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FIELDS VIROLOGY

SIXTH EDITION

EDITORS-IN-CHIEF

David M. Knipe

Peter M. Howley

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VOLUME ONE

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EDITORS-IN-CHIEF

David M. Knipe, PhD
Higgins Professor of Microbiology and Molecular Genetics
Department of Microbiology and Immunobiology
Chair, Harvard Program in Virology
Harvard Medical School
Boston, Massachusetts

Peter M. Howley, MD
Shattuck Professor of Pathological Anatomy
Department of Microbiology and Immunobiology
Harvard Medical School
Boston, Massachusetts

ASSOCIATE EDITORS

Jeffrey I. Cohen, MD
Chief
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Diane E. Griffin, MD, PhD
Professor and Chair
W. Harry Feinstone Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health
Baltimore, Maryland

Robert A. Lamb, PhD, ScD
John Evans Professor of Molecular and Cellular Biology
Investigator, Howard Hughes Medical Institute
Department of Molecular Biosciences
Northwestern University
Evanston, Illinois

Malcolm A. Martin, MD
Chief
Laboratory of Molecular Microbiology
Division of Intramural Research
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Vincent R. Racaniello, PhD
Higgins Professor
Department of Microbiology and Immunology
Columbia University
College of Physicians and Surgeons
New York, New York

Bernard Roizman, ScD
Joseph Regenstein Distinguished Service Professor
Departments of Microbiology and Molecular Genetics and Cell Biology
University of Chicago
Chicago, Illinois

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Stephen E. Straus, 1946–2007

Steve Straus was the consummate physician–scientist with broad interests in the basic science and clinical aspects of viral and immunological diseases and therefore was an ideal person to serve as clinical virology editor for *Fields Virology*. We were fortunate to work with him in his role as associate editor for the third through fifth editions of *Fields Virology*. However, unfortunately, with Steve's premature death in 2007, we lost our friend, colleague, and fellow editor. Steve's medical training and accomplishments are detailed elsewhere (*J Infect Dis* 2007;196:963–964). His research interests were broad and included the molecular biology and pathogenesis of varicella-zoster and herpes simplex viruses, acyclovir suppression of oral and genital herpes simples viruses, antiviral drug resistance, clinical testing of herpes simplex virus and varicella zoster virus vaccines, chronic active Epstein–Barr virus, chronic fatigue syndrome, and autoimmune lymphoproliferative syndrome. Steve was one of the leading scientists in the National Institutes of Health intramural program, serving as chief of the Laboratory of Clinical Investigation at the National Institute of Allergy and Infectious Diseases and the founding director of the National Center for Complementary and Alternative Medicine.

Steve cowrote the chapter on varicella zoster virus, and additionally worked effectively as an associate editor, for the third to fifth editions of *Fields Virology*. He seemed to read and edit the chapters immediately upon their submission, amazing us with his ability to do all of this on top of his other responsibilities. Steve was diagnosed with brain cancer in 2004 but insisted on editing chapters for the fifth edition right through the compiling of the chapters. The book was published in early 2007, not long before his death in May 2007.

On behalf of everyone who contributed to the sixth edition of *Fields Virology*, we dedicate this book to the memory of Stephen E. Straus, MD.

CONTRIBUTORS

Carlos F. Arias, MD
Professor
Department of Developmental Genetics and Molecular Physiology
Instituto de Biotecnologia
Universidad Nacional Autonoma de Mexico
Cuernavaca, Morelos, Mexico

Ann M. Arvin, MD, PhD
Lucile Salter Packard Professor
Departments of Pediatrics and Microbiology and Immunology
Stanford University School of Medicine
Stanford, California

Justin R. Bailey, MD, PhD
Assistant Professor
Department of Medicine
Johns Hopkins University School of Medicine
Baltimore, Maryland

Arnold J. Berk, MD
Professor and UCLA Presidential Chair in Molecular Cell Biology
Molecular Biology Institute
Department of Microbiology, Immunology, and Molecular Genetics
University of California, Los Angeles
Los Angeles, California

