A Mouse Model of Chronic Pulmonary Infection with *Pseudomonas aeruginosa* and *Pseudomonas cepacia*

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ABSTRACT. A mouse model of chronic pulmonary infection with either *Pseudomonas aeruginosa* or *Pseudomonas cepacia* was developed to compare bacteriologic and pathologic features of these infections. Experimental pneumonia was established in Swiss mice by transoral intratracheal inoculation of 10^3–10^4 colony-forming units of mucoid *P. aeruginosa* or *P. cepacia* enmeshed in agarose beads. Unilateral infection with either strain was tolerated without morbidity. By 10 days postinoculation, the mean colony-forming units per infected lung was 3.8×10^5 for *P. aeruginosa* and 1.0×10^5 for *P. cepacia*. Bacterial counts remained stable through 21 days with no significant difference between organisms. Acute and chronic inflammatory histopathologic changes similar to many found in the lungs of cystic fibrosis patients were present in 95% of lung specimens. The changes occurred with both organisms but were more extensive with mucoid *P. aeruginosa*. This model represents an important tool for study of the contribution of complement, antibody, and adoptive transfer of T cell-mediated immunity to the pathogenesis of chronic pneumonia with Pseudomonas species, and represents the first successful model of chronic pulmonary infection with *P. cepacia*. (*Pediatr Res* 22: 698–702, 1987)

Abbreviations

PA, *Pseudomonas aeruginosa*
PC, *Pseudomonas cepacia*
CFU, colony-forming units
CF, cystic fibrosis
PBS, phosphate-buffered saline
SDC, sodium desoxycholate
LPS, lipopolysaccharide

Chronic pulmonary infection with Pseudomonas is a major factor contributing to the morbidity and mortality of at least 75% of patients with CF (1). For two decades, PA, frequently expressing a mucoid surface exopolysaccharide, has been the major species colonizing the respiratory tract of these patients (2, 3). Recently, some CF centers have reported an increasing number of infections with PC which may occur in association with deteriorating pulmonary function and death (4–6). Little is known about the biologic behavior and pathologic determinants of PC in human or experimental animal infections.

Direct assessment of Pseudomonas lung infections in CF pa-

tients has been limited by the fact that invasive procedures such as bronchoscopy are needed to obtain lung lavage specimens. Since no animal model of CF exists, experimental models of Pseudomonas lung infection have been developed to investigate host-microbial interactions. In mice, long-term pulmonary infections previously have not been achieved in the absence of immunosuppression. In normal animals, initiation of infection by aerosolization or tracheal inoculation of organisms in saline produces transient pulmonary colonization accompanied by mild inflammatory reaction, or acute pneumonia which is fatal within 24 to 48 h (7–10). Cash *et al.* (11) developed a model of chronic PA lung infection in rats using bacteria-impregnated agar beads. In this model, there is proliferation of organisms and histologic damage similar to that observed in the lungs of CF patients. This model has been adapted to larger animals such as guinea pigs (12) and cats (13), but not to smaller animals. The availability of genetically defined strains as well as specific immunochemical reagents makes the mouse a theoretically ideal host for the study of immune response to chronic pulmonary infection.

The experiments reported herein fulfill two major goals. First, the technique of the agar bead method has been adapted to establish a mouse model of chronic pulmonary infection with Pseudomonas. Second, this model was employed to compare the bacteriology and histopathology of experimental PA and PC infections.

MATERIALS AND METHODS

Bacterial strains. PA strain 2192 (14) was a gift from Gerald B. Pier, Ph.D., (Channing Laboratory, Harvard Medical School, Boston, MA). This strain has a markedly mucoid appearance when grown on blood agar. The strain of PC S-1 was isolated originally at Texas Children's Hospital, Houston, TX from the sputum of an adolescent male in stable clinical condition with CF. In addition to these two strains, a nonmucoid revertant of PA 2192 (from Dr. Pier), PAO-1 (a gift from Barbara H. Iglewski, Ph.D., University of Rochester, Rochester, NY), and two additional PC isolates from patients with CF were employed in some experiments. Strains were stored at −80° C in trypticase soy broth supplemented with glycerol.

