomas presumably arising from thymic T cells have been described.[18] Lymph nodes and splenic lymphoid follicles also are dysplastic. Functional B cells are essentially nonexistent, yet normal numbers of B cell precursors (transformable by Abelson murine leukemia virus) are present.[19] Homozygous *scid* mice are hypogammaglobulinemic, do not produce specific antibody against several T-independent antigens, and fail to reject skin allografts.[17] Bosma et al[20] have described as "leaky" a class of *scid* mice with elevated serum immunoglobulins. Approximately 14% of 3- to 4-month-old *scid* mice had serum Ig-*kappa* concentrations of 0.1 to 6.4 mg/ml; the remaining 86% of *scid* mice had levels of serum Ig-*kappa* < 0.01 mg/ml.

Based on bone marrow reconstitution studies, Dorshkind et al[21] concluded that the lymphoid microenvironment of *scid* mice is conducive to lymphocyte differentiation and that the *scid* mutation specifically impairs the differentiation of stem cells into mature lymphocytes. Recent reports have suggested that the *scid* mutation may adversely affect the recombinase system, resulting in abnormal *Igh* and *TCRβ* gene rearrangements.[22,23] The immune defects of *scid* mice do not include cells of the myeloid lineage[21] or natural killer (NK) cells,[24,25] and the function of splenic antigen-presenting cells (APC) in *scid* mice is qualitatively and quantitatively similar to that in control mice.[26]

The *scid* mutation was first identified in the BALB/c congenic inbred mouse strain C.B-17 (BALB/c.C57BL/Ka-*Igh-1*^{b}/Icr) at the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA.[17] The mutation *scid* has recently been mapped to chromosome 16 with marker genes *md* and *Igl-1*.[27] In our laboratory, the *scid* mutation has been maintained on this inbred strain and also has been transferred to several other strains, including AKR/J, BALB/cByJ, C3H/HeSnJ, and C57BL/6J. The immunologic and pathologic phenotypes associated with this mutation do not appear to be greatly altered by background genomic differences.

This paper describes the natural history and pathobiology of pneumocystosis in *scid* mice. All mice homozygous for the *scid* mutation, bred and raised under the nonaxenic conditions described herein, spontaneously develop Pc pneumonia. The predictability, unequivocal expression, high morbidity, and well-defined genetic basis make *scid* mice the experimental model of choice for *in vivo* studies of spontaneous Pc pneumonitis. As an indication of the utility of this model system for further experimental studies, the effects of immunologic reconstitution of *scid* mice on spontaneous pneumocystosis are reported.

Methods

Mice

The C.B-17/Icr-*scid/scid* (referred to as *scid* mice) and congenic C.B-17/Icr-+/+ control mice were obtained from the breeding colony of the Institute for Cancer Research, Philadelphia, PA. They have since been maintained in both barrier and conventional research mouse colonies at The Jackson Laboratory. The congenic strains AKR/J-*scid,* BALB/cByJ-*scid,* C3H/HeSnJ-*scid,* and C57BL/6J-*scid* were derived by 10, 6, 10, and 10 backcrosses of C.B-17-*scid* mice to AKR/J, BALB/cByJ, C3H/HeSnJ, and C57BL/6J, respectively, followed by intercrossing and continued breeding of *scid* homozygotes. Conventionally housed mice received Old Guilford #96W pelleted chow (not pasteurized) (Emory Morse Co., Old Guilford, CT) and chlorinated water *ad libitum,* and were housed in polycarbonate boxes (1 to 5 mice per 5- × 6- × 11-inch) covered with rectangular sheets of Lexon filter material. Sterilized pine bedding was provided. During the time of this study, conventionally housed mice were tested and shown to be free of the following microorganisms: *Citrobacter spp, Pseudomonas aeruginosa, Salmonella spp, Staphylococcus aureus, Klebsiella spp, Bordetella bronchiseptica, Corynebacterium kutcheri, Streptobacillus moniliformis, Pasteurella multocida*, and *Mycoplasma spp.* These mice also were free of ectoparasites and endoparasites and 14 species of murine viruses, including Pneumonia Virus of Mice (PVM). Barrier-housed mice were fed Wayne Sterilizable Rodent Blocks (Allied Mills, Chicago, IL), and were provided acidified water (supplemented with vitamin K) *ad libitum.* Filter bonnets were used in lieu of Lexon filter covers. Mice introduced into this facility were caesarian-derived and foster nursed on mice known to be free of the above organisms. *Proteus spp* was present only in the conventional facility during the time of this study. *Pasteurella pneumotropica* was present in the conventional room and intermittently present in the barrier room.

Pneumocystis carinii organisms, while easily identified histologically in *scid* mice, are found only extremely rarely in any of the immunologically competent mice housed in either facility. However, in studies in progress using an enzyme-linked immunosorbent assay (ELISA) system (see below), we have identified significant titers of naturally acquired anti-Pc antibody in sera of immunocompetent mice (data not shown). *Candida spp* and other fungi were not found in Grocott's methenamine silver (GMS)-stained sections of *scid* lungs.

An assessment of the overall health of *scid* mice was made before necropsy. Clinical scores ranged from 0 (no apparent illness) to 4 (moribund). In general, the following attributes were used in assignment of these scores: 1: 10% to 15% reduction of control body weight (BW), reduced exploratory activity, unkempt appearance of coat; 2: 20% to 25% reduction of BW, coat extremely ruffled, modest kyphotic posture, head carried low; 3: 30% to 35% reduction of BW, extreme kyphotic posture, frank dyspnea, motor activity extremely reduced; 4: 40% to 50% reduction of BW, dyspnea may be paroxysmal, lack of motor activity when prodded, cyanosis often observed (easily judged in albino inbred strains such as C.B-17 by examination of the pinna and volar aspects of the fore and hind feet).

Packed Erythrocyte Volumes

Hematocrit percent (Hct%) was determined using a Clay-Adams Micro-Hematocrit Reader (Clay-Adams Co., Parsipany, NJ) after a 5-minute centrifugation (using an IEC Model MB centrifuge; International Equipment Co., Boston, MA) of standard 75-mm heparinized micro hematocrit capillary tubes. Individual mice were routinely bled from the retro-orbital sinus during the same time of day (8:00 A.M. to 11:00 A.M.).