Kenneth I. Berns, MD, PhD
Distinguished Professor Emeritus
Department of Molecular Genetics and Microbiology
University of Florida College of Medicine
Gainesville, Florida

Thomas J. Braciale, MD, PhD
Beirne B. Carter Professor in Immunology
Director, Carter Immunology Center
Professor of Pathology and Molecular Medicine
Professor of Microbiology, Immunology, and Cancer Biology
University of Virginia School of Medicine
Charlottesville, Virginia

Thomas Briese, PhD
Associate Professor
Department of Epidemiology
Associate Director
Center for Infection and Immunity
Mailman School of Public Health
Columbia University
New York, New York

Christopher C. Broder, PhD
Professor and Emerging Infectious Diseases
Graduate Program Director
Department of Microbiology and Immunology
Uniformed Services University
Bethesda, Maryland

Michael J. Buchmeier, PhD
Deputy Director
Pacific Southwest Center for Biodefense and Emerging Infectious Diseases
Professor
Division of Infectious Disease
Professor
Department of Molecular Biology and Biochemistry
University of California, Irvine
Irvine, California

Dennis R. Burton, PhD
Professor
Department of Immunology and Microbial Science
Center for HIV/AIDS Vaccine Immunology and Immunogen Disccovery and IAVI Neutralizing Consortium
Scripps Research Institute
La Jolla, California

Kathryn M. Carbone, MD
Deputy Scientific Director
Division of Intramural Research
National Institute of Dental and Craniofacial Research
National Institutes of Health
Bethesda, Maryland

Ethel Cesarman, MD, PhD
Professor
Department of Pathology and Laboratory Medicine
Weill Cornell Medical College
New York, New York

Donald M. Coen, PhD
Professor
Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School
Boston, Massachusetts

Jeffrey I. Cohen, MD
Chief
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Peter L. Collins, PhD
Chief
RNA Viruses Section
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Philippe Colson, PharmD, PhD
Associate Professor
URMITE UM 63 CNRS 7278 IRD 198 INSERM U1095
Aix-Marseille Université
Facultés de Médecine et de Pharmacie
Pôle des Maladies Infectieuses et Tropicales Clinique et Biologique
Fédération de Bactériologie-Hygiène-Virologie
IHU Méditerranée Infection
Centre Hospitalo-Universitaire Timone, Assistance Publique-Hôpitaux de Marseille
Marseille, France

Richard C. Condit, PhD
Professor
Department of Molecular Genetics and Microbiology
University of Florida
Gainesville, Florida

James E. Crowe Jr., MD
Ingram Professor of Research
Departments of Pediatrics and Pathology, Microbiology, and Immunology
Director, Vanderbilt Vaccine Center
Vanderbilt University
Nashville, Tennessee

Blossom A. Damania, PhD
Professor
Department of Microbiology and Immunology
Lineberger Comprehensive Cancer Center
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

Inger K. Damon, MD, PhD
Chief
Poxvirus and Rabies Branch
Division of High-Consequence Pathogens and Pathology
Centers for Disease Control and Prevention
Atlanta, Georgia

Juan-Carlos de la Torre, PhD
Professor
Department of Immunology and Microbial Science
The Scripps Research Institute
La Jolla, California

James A. DeCaprio, MD
Associate Professor of Medicine
Department of Medicine
Harvard Medical School
Associate Professor of Medicine
Dana-Farber Cancer Institute
Boston, Massachusetts

Terence S. Dermody, MD
Dorothy Overall Wells Professor of Pediatrics and Pathology, Microbiology, and Immunology
Director, Division of Pediatric Infectious Diseases
Director, Medical Scientist Training Program
Lamb Center for Pediatric Research
Vanderbilt University School of Medicine
Nashville, Tennessee

Ronald C. Desrosiers, PhD
Professor
New England Primate Research Center
Harvard Medical School
Southborough, Massachusetts

Michael S. Diamond, MD, PhD
Professor
Departments of Medicine, Molecular Microbiology, and Pathology and Immunology
Washington University School of Medicine
St. Louis, Missouri

Daniel DiMaio, MD
Waldemar Von Zedtwitz Professor
Department of Genetics
Yale School of Medicine
Scientific Director
Yale Cancer Center
Yale University
New Haven, Connecticut