Experimental animals. Three groups of 12 to 15 Swiss CD-1 outbred female mice, 6 to 10 wk old and free of antibody to pathogenic murine viruses (Charles River Laboratories, Wilmington, MA) were used for each experiment. These animals were separated from other animals and were housed in the laboratory with standard care and feeding conditions.

Experimental model of infection. A suspension of bacteria-impregnated agarose beads was made using slight modifications of previously described methods (11, 15). The bacteria strain to be used was grown for 18 h to late log phase in tryptic soy broth

(Baltimore Biologic Laboratories Microbiology Systems, Cockeysville, MD). A 25-ml aliquot of a mixture containing 0.1 ml of the tryptic soy broth culture and 50 ml of 2% agarose (w/v) in PBS, pH 7.2, was pipetted forcefully into 150 ml of heavy mineral oil at 50° C as the oil was stirred rapidly with a magnetic stirring bar. Stirring was continued at 24° C for 6 min followed by cooling of the mixture over 10 min. The bacteria-containing agarose beads formed were 100–200 μ in diameter. The oil-agarose mixture then was washed with 0.5% SDC in PBS. The oil and SDC layers were removed by aspiration, and the washing was repeated using 0.25% SDC in PBS, followed by three washes with PBS. The washed beads were resuspended in PBS to 2–4 × 10^4 CFU/ml. Actual bacterial counts were confirmed by serial dilution on blood agar plates of a hand homogenized suspension.

Mice were anesthetized lightly with ketamine hydrochloride (30 mg/kg) given intraperitoneally. The trachea was directly visualized by ventral midline, cervical incision. After orotracheal intubation with a 22-gauge bead-tipped stainless steel needle (Perfektum Company, New York, NY), 0.05 ml of the bead suspension was inoculated into either both lungs or the right lung. Needles were bent at a 30° angle to allow selective intubation of the right lung, and preliminary experiments employing methylene blue dye or agarose beads validated selective intubation of the right lung in more than 90% of animals. After inoculation, the incision was closed with suture. No animals developed wound infections and healing occurred in 2 to 3 days.

Bacteriology and histopathology. Animals were anesthetized with ether and sacrificed by retroorbital and cardiac exsanguination at 10 or 21 days after inoculation. The lungs and lower trachea were excised aseptically and selected randomly either for bacteriologic or histopathologic study. Lungs for bacteriology were homogenized by a Polytron Homogenizer (Brinkman Instruments, Inc., Westbury, NY) and homogenates were cultured quantitatively by serial dilution on blood agar plates. PA and PC were identified by Gram stain, colony morphology, oxidase reaction, and the rapid-NFT (DMS Laboratories, Flemington, NJ). Gram-positive organisms were identified by Gram stain, colony morphology, and catalase tests. Gram-negative fermentative bacilli were identified using the Rapid-E test (DMS Laboratories). Lungs for histopathologic study were removed *en bloc* and the trachea cannulated with a 20-gauge needle. They were fixed in 10% buffered formalin using an inflation-fixation apparatus constructed for the project and designed to deliver 15 to 20 cm of continuous intrapulmonary pressure. After 24 h of fixation, the lungs were sectioned and stained with hematoxylin and eosin. Coded sections were assessed blindly for extent of inflammation, bronchial epithelial alterations, alveolar changes, and fibrosis.

Statistical analysis. Because normal distributions for the measurements could not be assumed, nonparametric tests were used for statistical analysis of the data. A two-tailed Mann-Whitney U test was used to compare colony counts between infections with PA 2192 mucoid and PC S-1 at both 10 and 21 days. The χ^2 test with Yates correction was used to compare deaths in animals infected with PA and PC.

RESULTS

Animal survival. The inoculation procedure was tolerated well by most animals. Occasionally, a mouse developed asphyxiation from the thick bead suspension, and these animals were excluded from analysis. All other animals were mobile and alert within minutes after the procedure.