Histopathology

At necropsy, all tissues were fixed for approximately 24 hours in Bouin's solution, transferred to 70% ethanol, trimmed, embedded in paraffin, and sectioned at 5 μm. Lungs were not inflated at necropsy. Serial lung sections were routinely stained with 1) hematoxylin and eosin (H & E), 2) Grocott's methenamine silver (GMS) and light green (LG), or 3) Giemsa. Other tissues were generally stained with H & E only. For quantitation of Pc cysts, the left lung was fixed, and 5-μm sections were cut at the midpoint and perpendicular to the long axis. Sections were stained with GMS-LG. The density of GMS staining was monitored for consistency by resectioning and staining a control heavily Pc-infected lung. A Leitz Orthoplan microscope (Ernst Leitz, Wetzlar, GMBH) equipped with a 63× plano objective and 10× widefield eyepieces fitted with a large (0.0187 mm^2) rectangular reticle was used. All longitudinally contiguous rectangular fields were examined and unambiguous cysts counted. Alternate left–right shifting on the horizontal plane was used when encountering areas with significant (≥10%) nonparenchymal tissue (bronchioles, large vessels, etc.) Typically, 25 to 35 fields per case were examined (0.47 to 0.65 mm^2 = total area scanned), and the cyst density (cysts per mm^2) was calculated.

Bone Marrow Reconstitution

Cells were flushed from femurs and tibias of donor mice with Earle's balanced salt solution (EBSS; Gibco, Grand Island, NY) supplemented with 2% fetal bovine serum (FBS). Cells were washed twice in EBSS-2% FBS and resuspended in 0.9% NaCl at a concentration of 1.4×10^7 viable cells per milliliter. Recipient *scid* mice were injected intravenously (tail vein) with 3.5×10^6 cells in 0.25 ml. As indicated in earlier reports,[21] functional donor-derived B and T cells were detectable in *scid* mice reconstituted with normal bone marrow cells. Irradiation of the recipients was not necessary for such reconstitution.

Quantitation of Serum IgM levels by ELISA

Standard ELISA methods, using 96-well polystyrene microtitration plates, were used.[28] The principal reagents were used in the following sequence: 1 μg/well coating of goat anti-mouse IgM immunoglobulin (Southern Biotechnology, Birmingham, AL); 200 μl/well of blocking solution of 1% bovine serum albumin in phosphate-buffered saline (PBS); 50 μl/well of appropriately diluted test serum samples or purified myeloma-derived IgM immunoglobulin standards (TEPC 183, Litton Bionetics, Kensington, MD); 50 μl/well of a 1/500 dilution of alkaline phosphatase-conjugated goat immunoglobulin against mouse kappa light chain (Southern Biotechnology); and 50 μl/well of a 1 mg/ml solution of *p*-Nitrophenyl phosphate substrate. After the final incubation, the absorbance (at 405 nm) of each well was measured and the absolute levels of IgM were determined by linear interpolation between points on the standard curve.

Statistical Analysis

Results were expressed as the arithmetic mean ± standard error of the mean. Two-tailed Student's *t*-tests (for

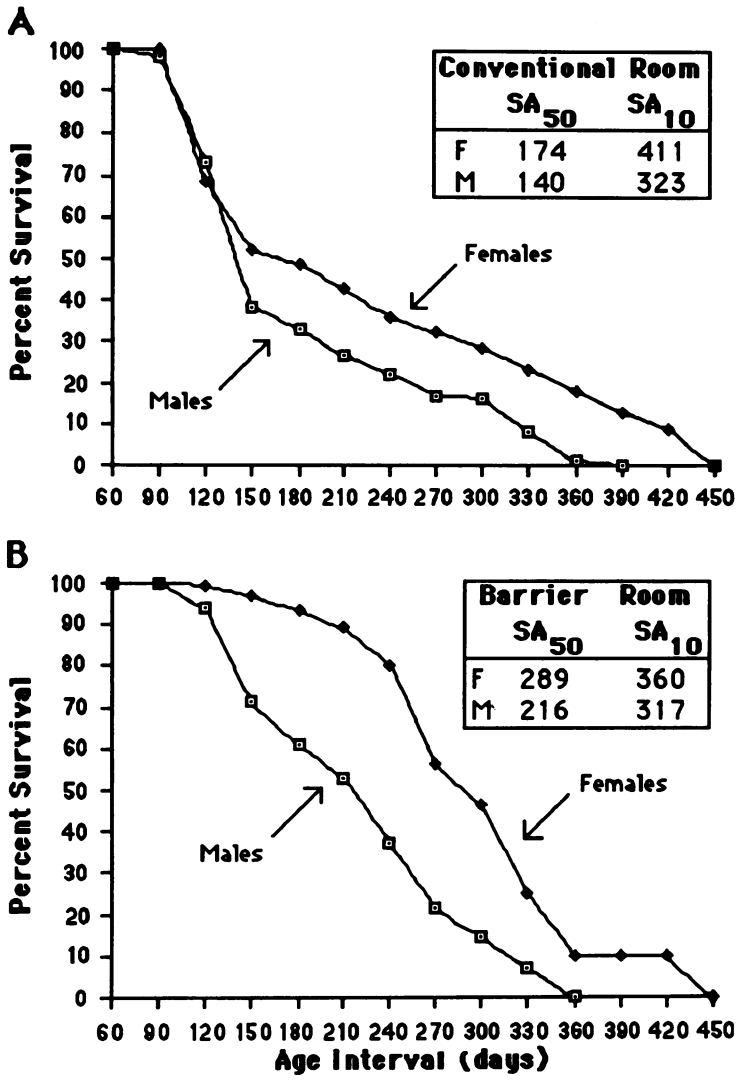

Figure 1. *Cumulative percentage of survival of C.B-17-*scid *mice maintained in either a* A: *conventional or* B: *barrier mouse room. The survival age (SA) at which 50% and 10% of the female and male mice remained alive is indicated. The number of mice at risk is noted in Results.*

comparison of unpaired samples) were performed. *P* values ≤ 0.05 were considered to be significant.

Results

Longevity

Survival data was acquired from populations of *scid* breeders and offspring mice specifically held for lifespan analysis and pathology. During these studies, 535 C.B-17-*scid* mice in all were followed: 89 male and 121 female C.B-17-*scid* mice from the barrier facility and 149 male and 176 females from the conventional animal room. Because some mice were intentionally diverted from the aging population to specific experiments, an established interval technique[29] based on 'adjusted populations at risk' was used to eliminate the effect of diverting animals from the study group and to describe the cumulative percent survival of these mouse stocks (Figure 1). From this data the ages (survival ages, SA) at which 50% and 10% of the individuals from each group were still living (SA_{50}, SA_{10}) were determined. *Scid* mice from conventional quarters had a dramatic reduction in median lifespan (35% to 40% lower SA_{50}s) compared with those mice housed in the barrier facility. Male *scid* mice from both barrier and conventional animal facilities died earlier than corresponding female mice. As described in more detail below, 20 ill *scid* mice underwent comprehensive necropsy (Table 1), and the lungs of 31 others also were examined histologically during the course of these studies, and death was attributed to pulmonary insufficiency due to interstitial pneumonitis characteristic of *P. carinii* infection.