John H. Elder, PhD
Professor
Department of Immunology and Microbial Science
The Scripps Research Institute
La Jolla, California

Richard M. Elliott, DPhil
Chair of Infectious Diseases
Centre for Virus Research
University of Glasgow
Glasgow, Scotland, United Kingdom

Suzanne U. Emerson, PhD
Chief
Molecular Hepatitis Laboratory
Laboratory of Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Lynn W. Enquist, PhD
Professor
Department of Molecular Biology
Princeton University
Princeton, New Jersey

Mary K. Estes, PhD
Professor
Department of Molecular Virology and Microbiology
Baylor College of Medicine
Houston, Texas

David T. Evans, PhD
Associate Professor
Department of Microbiology and Immunobiology
Harvard Medical School
Southborough, Massachusetts

Hung Fan, MD
Professor and Director
Cancer Research Institute
Department of Molecular Biology and Biochemistry
University of California, Irvine
Irvine, California

Patrizia Farci, MD
Senior Investigator and Chief
Hepatic Pathogenesis Section, Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Heinz Feldmann, MD
Chief
Laboratory of Virology
Division of Intramural Research
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Hamilton, Montana

Eric O. Freed, PhD
Head, Virus-Cell Interaction Section
HIV Drug Resistance Program
Frederick National Laboratory for Cancer Research
National Cancer Institute
National Institutes of Health
Frederick, Maryland

Paul D. Friesen, PhD
Professor
Institute for Molecular Virology
University of Wisconsin, Madison
Madison, Wisconsin

Masahiro Fujii, MD, PhD
Professor
Division of Virology
Niigata University Graduate School of Medical and Dental Sciences
Niigata, Japan

Thomas W. Geisbert, PhD
Professor
Departments of Microbiology and Immunology
University of Texas Medical Branch at Galveston
Galveston, Texas

James E. Gern, MD
Professor of Pediatrics and Medicine
Department of Pediatrics
University of Wisconsin School of Medicine and Public Health
Medical Staff, Department of Pediatrics
University of Wisconsin Hospital and Clinics
American Family Children's Hospital
Madison, Wisconsin

Don Gilden, MD
Louise Baum Endowed Chair and Professor
Department of Neurology
University of Colorado School of Medicine
Neurologist
University of Colorado Denver
Aurora, Colorado

Stephen P. Goff, PhD
Higgins Professor of Biochemistry
Department of Biochemistry and Molecular Biophysics
Columbia University and Howard Hughes Medical Institute
New York, New York

Barney S. Graham, MD, PhD
Senior Investigator
Chief, Viral Pathogenesis Laboratory
Chief, Clinical Trials Core
Vaccine Research Center
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Kim Y. Green, PhD
Senior Investigator
Caliciviruses Section, Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Harry B. Greenberg, MD
Joseph D. Grant Professor
Departments of Medicine and Microbiology and Immunology
Stanford University School of Medicine
Stanford, California

Diane E. Griffin, MD, PhD
Professor and Chair
W. Harry Feinstone Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health
Baltimore, Maryland

Paul D. Griffiths, MD, DSc
Professor of Virology
Centre for Virology
University College London
Professor of Virology
Royal Free London NHS Foundation Trust
London, United Kingdom

Young S. Hahn, PhD
Professor
Department of Microbiology, Immunology, and Cancer Biology
University of Virginia School of Medicine
Charlottesville, Virginia

Stephen C. Harrison, PhD
Giovanni Armenise–Harvard Professor of Basic Biomedical Sciences
Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School and Howard Hughes Medical Institute
Boston, Massachusetts

Mark T. Heise, PhD
Associate Professor
Department of Genetics, Microbiology, and Immunology
University of North Carolina
Chapel Hill, North Carolina

Ari Helenius, PhD
Professor
Institute of Biochemistry
ETH Zurich
Zurich, Switzerland

Roger W. Hendrix, PhD
Distinguished Professor
Department of Biological Science
University of Pittsburgh
Pittsburgh, Pennsylvania