Although cultures of other organs were not obtained, no animals developed clinical or macroscopic evidence of infections or other abnormalities in extrapulmonary sites. Retroorbital blood cultures obtained in selected animals at 30 min and 4, 8, 24, 48, 72, and 96 h after pulmonary inoculation were consistently negative for PA 2192 mucoid and PC S-1.

A number of animals inoculated bilaterally with either PA or PC died spontaneously before the end of the intended period of observation. Animals with bilateral pneumonia were observed only for survival; no culture or histologic studies were performed prior to death. Death from bilateral infection was significantly more common in animals inoculated with PA compared with PC (43 *versus* 15%, χ^2 = 16.4, p < 0.001). All spontaneous deaths occurred 4 to 9 days after pulmonary challenge. These animals became symptomatic 24 to 48 h prior to death. All animals that died spontaneously had severe, bilateral pneumonia which affected all lobes of both lungs. Cultures of lung homogenates from animals selected because death was observed yielded 10^6–10^9 CFU/lungs of the challenge organism. Since most of these deaths were not observed, meaningful histopathologic study of these animal's lungs could not be performed. No animal with unilateral lung infection died before experimental sacrifice. The animals with bilateral pneumonia were excluded from further bacteriologic and histopathologic analysis.

Bacteriology. The lung homogenates generally yielded the challenge organism in pure culture. Isolated colonies of staphylococci, α-hemolytic streptococci or Proteus species were recovered occasionally. The biochemical profiles of the strains of PA and PC recovered from lung homogenates were identical to those of the inoculating strains. In no case did mucoid PA revert to a nonmucoid morphology.

The mean challenge doses for PA 2192 mucoid and PA S-1, and the mean CFU per lung homogenate are shown in Table 1. For each organism, the mean bacterial count increased almost two logs from inoculation to day 10, and then remained relatively stable through day 21. The degree of bacterial proliferation was similar for PA and PC at either time interval (10 days: U = 1.22, p = 0.22; 21 days: U = 1.61, p = 0.11). Rarely, animals infected with either organism had sterile lung homogenates despite the presence of obvious lobar pneumonia by gross inspection.

Pathology. Macroscopic changes were apparent in 95% of the animals infected with either organism, and ranged from hyperemia to frank consolidation of lung tissue. Areas of consolidation were usually unilateral and affected one or two of the right lobes. The consolidated areas were dark red-brown, with a smooth but firm surface and were difficult to inflate with formalin. Fibrinous pleural adhesions frequently were present. There were no qualitative differences in gross lung appearance between PA 2192 mucoid and PC S-1-infected animals, or between animals sacrificed at 10 and 21 days.

Preliminary experiments showed that instillation of sterile agar beads into the mouse lung caused no local inflammatory reaction (Fig. 1). In contrast, virtually all animals inoculated with bacteria-impregnated agar beads demonstrated both bronchial and parenchymal changes microscopically. Frequently, agar beads containing microcolonies of organisms, which were surrounded by inflammatory cells, could be seen within large or small airways (Fig. 2). The ratio of neutrophils to macrophages in this airway exudate was 8–10:1. These cells did not contain any phagocytosed bacteria. They were particularly dense near the microcolonies and in some cases appeared to invade through the surface of the agar bead (Fig. 3). There were no differences in inflammatory cell numbers or ratios between PA mucoid 2192 and PC S-1-infected lungs. Some lung sections revealed bronchial epithelial hyperplasia, while others had focal epithelial ulcerations and wide-spread squamous metaplasia. Bronchiectasis was a common finding. Proliferation of bronchus-associated lymphoid tissue occurred frequently in the peribronchial area adjacent to bead-containing airways, and consisted almost entirely of small lymphocytes. Assessment of a limited number of animals at 4 wk after inoculation revealed similar histopathologic findings.