For comparison, 17 male and 26 female C.B-17-+/+ control mice were set aside in conventional animal quarters for lifespan analysis and pathology. At the time of this writing, the age range of this population is from 389 to 486 days of age, and only three (6.5%) of these 43 control mice have died so far.

Table 1. *Pulmonary Histopathology and Quantitation of* Pneumocystis carinii *Cysts in C.B-17-*scid *mice* *

Mouse number	Sex	Body weight†	Pc cyst density‡	Foamy exudate§	Mononuclear inflammation§	PMN inflammation		Multinucleated giant cells¶	Bronchiolar free cells#**	Alveolar D. epithelial cells#
						Distribution	Extent‖			
20	F	11.7	237	++++	++++	Diffuse	++	+	+++ (M)	++
2	F	12.3	93	++++	+++	Diffuse	++	−	−	++
19	F	13.4	65	++++	++++	Diffuse	++++	++	++++ (M, P, E)	++
1	F	13.7	254	+++++	+++	Multifocal	++	−	−	++
17	F	14.0	155	++++	++++	Diffuse	++++	++++	+++ (P, E)	+++
8	F	14.3	43	+	++	Diffuse	+++	−	++ (E)	++
18	F	14.4	38	+++	++++	Diffuse	++++	+++	+++ (P, E, R)	+++
5	F	14.7	18	++	++	Diffuse	++	−	NA	−
7	F	15.7	109	++	++	Diffuse	+++	++	−	+++
16	F	18.5	172	+++	+++	Diffuse	+++	++++	+++ (E)	+++
6	F	18.7	11	+++	++	Diffuse	+++	+	+	+++
4	M	15.4	610	++++	++	Diffuse	+++	−	+	++
3	M	16.3	544	+++	++	Diffuse	+++	+	+++ (E)	++
9	M	19.9	136	+++	++	Multifocal	++	−	++ (E, P, R)	++
10	M	23.1	247	+	++	Diffuse	++	−	−	++
15	M	27.3	570	+	+		+	−	+	++
14	M	27.6	413	++	++	Diffuse	++	−	++ (E)	++
13	M	27.7	314	+	++		+	−	−	++
12	M	27.9	562	+++	+++	Multifocal	++	−	++ (E)	++
11	M	29.5	337	+	+		−	−	−	++

* All mice between 105 and 140 days of age and reared in conventional animal room.
† Body weight in grams.
‡ PC cyst density. Counts of cysts in GMS-stained 5 μm sections with 63× objective (per mm²).
§ Foamy matrix − (none), + (trace: 1–10% alveoli), ++ (slight: 11–25%), +++ (moderate: 26–50%), ++++ (heavy: 51–75%), +++++ (severe: >75%).
‖ Inflammatory cells, − (none), + (rare: <5 per 40× field), ++ (occasional: 6–20), +++ (moderate: 21–50), ++++ (abundant: >50).
¶ Multinucleated giant cells. − (none), + (rare: 1–2 per section), ++ (occasional: 3–6, +++ (moderate: 7–10), ++++ (abundant: >10).
# Presence of free cells in bronchioles or desquamated epithelial cells in alveoli. − (none), + (rare), ++ (occasional), +++ (moderate), ++++ (abundant).
** Free cell types. M (macrophages), P (polymorphonuclear leukocytes), E (desquamated epithelial cells), R (erythrocytes). NA (no bronchioles in section).

Reduced longevity also was documented for *scid* homozygotes on the inbred strain backgrounds AKR/J, BALB/cBy, C3H/HeSnJ, and C57BL/6J as compared with congenic +/+ controls (data not shown). For example, the SA_{50}s of 16 male and 31 female C3H/HeSnJ-*scid* mice followed for lifespan in the barrier animal facility were 169 and 225 days, respectively, as compared with average lifespans of congenic C3H/HeSnJ-+/+ mice older than 2 years.

Clinical Observations

A series of 28 C.B-17-*scid* mice maintained in a conventional mouse room was necropsied for purposes of *P. carinii* isolation, cyst quantitation, and histopathologic examination. The mice selected had an age range (92 to 168 days) embracing the median lifespan indicated above. Clinical scores were assigned as described in Materials and Methods.

Body weight

By far the most obvious sign of morbidity was loss of body weight. Female and male *scid* mice were 0.4 and 0.3 times as heavy, respectively, as their similarly aged congenic normal controls (Table 2).

Lung Weights

The lungs of clinically ill (grades 3 to 4) *scid* mice were strikingly enlarged and had a grayish, often mottled appearance. The lungs of 14 of the above 28 *scid* mice were weighed. For comparison, the lungs from a total of 20 C.B-17-+/+ control mice were removed and weighed (in

Table 2. *Body and Lung Weight in C.B-17-*scid *and Control Mice*

Genotype	Sex	Body weight (g)	Lung weight (mg)
scid/scid	F	15.3 ± 0.7 [15]*	451 ± 28 [9]
	M	21.9 ± 1.6 [13] (92–168 d)†	356 ± 57 [5] (92–147 d)
+/+	F	24.9 ± 0.6 [7]	127–140‡
	M	30.4 ± 1.0 [7] (119–154 d)	(51–179 d)

* Mean weight ± SE [no. of mice].
† Age range of group in days.
‡ Lung weights of control mice (a total of 10 females and 10 males) were determined by mass weighings on three separate occasions. The three average values were 127, 135, and 140 mg.

three mass weighings). The lungs of the *scid* homozygotes were more than 2.5 times as heavy as those of similarly aged C.B-17-+/+ controls (Table 2).

Packed Erythrocyte Volumes

Moribund *scid* mice had a cyanotic appearance, and their whole blood was markedly darker and more viscous than that of control mice. Because secondary polycythemia often is associated with respiratory diseases affecting alveolocapillary exchange of oxygen, 27 male and female C.B-17-*scid* mice between 51 and 360 days of age from the conventional animal room were bled from the retro-orbital sinus, and packed erythrocyte volumes (hematocrit percent, Hct%) were determined. The Hct% of these mice was elevated from near normal to polycythemic levels. As indicated in Figure 2, all *scid* mice with clinical scores greater than 0 (ie, with visual signs associated with pneumocystosis) had significantly ($P < 0.01$) elevated hematocrit levels, with the extent of elevation correlated positively with clinical score. Moribund (group 4) *scid* mice had an average hematocrit of 62.8% ± 1.3%, a striking increase ($P < 0.001$) compared with the average Hct of 50.2% ± 0.4% in a group of 23 congenic +/+ controls.