Christiane Herden, Prof Dr habil
Full Professor
Institute of Veterinary Pathology
Justus-Liebig-University Giessen
Giessen, Germany

Tom C. Hobman, PhD
Professor
Department of Cell Biology
University of Alberta
Edmonton, Alberta, Canada

F. Blaine Hollinger, MD
Professor of Medicine, Molecular Virology, and Epidemiology
Director, Eugene B. Casey Hepatitis Research Center
Departments of Molecular Virology and Microbiology and Medicine
Baylor College of Medicine
Houston, Texas

Edward C. Holmes, MD
NHMRC Australia Fellow
School of Biological Sciences and Sydney Medical School
The University of Sydney
Sydney, New South Wales, Australia

Peter M. Howley, MD
Shattuck Professor of Pathological Anatomy
Department of Microbiology and Immunobiology
Harvard Medical School
Boston, Massachusetts

Eric Hunter, PhD
Professor
Department of Pathology and Laboratory Medicine
Emory University
Atlanta, Georgia

Michael J. Imperiale, PhD
Arthur F. Thurnau Professor
Department of Microbiology and Immunology
University of Michigan
Ann Arbor, Michigan

Michael G. Ison, MD, MS, FIDSA
Associate Professor
Divisions of Infectious Diseases and Organ Transplantation
Northwestern University Feinberg School of Medicine
Director
Northwestern University Comprehensive Transplant Center
Northwestern Memorial Hospital
Chicago, Illinois

Akiko Iwasaki, PhD
Professor
Departments of Immunobiology and Molecular, Cellular, and Developmental Biology
Yale University School of Medicine
New Haven, Connecticut

Ruth A. Karron, MD
Professor
Department of International Health
Director, Center for Immunization Research
Johns Hopkins Bloomberg School of Public Health
Baltimore, Maryland

Yoshihiro Kawaoka, DVM, PhD
Professor
Influenza Research Institute
Department of Pathobiological Sciences
School of Veterinary Medicine
University of Wisconsin, Madison
Madison, Wisconsin
Director and Professor
International Research Center for Infectious Diseases
Division of Virology, Department of Microbiology and Immunology
Institute of Medical Sciences
University of Tokyo
Tokyo, Japan

Elliott Kieff, MD, PhD
Albee Professor of Microbiology and Immunobiology
Harvard Medical School
Channing Laboratory
Albee Professor of Infectious Diseases
Department of Medicine
Brigham and Women's Hospital
Boston, Massachusetts

Marjolein Kikkert, PhD
Associate Professor
Department of Medical Microbiology
Leiden University Medical Center
Leiden, The Netherlands

David M. Knipe, PhD
Higgins Professor of Microbiology and Molecular Genetics
Department of Microbiology and Immunobiology
Chair, Harvard Program in Virology
Harvard Medical School
Boston, Massachusetts

Richard A. Koup, MD
Chief
Immunology Laboratory
Vaccine Research Center
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Richard J. Kuhn, PhD
Professor and Head
Department of Biological Sciences
Purdue University
West Lafayette, Indiana

Daniel R. Kuritzkes, MD
Professor of Medicine
Harvard Medical School
Chief, Division of Infectious Diseases
Brigham and Women's Hospital
Boston, Massachusetts

Ivan V. Kuzmin, PhD
Director
Aravan, LLC (Biomedical Consulting)
Lilburn, Georgia

Robert A. Lamb, PhD, ScD
John Evans Professor of Molecular and Cellular Biology
Investigator, Howard Hughes Medical Institute
Department of Molecular Biosciences
Northwestern University
Evanston, Illinois

Julie E. Ledgerwood, DO
Senior Clinician
Deputy Chief, Clinical Trials Core
Vaccine Research Center
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Dirk Lindemann, PhD
Professor
Institute of Virology
Technische Universität Dresden
Dresden, Germany

Brett D. Lindenbach, PhD
Associate Professor
Department of Microbial Pathogenesis
Yale University School of Medicine
New Haven, Connecticut

W. Ian Lipkin, MD
John Snow Professor of Epidemiology and Director
Center for Infection and Immunity
Mailman School of Public Health
Professor of Pathology and Neurology
College of Physicians and Surgeons
Columbia University
New York, New York