A wide variety of parenchymal changes occurred, including alveolar infiltrates and interstitial inflammation. Frequently, a dense necrotizing and granulomatous reaction occurred with loss of normal architecture. Early fibrosis was seen in 10-day lung sections but was more common at 21 days. While individual bacteria and bacterial microcolonies could be visualized readily

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Table 1. *Bacteriologic parameters during chronic pneumonia in mice*

Organism/strain	Length of infection (days)	No. of animals*	Challenge dose† (CFU × 10³)	Bacterial persistence‡ (CFU × 10⁵)
PA/2192 mucoid	10	42	4.6 ± 1.1	3.8 ± 1.9
PC/S-1	10	45	5.4 ± 1.8	1.0 ± 0.4
PA/2192 mucoid	21	45	4.4 ± 1.3	2.3 ± 1.6
PC/S-1	21	43	4.4 ± 0.8	1.7 ± 0.6

* Sum of animals from three separate experimental groups.

† Geometric mean (± SE) of CFU/0.05 ml of inoculum suspension of beads for three groups of animals.

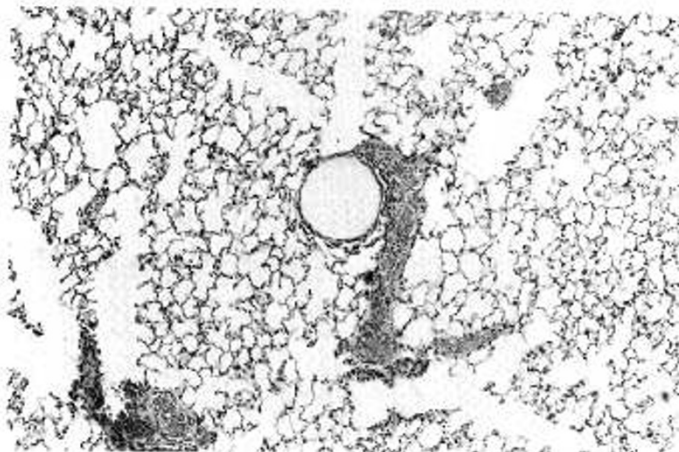‡ Geometric mean (± SE) of CFU in lung homogenate per animal.

Fig. 1. Lung of an animal with sterile beads for 10 days. There is no surrounding inflammatory reaction (hematoxylin and eosin, × 150).

within the beads, no free bacteria were seen by light microscopy within the airways or the parenchyma. In general, the blood vessels had normal architecture and were not inflamed.

The appearance of the agar beads, airway inflammation, numbers and types of inflammatory cells, and parenchymal infiltrates caused by PA 2192 mucoid and PC S-1 were indistinguishable. However, the parenchymal damage caused by PA 2192 mucoid was 50% more extensive by area, spreading more distally from bead-containing airways. Results with PA 2192 nonmucoid revertant and PAO-1 were identical to those with PA 2192 mucoid, and those with our other two PC strains were indistinguishable from PC S-1.