Histopathology

Pulmonary Histopathology

In general, the pulmonary lesion of *scid* mice was similar to pneumocystosis as described in patients with immunodeficiency diseases and in cortisone-induced immunodeficiency states in laboratory animals. The hallmarks of this lesion included: masses of basophilic staining *P. carinii* trophozoites enmeshed in an eosinophilic foamy matrix of coalesced macrophages and exudate filling alveolar spaces; thickened alveolar septa with prolific hypertrophic alveolar epithelial cells and infiltrations of macrophages; and on GMS-stained sections, single or clustered Pc cysts. Usually, the earliest pathologic changes (scant trophozoite-laden foamy matrix with occasional free macrophages) occurred as small isolated subpleural foci. In advanced lesions, consolidation was either focal or diffuse, but never involved entire lobes (85% to 90% of pulmonary parenchyma was the maximum level of consolidation observed).

A complete light microscopic histopathologic analysis was conducted on a set of 20 C.B-17-*scid* mice at about the median survival age (between 105 and 140 days) of this stock (Table 1). These mice, raised in a conventional animal room, were a subset of a larger group of mice killed for preparing lung extracts containing *P. carinii* and for Pc cyst quantitation. The cardinal features of pneumocystosis as indicated in Table 1 are shown photographically in Figure 3. All *scid* mice in this set had significant detectable pneumocystosis, although based on the scoring system used, females had an increased level of both alveolar consolidation with foamy exudate (Figure 3A) and cellular inflammatory component. The predominant inflammatory cells were large, often foamy, macrophages (Figure 3B). These appeared as either free cells within alveolar spaces or as infiltrating cells in the alveolar septa. Increased numbers of large alveolar epithelial cells also contributed to the often greatly thickened interstitium (Figure 3C). There were no to very few small, well-differentiated mononuclear cells in the lesions evaluated. No peribronchial or perivascular cuffing by lymphocytes was observed. No plasma cells were identified. Polymorphonuclear leukocytes (PMN) appeared in great numbers in some individuals to none or trace levels in others. When present, they were usually diffusely distributed, generally residing in alveolar spaces (Figure 3B). There were no instances of necrotizing or pyogenic inflammation. The alveolar spaces (in portions of lung that were not consolidated) often had considerable numbers of desquamated epithelial cells. Additionally, some alveoli often contained proteinaceous or mucoid fluid exudate. Desquamated epithelium (individual cells or small sheets or clusters) as well as macrophages, PMN, and erythrocytes were identified within bronchioles (Figure 3D). Approximately two thirds of these 20 individuals had some bronchiolar free cells. Multinucleated giant cells were observed in seven of 11 females, but only one of nine males (Figure 3E). These cells were typically found in widely scattered locations within the thickened alveolar septa usually just beneath the pleural surface. Pleuritis was not observed in this series. Intra-alveolar hemorrhage and erythrophagocytosis, a common feature of PVM (pneumonia virus of mice)-infected mice,[30] was rarely observed. In GMS-stained sections, Pc cysts appeared as round bodies (approximately 4- to 5-μm diameter), staining gray/black with increasing density at the periphery (Figure 3F). Frequently, cysts were found to contain multiple (one to eight) intracystic bodies. Also, characteristic crescent-shaped excysted forms were commonly observed. The inflammatory lesions were more extensive in females compared with males, whereas males of this set had a fourfold greater density of cysts than females (415 ± 56 and 109 ± 26 cysts per mm^2, respectively [$P < 0.001$]). Among the histologic characteristics observed and scored, none of the pairs of endpoints (eg, cyst density:foamy exudate) showed a strong (correlation coefficient, $R^2 \geq 0.60$) relationship.

Nonpulmonary Histopathology

The thymus, cervical lymph nodes (and others if found), and spleen were saved at necropsy. The spleens

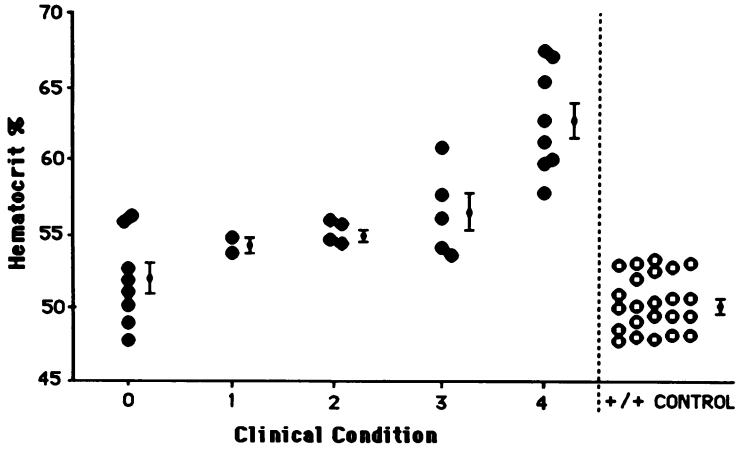

Figure 2. *Packed erythrocyte volume (hematocrit percentage) of individual male and female C.B-17-*scid *mice at various degrees (see Materials and Methods) of clinically apparent pneumocystosis, grade 0 = negative to grade 4 = moribund (closed circles). Twenty-three male and female C.B-17-+/+ controls (open circles). Mean ± SE indicated.*

had active red pulp with both extramedullary erythropoiesis and granulopoiesis evident. The thymus, lymph nodes, and splenic white pulp were atypical, with ill-defined cortices or periarteriolar lymphoid sheaths. Essentially no well-differentiated lymphocytes were evident. Macrophages and fibroblasts were loosely distributed around such landmarks as the subcapsular sinusoids of lymph nodes and central arterioles of the splenic lymphatic tissue. Among 67 *scid* mice necropsied between 82 and 281 days of age, we found one thymic lymphoma in a 239-day-old female. No other tumor was found in this series. Nonreticuloendothelial tissues saved at necropsy included salivary glands, heart and mediastinal vessels, liver, pancreas, kidney, adrenals, and occasionally upper and lower gut, esophagus, trachea, and uterus. A thorough histologic examination of these tissues from three C.B-17-*scid* mice with advanced pneumocystosis revealed no Pc cysts or other significant microscopic lesions.

Ontogeny of Pc Cyst Development

A series of 23 C.B-17-*scid* mice from 7 to 340 days of age from the barrier animal room were necropsied and evaluated for density of Pc cysts as described above. The relationship between age and cyst density is illustrated in Figure 4. No cysts were detected in the single 7-day-old female pup. In the eight *scid* mice ≤60 days of age, the mean density of Pc cysts was 14 ± 5 per mm^2. In comparison, the 15 *scid* mice over 98 days of age had an average cyst density of 171 ± 35 per mm^2 ($P < 0.005$). Clearly, the burden of Pc cysts was acquired over time, with the most remarkable increase occurring after 60 days of age. Grocott's-methenamine-silver–stained sections of lungs from 11 C.B-17-+/+ mice ranging in age from 1 to 11 months were carefully scrutinized for Pc cysts and none was found.