Richard M. Longnecker, PhD
Dan and Bertha Spear Research Professor
Department of Microbiology and Immunology
Northwestern University Medical School
Chicago, Illinois

Douglas R. Lowy, MD
Chief
Laboratory of Cellular Oncology
Center for Cancer Research
Deputy Director, National Cancer Institute
Bethesda, Maryland

Douglas S. Lyles, PhD
Professor and Chair
Department of Biochemistry
Wake Forest School of Medicine
Winston-Salem, North Carolina

John S. Mackenzie, PhD
Professor of Tropical Infectious Diseases
Faculty of Health Sciences
Curtin University
Perth, Western Australia
Honorary Senior Principal Fellow
Burnet Institute
Melbourne, Australia

Eugene O. Major, PhD
Senior Investigator
Laboratory of Molecular Medicine and Neuroscience
National Institute of Neurological Disorders and Stroke
National Institutes of Health
Bethesda, Maryland

Annette Martin, PhD
Senior Scientist and Principal Investigator
Department of Virology
Institut Pasteur
Paris, France

Malcolm A. Martin, MD
Chief
Laboratory of Molecular Microbiology
Division of Intramural Research
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

William S. Mason, PhD
Professor
Department of Virology
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Paul S. Masters, PhD
Chief
Laboratory of Viral Replication and Vector Biology
Division of Infectious Diseases
Wadsworth Center
New York State Department of Health
Associate Professor
Department of Biomedical Sciences
School of Public Health
University at Albany
State University of New York
Albany, New York

Masao Matsuoka, MD, PhD
Professor and Director
Institute for Virus Research
Kyoto University
Kyoto, Japan

Ruslan Medzhitov, PhD
David W. Wallace Professor of Immunobiology
Investigator, Howard Hughes Medical Institute
Chevy Chase, MD

Ernesto Méndez, PhD (deceased)
Associate Professor
Department of Developmental Genetics and Molecular Physiology
Instituto de Biotecnologia
Universidad Nacional Autonoma de Mexico
Cuernavaca, Morelos, Mexico

Xiang-Jin Meng, MD, PhD
Professor of Molecular Virology
Department of Biomedical Sciences and Pathobiology
Virginia Polytechnic Institute and State University
Blacksburg, Virginia

W. Allen Miller, PhD
Professor
Department of Plant Pathology and Microbiology
Iowa State University
Ames, Iowa

Edward S. Mocarski Jr., PhD
Robert W. Woodruff Professor
Department of Microbiology and Immunology
Emory University School of Medicine
Atlanta, Georgia

Yasuko Mori, MD, PhD
Professor
Department of Microbiology and Infectious Diseases
Division of Clinical Virology
Kobe University Graduate School of Medicine
Kobe, Japan

Bernard Moss, MD, PhD
Chief
Laboratory of Viral Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

William J. Moss, MD, MPH
Professor
Departments of Epidemiology, International Health, and Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health
Baltimore, Maryland

Catherine L. Murray, PhD
Novartis Institutes for BioMedical Research
Infectious Disease Area
Emeryville, California

Neal Nathanson, MD
Associate Dean, Global Health Programs
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania

Gabriele Neumann, PhD
Research Professor
Influenza Research Institute
Department of Pathobiological Sciences
School of Veterinary Medicine
University of Wisconsin, Madison
Madison, Wisconsin

M. Steven Oberste, PhD
Chief
Polio and Picornavirus Laboratory Branch
Centers for Disease Control and Prevention
Atlanta, Georgia

Peter Palese, PhD
Professor and Chair
Department of Microbiology
Mount Sinai School of Medicine
New York, New York

Mark A. Pallansch, PhD
Director
Division of Viral Diseases
Centers for Disease Control and Prevention
Atlanta, Georgia

Ann C. Palmenberg, PhD
Professor
Institute for Molecular Virology
University of Wisconsin, Madison
Madison, Wisconsin

John S. L. Parker, BVMS, PhD
Associate Professor
Baker Institute for Animal Health
Cornell University
Ithaca, New York