DISCUSSION

The agar bead model of chronic pneumonia has been adapted successfully to several animal species (12, 13). Previous studies using this model have examined the ability of certain Pseudomonas products to either promote pulmonary tissue damage or to be used as vaccines. Except for measurements of antibody titers to certain bacterial products, studies of the host's immunologic responses to chronic pulmonary infection are lacking. Several characteristics of the mouse make it a unique host for immunologic studies. First, the genetic diversity of mouse strains will enhance the study of the role of specific bacterial products and components of the host response in pulmonary pathogenesis. For instance, the contributions of LPS and surface polysaccharides to tissue damage and animal protection can be examined in mouse strains with genetically determined exaggerated or diminished response to these antigens. Similarly, the importance of complement in bacterial clearance and immune complex formation can be determined using normal animals and congenitally complement-deficient mutants. Second, a much wider array of immunochemical reagents are available for the mouse than other animals, augmenting the ability to determine the
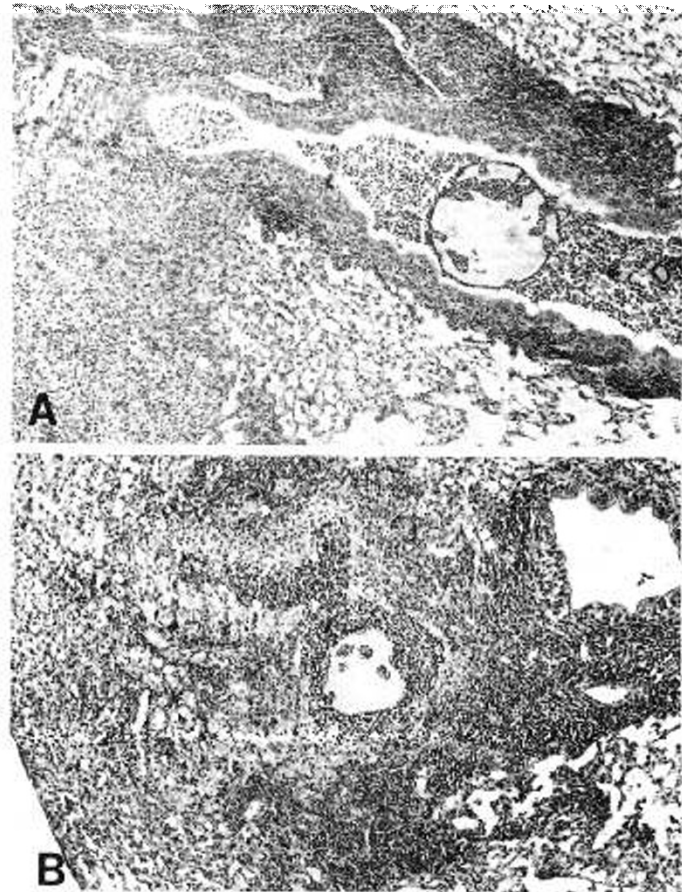

Fig. 2. Lung from animals infected for 10 days with agar beads containing PA 2192 mucoid (*A*) and PC S-1 (*B*). The airways are filled with an exudate consisting predominately of neutrophils with some macrophages. The surrounding parenchyma is obscured by a mononuclear cell inflammatory response. Focal destruction and squamous metaplasia of the bronchiolar epithelium can be seen (*A*). The airway epithelium is ulcerated widely (*B*). The degree of inflammation seen (*B*) is unusually severe for infection with PC S-1 (hematoxylin and eosin, × 150).

importance of specific antibody subclasses and leukocyte cell surface components in host pulmonary defense. Finally, since previous studies have demonstrated an important contribution of T cell-mediated immunity against Pseudomonas surface polysaccharide in mice (16), studies of protection against lung infection using adoptive transfer of leukocytes could be performed in a mouse model of infection.

Before experiments suggested by these characteristics can be performed, a mouse model of chronic lung infection must be defined. Previous attempts using aerosolization (10) or direct tracheal instillation of organisms in saline (7, 9) have produced,
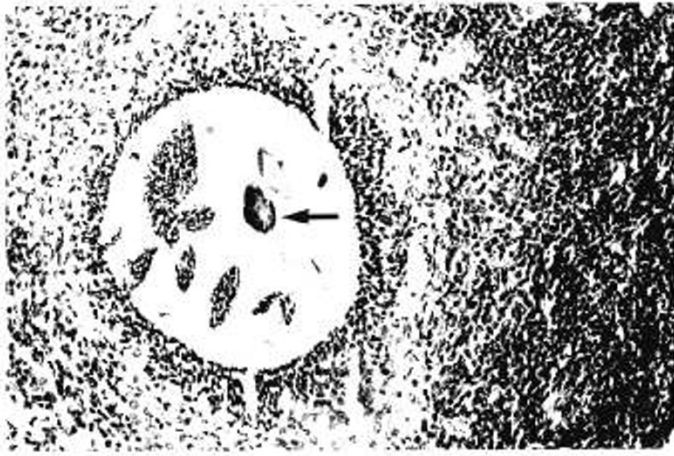
Fig. 3. An agar bead containing PC S-1 in a mouse infected for 21 days. Several microcolonies (*arrow*) can be seen, some of which are covered with neutrophils. At higher power, individual organisms are seen easily within these microcolonies. The bronchial epithelium is completely destroyed (hematoxylin and eosin, × 300).