Effect of Normal Bone Marrow Transplantation on Pneumocystosis

Reconstitution of homozygous *scid* mice with 5 to 10 × 10^6 congenic +/+ bone marrow cells was undertaken on several occasions to provide immunologically competent *scid* breeders. The experience of such efforts was that, while approximately one third of such recipients survived and bred up to full terms approaching 1 year, the other two thirds died about 1 month after reconstitution.

To further study the pathobiology of this response to transplantation of normal marrow, a series of C3H/HeSnJ-*scid* mice were reconstituted with 5 × 10^6 congenic +/+ or *scid* bone marrow cells and then serially killed and evaluated. (The C3H/HeSnJ-*scid* mice were used in this experiment simply because this stock was abundant at the time. Similar data [not shown] have also been obtained with smaller numbers of C.B-17-*scid* and BALB/cBy-*scid* mice). Twenty-four recipients (age range, 42 to 70 days at time of cell transfer) of +/+ marrow and 15 recipients (age range, 57 to 70 days at transfer) of *scid* marrow were studied. Eight of 24 (33%) of the +/+ → *scid* group died (from 29 to 41 days after the transplant), while none of 15 of the *scid* → *scid* group died. Of the survivors, mice were killed at intervals from 23 to 49 days after transplant.

Pulmonary Pathology

+/+ *Marrow-reconstituted* scid *Mice*

As indicated in Figure 5A, at 30 days after transplantation, the lung weight of +/+ → *scid* recipients was more than twice that of *scid* → *scid* recipients. This was followed by a return to initial lung weight in +/+ → *scid* recipients by 49 days after transplant. At 30 days after transplant, the lungs of +/+ → *scid* mice had a formidable mononuclear inflammatory reaction (Table 3). There

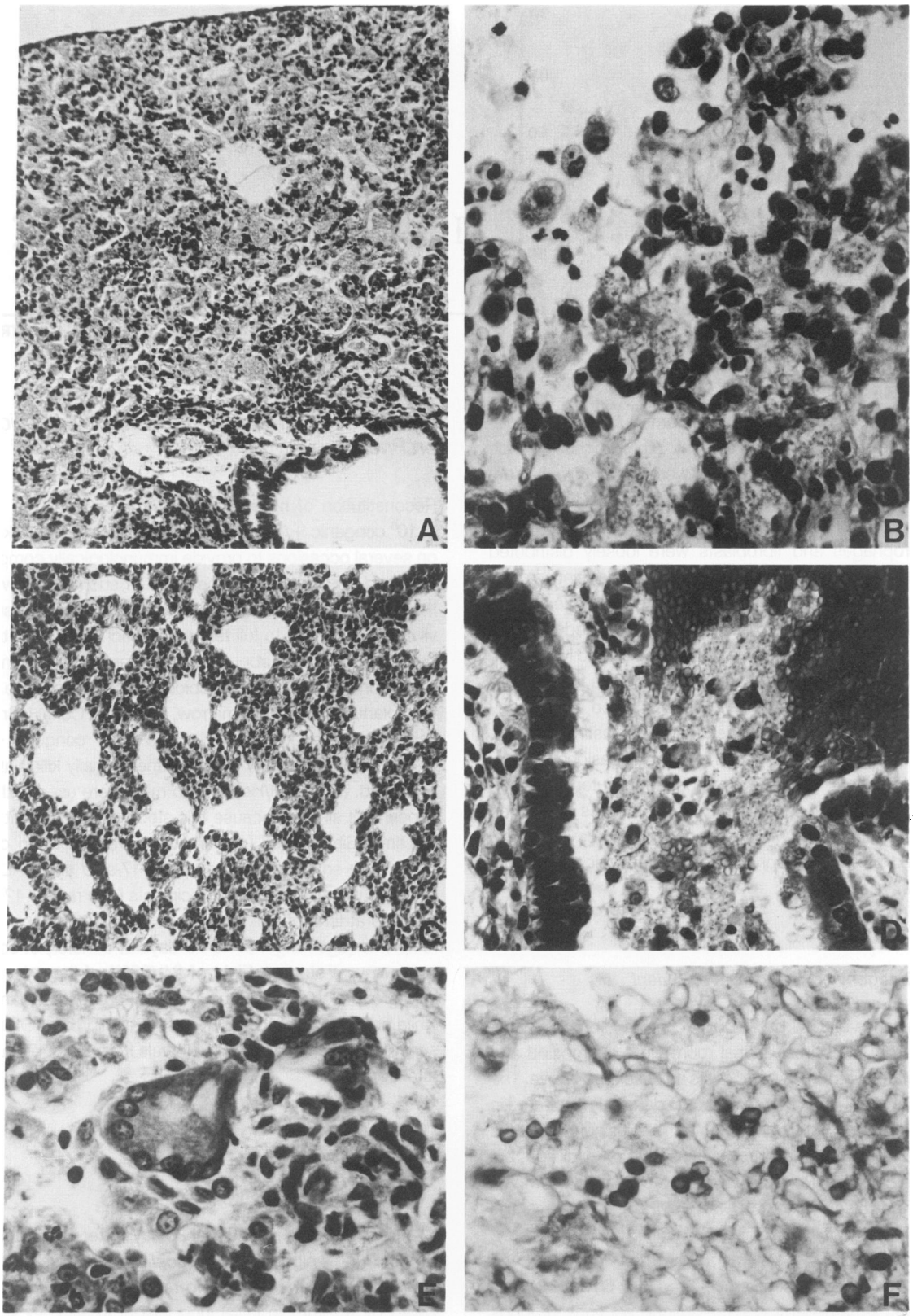

Figure 3. *Microscopic lesions associated with Pc pneumonitis in individual* scid *mice identified in Table 1.* **A:** *Female 1. Nearly complete consolidation of alveoli with Pc trophozoite-rich foamy exudate. Macrophages were widely scattered, primarily within the alveoli. Bronchioles were free of formed elements. (×34).* **B:** *Female 19. Basophilic stippling of trophozoites observed in alveoli with admixed free macrophages and polymorphonuclear leukocytes. (×134).* **C:** *Female 6. Extensive interstitial thickening composed primarily of macrophages, hyperplastic and defunct alveolar epithelial cells, with some fibroblasts. Some alveoli were completely free of Pc, giving a Swiss-cheese appearance. (×34).* **D:** *Female 17. Bronchiole with desquamated epithelium (lower right), macrophages, foamy exudate, hemorrhage, and scattered PMN. (×105).* **E:** *Female 16. Multinucleated giant cell in the midst of predominantly mononuclear inflammatory cells. (×134).* **A–E:** *Giemsa.* **F:** *Male 4. Focus of large numbers of Pc cysts. (×211). GMS-LG.*