Griffith D. Parks, PhD
Professor and Chair
Department of Microbiology and Immunology
Wake Forest School of Medicine
Winston-Salem, North Carolina

Colin R. Parrish, PhD
Professor of Virology
Baker Institute for Animal Health
College of Veterinary Medicine
Cornell University
Ithaca, New York

Robert F. Pass, MD
Professor
Departments of Pediatrics and Microbiology
University of Alabama at Birmingham
Director, Hospital Medicine
Department of Pediatrics
Children's of Alabama
Birmingham, Alabama

Philip E. Pellett, PhD
Professor
Department of Immunology and Microbiology
Wayne State University
Detroit, Michigan

Stanley Perlman, MD, PhD
Professor
Departments of Microbiology and Pediatrics
University of Iowa
Iowa City, Iowa

Clarence J. Peters, MD
Professor
Department of Microbiology and Immunology
University of Texas Medical Branch
Galveston, Texas

Theodore C. Pierson, PhD
Senior Investigator
Laboratory of Viral Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Stanley B. Prusiner, MD
Director
Institute for Neurodegenerative Diseases
Professor
Department of Neurology
University of California, San Francisco
San Francisco, California

Robert H. Purcell, MD (Retired)
Distinguished Investigator
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Vincent R. Racaniello, PhD
Higgins Professor
Department of Microbiology and Immunology
Columbia University
College of Physicians and Surgeons
New York, New York

Didier Raoult, Professor, MD, PhD
Professor
URMITE, Inserm 1095
Aix Marselle Université
Professor
Pôle Infectieux
CHU de la Timone
Marseille, France

Stuart C. Ray, MD
Professor
Fellowship Program Director
Department of Medicine, Infectious Diseases
Johns Hopkins Medical Institutions
Baltimore, Maryland

Axel Rethwilm, MD
Head of Institute
Institute for Virology
University of Wuerzburg
Wuerzburg, Germany

Charles M. Rice, PhD
Professor and Head
Laboratory of Virology and Infectious Disease
Rockefeller University
New York, New York

Douglas D. Richman, MD
Florence Seeley Riford Distinguished Professor
Director, Center for AIDS Research
Departments of Pathology and Medicine
University of California, San Diego
La Jolla, California

Jürgen A. Richt, DVM, PhD
Regents Distinguished Professor
Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine
Kansas State University
Manhattan, Kansas

Bernard Roizman, ScD
Joseph Regenstein Distinguished Service Professor
Departments of Microbiology and Molecular Genetics
and Cell Biology
University of Chicago
Chicago, Illinois

Polly Roy, MSc, PhD, FMed Sci
Professor
Department of Infectious and Tropical Diseases
London School of Hygiene and Tropical Medicine
London, United Kingdom

Steven A. Rubin, PhD
Senior Investigator
Center for Biologics Evaluation and Research
U.S. Food and Drug Administration
Bethesda, Maryland

Charles E. Rupprecht, VMD, MS, PhD
Director of Research
The Global Alliance for Rabies Control
Manhattan, Kansas

Anthony Sanchez, PhD
Associate Director for Laboratory Science
Division of High-Consequence Pathogens and Pathology
Centers for Disease Control and Prevention
Atlanta, Georgia

Christian J. Sauder, PhD
Staff Scientist
Center for Biologics Evaluation and Research
U.S. Food and Drug Administration
Bethesda, Maryland

John T. Schiller, PhD
Senior Investigator
Laboratory of Cellular Oncology
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

Connie S. Schmaljohn, PhD
Senior Scientist, Infectious Diseases
U.S. Army Medical Research Institute of Infectious Diseases
Ft. Detrick, Frederick, Maryland

Christoph Seeger, PhD
Professor
Department of Virology
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Megan L. Shaw, PhD
Associate Professor
Department of Microbiology
Mount Sinai School of Medicine
New York, New York

Thomas Shenk, PhD
Professor
Department of Molecular Biology
Princeton University
Princeton, New Jersey

Barbara Sherry, PhD
Professor
Department of Molecular Biomedical Sciences
North Carolina State University
Raleigh, North Carolina