depending on the inoculum, either transient, mild inflammatory reactions with complete bacterial clearance, or acute fatal pneumonia. The present experiments have demonstrated that mice subjected to unilateral transtracheal inoculation with Pseudomonas-impregnated agar beads could maintain an inflammatory response for at least 3 wk. The results were similar to those observed in larger animals (11, 12). Bacterial counts increased in the days following inoculation by 100-fold, and then remained stable throughout the remaining course of infection. The histopathologic lesions seen in mice were similar to some found at autopsy in CF patients with chronic pulmonary disease (17). Both naturally occurring and experimental infections frequently manifest bronchopneumonia, bronchiectasis, mucus plugging, epithelial metaplasia, fibrosis, and alveolar exudates with acute and chronic inflammatory cells. Lymphoid hyperplasia, which was prominent in the infected animals, also is noted frequently in the lungs of CF patients and is believed to be a consequence of chronic antigenic stimulation (18). Pulmonary vascular inflammatory lesions, which are common among patients with acute bacteremic Pseudomonas pneumonia, were not observed in CF patients or in chronically infected animals.

Although PA has been the predominant pathogen colonizing the lungs of CF patients during the last two decades, PC recently has emerged as a serious cause of morbidity and mortality. Some CF centers report a prevalence of PC colonization of 15 to 20% (5, 6). Pulmonary acquisition of PC may be asymptomatic, but it is often followed by a gradual or very rapid decline in clinical status. Systemic signs of infection and bacteremia, which are rarely associated with PA infection in CF patients, occur commonly in patients colonized with PC (6). The pathogenesis of PC in CF patients is unknown, and the host immune response to PC has not been investigated.

No previous studies of experimental PC pneumonia have been reported. PC pneumonia is not easily established in the guinea pig and chronic infection in the rat has been described only superficially (19). In our model the findings with PC were similar to those with PA. The increase in bacterial CFU was the same for the two organisms, and both appeared to grow in microcolonies within the agar beads. Using electron microscopy, Lam *et al.* (20) showed that PA obtained from bronchial mucus of CF patients and from rats with agar-bead-induced chronic pneumonia formed microcolonies that were enveloped in a fibrous anionic matrix. These investigators postulated that this form of growth protected the organism from host defense mechanisms, but also limited its toxicity and dissemination (21). Although PC

growth has not been studied similarly, we were able to demonstrate microcolony growth within agar beads by light microscopy.

The pathologic processes associated with PA and PC were qualitatively the same, although they were slightly more extensive in the PA groups of animals. The increased lethality of bilateral pneumonia with PA compared to PC is consistent with the more extensive disease also seen in unilateral infection. The differences observed between PA 2192 and PC S-1 may be a reflection of strain rather than species characteristics. In smaller groups of mice, the results with two additional strains of PA were indistinguishable from those of mucoid PA 2192, while two other strains of PC obtained from CF patients yielded results identical to those with PC S-1. For more meaningful comparisons to be made, future studies employing more well-defined strains of PA and PC will have to be undertaken. In this way, the role of specific virulence factors such as rough *versus* smooth LPS, mucoid polysaccharide, and specific proteases and toxins, could be defined.

One disadvantage of the agar-bead infection model is that it does not simulate the natural history of Pseudomonas infection in CF from airway colonization to lung injury. However, the type of tissue damage and the host response to both PA and PC in the mouse are similar to those that occur in CF patients. Therefore, this model can be used with normal animals as well as those with diminished responses to LPS in the evaluation of the contributions of complement, antibodies of various classes and subclasses, cell-mediated immune function, and immune complex deposition to protection from or promotion of tissue damage in chronic Pseudomonas pulmonary infection.