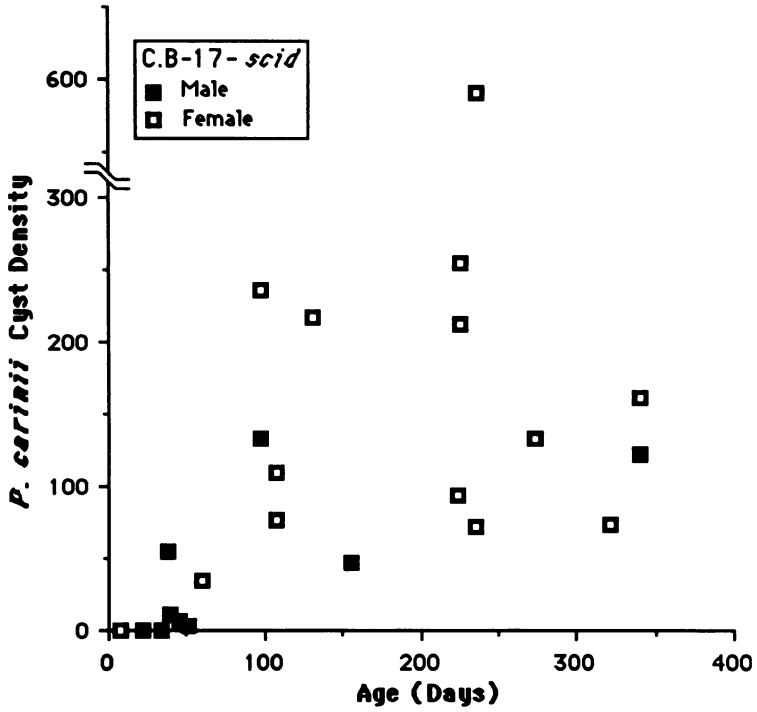

Figure 4. *Ontogeny of development of Pc cysts in C.B-17-*scid *mice. Cyst density expressed as cysts/sq mm (5 μm GMS-stained sections; see text). Closed squares, male; open squares, female.*

were some foci of typical pneumocystosis with alveolar consolidation and septal thickening (Figure 6A). However, the majority of alveoli contained massive numbers of free macrophages (Figure 6B), especially large, foamy macrophages (Figure 6C). Other alveoli were filled with edema fluid (Figure 6D). Unlike untreated *scid* homozygotes, these +/+ reconstituted *scid* mice had prominent perivascular and peribronchial infiltration by well-differentiated lymphocytes (Figure 6B). By 49 days after transplant, no foamy matrix was found, and the number of macrophages was greatly reduced (Table 3). Small bands of perivascular and peribronchiolar lymphocyte cuffing remained, and the alveolar interstitium had some residual epithelial hyperplasia and hypertrophy, but the air spaces were patent (Figure 6E). The density of Pc cysts in the lungs of recipients of +/+ marrow dramatically fell from a high at 23 days after transplant to none detectable at 49 days after transplant (Figure 5B).

Scid *Marrow-reconstituted* scid *Mice (Controls)*

In contrast to recipients of +/+ marrow, the lungs of *scid* recipients of *scid* marrow increased in mass from 137 mg to 300 mg between 23 and 49 days after transplant (Figure 5A). At 49 days after transplant, *scid* → *scid* mice (106 to 119 total days of age) had marked interstitial thickening, significant levels of 'foamy matrix,' and large numbers of inflammatory cells consisting predominantly of macrophages and some PMN (similar to that shown in Figure 4A through C). The number of Pc cysts correspondingly rose from a density of 186/mm^2 at 23 days to a mean range of 303 to 361/mm^2 from 30 to 49 days after transplant (Figure 5B).

Immunologic Reconstitution

The spleens of both groups of C3H/HeSnJ-*scid* recipients were examined histologically (not shown). To summarize these findings, by 49 days after transplant, the spleens of +/+ → *scid* mice had large lymphoid follicles that were well populated with mature lymphocytes, and foci of germinal activity were found. In contrast, the spleens of *scid* → *scid* transplant recipients had underdeveloped white pulp. Lymphoid follicles, as defined by such architectural landmarks as the central arterioles and marginal zones subjacent to the red pulp, were only sparsely populated with lymphoidlike cells; the principle cell types were macrophages and fibroblasts.

Because IgM is the first immunoglobulin isotype expressed by developing B cells, serum IgM levels were measured to address the level of B lymphocyte reconstitution in these formerly severely B- (and T-) cell deficient mice. As measured by ELISA assay,[28] at 37 to 49 days after bone marrow transplantation, six +/+ → *scid* mice had 57-fold higher levels of serum IgM (243 ± 48 μg/ml) than did the eight still immunodeficient *scid* → *scid* control mice (4 ± 1 μg/ml) tested.

Discussion

In the present study, we have documented that untreated *scid* mice raised in two nonaxenic facilities and not receiv-

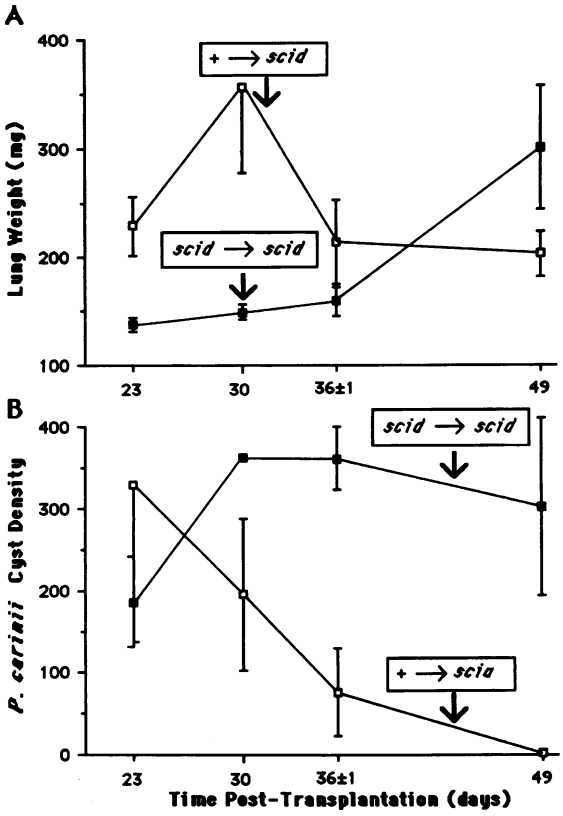

Figure 5. *Bone marrow reconstitution (C3H/HeSnJ-*scid *recipients).* A: *Lung weight (mg) and* B: *Pc cyst density (cysts/sq mm) at intervals following transplantation. Mean ± SE. Numbers of mice indicated in Table 3. Closed squares,* scid → scid *group; open squares,* + → scid *group.*

ing antimicrobial treatment developed profound Pc pneumonia and died within about 1 year of life. However, the longevity of *scid* mice raised in the barrier animal room was significantly greater than those raised in the conventional room. Based on knowledge of the flora associated with the two animal facilities described above, we suggest that coinfectious agents (secondary opportunistic pathogens) may exacerbate the clinical expression of pneumocystosis. Indeed, in ongoing collaborative studies to be reported elsewhere (CLS, JBR, and A. Smith, Yale University School of Medicine), we have found that inoculation with micro-organisms not usually associated with significant morbidity in immunocompetent mice causes heightened Pc-associated pulmonary pathology, increased density of Pc cysts, and reduced survival of *scid* mice.