Eric J. Snijder, PhD
Professor of Molecular Virology
Department of Medical Microbiology
Leiden University Medical Center
Leiden, The Netherlands

Gregory A. Storch, MD
Ruth L. Siteman Professor of Pediatrics
Professor of Medicine and of Molecular Microbiology
Department of Pediatrics
Washington University in St. Louis School of Medicine
Medical Director, Clinical Laboratories
St. Louis Children's Hospital
St. Louis, Missouri

John M. Taylor, PhD
Professor Emeritus
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Prof. Dr. Heinz-Jürgen Thiel
Professor
Institute of Virology
Justus-Liebig University
Head of Institute
Veterinary Medicine
Giessen, Germany

David L. Thomas, MD, MPH
Chief
Division of Infectious Diseases
Stanhope Baine Jones Professor of Medicine
Johns Hopkins School of Medicine
Baltimore, Maryland

Herbert W. Virgin IV, MD, PhD
Edward Mallinckrodt Professor and Head
Department of Pathology and Immunology
Washington University School of Medicine
St. Louis, Missouri

David Wang, PhD
Associate Professor
Departments of Molecular Microbiology and Pathology and Immunology
Washington University in St. Louis
St. Louis, Missouri

Lin-Fa Wang, PhD
Science Leader
CSIRO Australian Animal Health Laboratory
Geelong, Australia
Director and Professor
Program in Emerging Infectious Diseases
Duke–NUS Graduate Medical School
Singapore

Sean Whelan, PhD
Professor
Department of Microbiology and Immunobiology
Harvard Medical School
Boston, Massachusetts

Steven A. Whitham, PhD
Professor
Department of Plant Pathology and Microbiology
Iowa State University
Ames, Iowa

Richard J. Whitley, MD
Distinguished Professor
Department of Pediatrics
Division Director
Department of Pediatric Infectious Diseases
Vice Chair
Department of Pediatrics
University of Alabama at Birmingham
Birmingham, Alabama

J. Lindsay Whitton, MD, PhD
Professor
Department of Immunology and Microbial Science
Scripps Research Institute
La Jolla, California

Reed B. Wickner, MD
Chief
Laboratory of Biochemistry and Genetics
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Maryland

William S.M. Wold, PhD
Professor and Chairman
Department of Molecular Microbiology and Immunology
Saint Louis University
St. Louis, Missouri

Peter F. Wright, MD
Professor
Department of Pediatrics
Geisel School of Medicine at Dartmouth University
Lebanon, New Hampshire

Koichi Yamanishi, MD, PhD
Director General
National Institute of Biomedical Innovation
Osaka, Japan

Fabien Zoulim, MD
Professor and Head of Laboratory
Viral Hepatitis Laboratory
INSERM Unit 1052
Professor and Head of Department
Department of Hepatology
Hospices Civils de Lyon
Lyon, France

PREFACE

In the early 1980s, Bernie Fields originated the idea of a virology reference textbook that combined the molecular aspects of viral replication with the medical features of viral infections. This broad view of virology reflected Bernie's own research, which applied molecular and genetic analyses to the study of viral pathogenesis, providing an important part of the foundation for the field of molecular pathogenesis. Bernie led the publication of the first three editions of *Virology* but unfortunately died soon after the third edition went into production. The third edition became *Fields Virology* in his memory, and it is fitting that the book continues to carry his name.

We are pleased that the printed book of the sixth edition of *Fields Virology* contains four-color art throughout and that an e-book version accompanies the printed book as well. We have increased the numbers of figures in each chapter, and with the color and availability of the figures from the e-book for use as slides, most chapters should have sufficient figures for slides for one lecture. There have been continued significant advances in virology since the previous edition 6 years ago, and all of the chapters have been updated to reflect these advances. Our increased knowledge of virology has caused us to use shortened lists of key references (up to 200 in most cases) in the printed book to save space, whereas complete reference lists appear as part of the e-book. We have retained the general organization of the earlier editions for the sixth edition of *Fields Virology*. Section I contains chapters on general aspects of virology, and Section II contains chapters on replication and medical aspects of specific virus families and specific viruses of medical importance. In Section I, we have added a new emphasis on virus discovery in the Diagnostic Virology chapter and emerging viruses in the Epidemiology chapter to address the interest in discovery of new viruses and emerging viruses. In Section II, we have added new chapters on circoviruses and mimiviruses and have added a new section on Chikungunya virus to the alphavirus chapter.