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Announcement

FASEB Summer Research Conferences

1987 Series Completed, 1989 Topic Proposals Sought

The sixth summer of the FASEB Summer Research Conference series has been completed. This summer there were 17 conferences held at two conference sites: Saxtons River, Vermont, and Copper Mountain, Colorado. The series was established to complement the large FASEB Annual Meeting. It was designed to provide quiet settings to encourage a relaxed exchange of information on the cutting edge of biomedical research. More than 1900 scientists from the United States and 18 foreign countries enjoyed the exciting science that was presented and discussed at the meetings in 1987.

Many of the 1987 conference participants requested that their conference topics be rescheduled in the future FASEB Summer Research Conference series. Topic proposals are required from potential organizers of these conferences, as well as from those proposing new topics for consideration by the Conference Advisory Committee at its fall meeting.

The committee is seeking new topic proposals on subjects of keen interest to biological and medical researchers for fields that require concentrated 5-day programs for adequate discussions on the latest developments. A topic proposal outline is available from Dr. Robert W. Krauss, FASEB Executive Office (301-530-7093) to assist in the submission of proposals for FASEB Summer Research Conferences in 1989 and 1990.

The topics for the 1988 series have been set and are outlined below. The Conference Advisory Committee will meet in October or November of this year to establish the 1989 schedule and a tentative schedule for 1990.

1988 FASEB Summer Research Conferences

Saxtons River, VT

Yeast RNA: Transcription, Splicing, Translation, Replication and Transposition   June 12–17
Chairperson: Reed B. Wickner, NIADDK, NIH

Retinoids   June 19–24
Chairperson: DeWitt S. Goodman, Columbia University College of P&S

Smooth Muscle   June 26–July 1
Chairperson: R. Kent Hermsmeyer, University of Nevada School of Medicine

Autoimmunity   July 3–8
Chairperson: Howard L. Weiner, Brigham and Women's Hospital

Phospholipases   July 10–15
Chairperson: Moseley Waite, Bowman Gray School of Medicine

Immunopharmacology   July 17–22
Chairperson: Timothy Sullivan, University of Texas Health Science Center-Dallas

Structure and Function of Cell Membranes   July 24–29
Chairperson: Philip L. Yeagle, SUNY at Buffalo

Somatic Cell Genetics   July 31–August 5
Chairperson: Geoffrey Wahl, The Salk Institute

Receptors   August 7–12
Chairperson: Richard Klausner, National Institutes of Health

Electrophysiological Mechanisms of Propagation in and Activation of Cardiac and Smooth Muscle   August 14–19
Chairperson: David R. Harder, Medical College of Wisconsin

Copper Mountain, CO

Neuroimmunomodulation   July 26–July1
Chairperson: Novera H. Spector, University of Alabama at Birmingham

Ultradian and Infradian Modulation of the Circadian System   July 3–8
Chairperson: Lawrence E. Scheving, University of Arkansas

Regulation of Gene Expression in Higher Animals by Hormones and Nutritional Substrates   July 10–15
Chairperson: George A. Scheele, The Rockefeller University

Molecular Biology of Infectious and Parasitic Diseases   July 17–22
Chairperson: Richard A. Young, Whitehead Institute for Biomedical Research

Trichothecene, Blue-Green Algal and Marine-Toxins: Mechanisms, Detection and Therapy   July 24–29
Chairperson: Adrianne Rogers, Boston University School of Medicine

Folic Acid, Vitamin B-12 and One-Carbon Metabolism   July 31–Aug 5
Chairperson: Raymond Blakely, St. Judes Children's Research Hospital

Endothelium and Cardiovascular Function   August 7–12
Chairperson: Paul M. Vanhoutte, Mayo Foundation

Neoplastic Transformation of Liver Cells   August 14–19
Chairperson: Snorri Thorgiersson, National Cancer Institute, NIH