The lungs of 30-day-old *scid* mice contained few Pc cysts and were essentially free of pulmonary disease, whereas by 100 days of age, the density of Pc cysts reached the level characteristic of clinically ill *scid* mice. At ages approximating the population median survival time, extensive Pc infection was evident from histopathologic analysis of lungs. Female *scid* mice had an increased level of trophic stage Pc organisms, epithelial proliferation, and chronic inflammatory response as compared with their age-matched male counterparts. The presence of multinucleated giant cells was common in females and rare in males. The density of Pc cysts was highly variable (by an order of magnitude or more) within a sex/age group, but appeared to be significantly higher in males than in females of the same age. Males raised in either barrier or conventional animal rooms had a modestly shorter lifespan than the corresponding group of females. Striking weight loss (approximately one third to one half of age- and sex-matched controls) was antecedent to death. The lungs of clinically ill *scid* mice were threefold to fourfold heavier than non-*scid* mice, an increase due primarily to increased cellularity (epithelial hyperplasia and inflammatory components), edema fluid, and Pc organisms.

Polycythemia (secondary erythrocytosis) associated with appropriate erythropoietin (EP) secretion due to tissue hypoxia is a well-described clinical finding in patients with chronic pulmonary diseases.[31] Normal mice maintained chronically in hypoxic chambers become polycythemic (hematocrits rising from approximately 44% to a maximum of 66% beginning by 20 days after initiation of hypoxia).[32] Under these conditions, polycythemia per

Table 3. *Histopathologic Assessment of Pneumocystosis in C3H/HeSnJ-*scid *Mice After Bone Marrow Transplantation*

	Time after transplant (days)*			
Donor → Recipient	23	30	36 ± 1	49
Foamy Matrix				
scid → *scid*	0.5 ± 0.5†	1.0	1.0 ± 0.0	1.8 ± 0.5
+ → *scid*	1.0 ± 0.0	0.7 ± 0.3	0.5 ± 0.2	0.0
Mononuclear Inflam.				
scid → *scid*	2.0 ± 0.0	2.0	2.0 ± 0.0	2.3 ± 0.3
+ → *scid*	3.0 ± 0.0	2.7 ± 0.9	3.0 ± 0.4	0.8 ± 0.3
No. Mice Tested				
scid → *scid*	2M	1M	3F	1M, 5F
+ → *scid*	2M	3M	1M, 5F	1M, 3F

* Recipients of +/+ marrow were 42 to 70 days and recipients of scid marrow were 57 to 70 days of age when transplanted with 5 million nucleated bone marrow cells. Recipients were not irradiated.

† Mean ± SE of histopathology scores as previously defined (see Table 1).

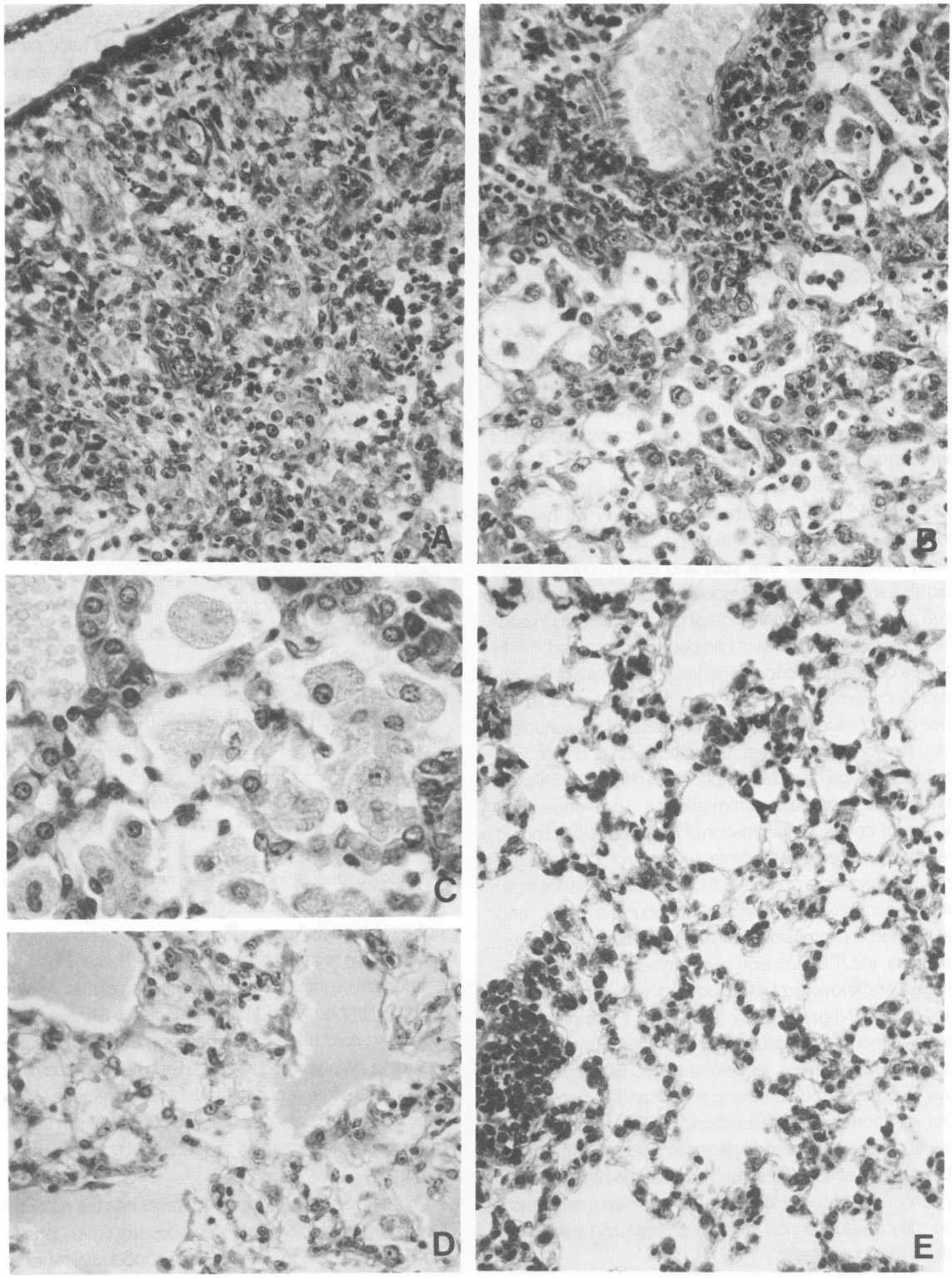

Figure 6. *Bone marrow reconstitution (C3H/HeSnJ-*scid *recipients). Histopathology of* + → scid *recipient mice.* **A:** *30 days after transplantation (+30). Relatively unchanged focus characterized by alveolar consolidation with foamy matrix, free macrophages, and hyperplastic alveolar epithelial cells. (×83).* **B:** *+30 days. Conspicuous abundance of free macrophages filling alveoli (foamy matrix essentially absent). Lymphocytes concentrated perivascularly with a few scattered among macrophages. (×83).* **C:** *+30 days. Macrophages extremely enlarged, foamy, vesiculated. (×134).* **D:** *+30 days. Pulmonary edema. Many alveoli were free of formed elements, but filled with proteinaceous or mucoid exudate. (×83).* **E:** *+49 days. Essentially no residual pneumocystosis. Alveolar interstitium was thin, but with slight epithelial hypertrophy. Macrophages not common. Small focus of lymphocytes (lower left) located adjacent to a terminal bronchiole. (×83).* **(A–E:** *Giemsa).*

sisted as long as hypoxic conditions were maintained. In the present study, hypoxia can be presumed based on the histopathologic features of the lungs and the significant polycythemia identified in clinically ill *scid* mice. Furthermore, other pathologic conditions associated with inappropriate EP secretion (eg, hepatic and renal carcinomas) have not been observed in *scid* mice.

In the absence of functional T and B lymphocytes, *scid* mice respond to Pc infection with vigorous responses by (primarily) mononuclear phagocytes and (secondarily) PMN. Macrophages were evident in large numbers and were phagocytically active based on finding cytoplasmic inclusions by light microscopy and abundant intracellular Pc organisms by transmission EM (data not shown). Rarely, spindle cells and scattered fibrotic foci were identified in the thickened alveolar septae, and multinucleated giant cells were found in lungs of *scid* mice with advanced pneumocystosis. Granulomata were not seen. Recent studies support the concept that host defenses against opportunistic pathogens may rely primarily on nonlymphocyte effector cells. For example, Mahanty et al[33] recently demonstrated that *scid* and Balb/c control mice had similar time courses of recovery from inoculated *Candida albicans*. The importance of phagocytic cells in control of systemic candidiasis has been documented.[34,35] In studies of *Listeria monocytogenes* (LM) infection, Deschryver-Kecskemeti et al[36] reported that the lymphoid tissues of *scid* mice underwent hyperplasia of mononuclear cells, including monocytes, dendritic cells, and cells with features of large granular lymphocytes/NK cells. This occurred without granuloma formation (a T-cell–dependent feature of chronic inflammation).[37] A remarkable finding was that, compared with uninfected controls, LM-infected *scid* mice had large numbers of class II major histocompatability complex (Ia)-positive mononuclear cells and heightened Ia expression in all organs, including gut, liver, pancreas, etc.[38] Expression of Ia and activation of macrophages are known to be associated with production of interferon (IFN)-gamma by activated T lymphocytes. These authors[36,37] speculated that NK cells may be the source of IFN-gamma in *scid* mice. C.B-17-*scid* mice have been reported as having an expanded pool of NK cells with normal *in vitro* function.[24,25] The relatively long clinical course of pneumocystosis in *scid* mice may be a tribute to the effectiveness of the inflammatory response by nonlymphoid cells. However, the morbid consequence also may be due in part to such chronic and excessive cellular responsiveness.

In the bone marrow reconstitution studies reported here, 30% to 50% of *scid* mice receiving normal bone marrow died or were judged as not likely to have survived past 30 to 40 days after transplantation, as a consequence of a prodigious pulmonary inflammatory response (presumably to a pre-existing, albeit silent, Pc infection). Essentially complete resolution of Pc pneumonitis was found among those marrow-treated *scid* mice surviving this period of deleterious hyperinflammatory response. Lymphocytes were found in the lungs of these treated mice, primarily in the perivascular spaces; small numbers also were diffusely scattered and intermixed with the massive numbers of intra-alveolar and interstitial macrophages. These lymphocytes apparently were critical to the resolution of pneumocystosis, possibly via macrophage-enhancing T-cell products (such as IFN-gamma, as described above in *Listeria*-infected *scid* mice). The presence of serum IgM was documented, but the occurrence and significance of anti-Pc antibody in decreasing the pathogenic burden of Pc awaits further study. Marrow reconstitution of younger (preweaning) *scid* mice would perhaps bypass the morbid phase just described. In lieu of this, treatment of *scid* mice (before and immediately after marrow transplantation) with anti-Pc drugs also may reduce morbidity. For example, a regimen of trimethoprim-sulfamethoxazole treatment of conventionally housed *scid* mice has been shown to have a positive effect on health and lifespan.[16] The issue of successfully restoring immune function to immunodeficient and opportunistically infected individuals is clearly of major importance to AIDS patients.

Custer et al,[18] while describing a 15% incidence of thymic lymphomas in C.B-17-*scid* mice by 65 weeks of age, did not report Pc pneumonitis. The *scid* mice in that study were produced from specific pathogen-free (SPF) breeders and were housed in microisolator cages, an indicator that Pc pneumonitis can be eliminated by germ-free derivation and maintenance.

Studies describing *scid*-Hu mice, in which homozygous *scid* mice have been transplanted with human peripheral blood leukocytes[39] or human fetal hematopoietic cells,[40] have recently been published. These xenografted chimeric mice support the differentiation of mature human T and B lymphocytes. In experiments by McCune et al,[40] untreated *scid* mice developed opportunistic infections, including Pc pneumonitis, and died by 4 months of age. In contrast, the reconstituted human immune system seems to have protected the *scid*-Hu mice from such opportunistic infections, as evidenced by survival to 17 months.

In summary, this manuscript describes the natural history and pathobiology of *Pneumocystis carinii* pneumonia in mutant *scid* mice. The unequivocal clinical expression of spontaneous pneumocystosis provides an excellent *in vivo* model for studying the pathogenesis, immunobiology, and treatment of human Pc pneumonia. The ability to combine (by appropriate matings) multiple single gene mutations or allotypic markers in a defined inbred strain provides a powerful experimental approach

for studying the mechanisms of host susceptibility/resistance to Pc or other infectious organisms.

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