Numerous chapters have been updated to include the latest information on outbreaks during the past 5 years, including pandemic H1N1 influenza, new adenovirus serotypes, noroviruses, human polyomaviruses, the re-emergence of West Nile virus in North America, novel coronaviruses, novel Coxsackie and rhino viruses, and other emerging and re-emerging viruses. Important advances in antivirals, including new hepatitis C virus protease inhibitors and HIV integrase inhibitors, have been described. As with the previous edition, we have continued to combine the medical and replication chapters into a single chapter to eliminate duplication and to present a more coherent presentation of that specific virus or virus family. The main emphasis continues to be on viruses of medical importance and interest; however, other viruses are described in specific cases where more is known about their mechanisms of replication or pathogenesis. Although not formally viruses, prions are still included in this edition for historical reasons and because of the intense interest in the infectious spongiform encephalopathies.

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David M. Knipe, PhD
Peter M. Howley, MD
Jeffrey I. Cohen, MD
Diane E. Griffin, MD, PhD
Robert A. Lamb, PhD, ScD
Malcolm A. Martin, MD
Vincent R. Racaniello, PhD
Bernard Roizman, ScD

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General Virology

SECTION I

CHAPTER 1

Lynn W. Enquist • Vincent R. Racaniello

Virology: From Contagium Fluidum to Virome

Virology has had a remarkable history. Even though humans did not realize viruses existed until the late 1880s, viral diseases have shaped the history and evolution of life on the planet. As far as we know, all living organisms, when studied carefully, are infected by viruses. These smallest microbes exert significant forces on every living thing, including themselves. The consequences of viral infections have not only altered human history, they have powerful effects on the entire ecosystem. As a result, virologists have gone to extraordinary lengths to study, understand, and eradicate these agents. It is noteworthy that just as the initial discovery of viruses required new technology (porcelain filters), uncovering the amazing biology underlying viral infections has gone hand in hand with new technology developments. Indeed, virologists have elucidated new principles of life processes and have been leaders in promoting new directions in science. For example, many of the concepts and tools of molecular biology and cell biology have been derived from the study of viruses and their host cells. This chapter is an attempt to review selected portions of this history as it relates to the development of new concepts in virology.

THE CONCEPT OF VIRUSES AS INFECTIOUS AGENTS

A diverse microbial world of bacteria, fungi, and protozoa had been widely accepted by the last half of the 19th century. An early proponent of the germ theory of disease was the noted German anatomist Jacob Henle of Gottingen (the discoverer of Henle's loop and the grandfather of 20th-century virologist Werner Henle). He hypothesized in 1840 that specific diseases were caused by infectious agents that were too small to be observed with the light microscope. However, he had no evidence for such entities, and consequently his ideas were not generally accepted. It would take the work of Louis Pasteur and Henle's student, Robert Koch, before it became evident that microbes could cause diseases.

Three major advances in microbiology came together to set the stage for the development of the concept of a submicroscopic agent that would come to be called a virus (e-Table 1.1). The first advance concerned spontaneous generation of organisms, which for years had been both supported and refuted by a variety of experiments. Louis Pasteur (1822–1895) used his swan-neck flasks to strike a mortal blow to the concept of spontaneous generation. Afterward Pasteur went on to study fermentation by different microbial agents. From his work he concluded that "different kinds of microbes are associated with different kinds of fermentations," and he soon extended this concept to diseases. Pasteur's reasoning strongly influenced Robert Koch (1843–1910), a student of Jacob Henle and a country doctor in a small German village. Koch developed solid media to isolate colonies of bacteria to produce pure cultures, and stains to visualize the microorganisms. With these tools in hand, Koch identified the bacterium that causes anthrax (*Bacillus anthracis,* 1876) and tuberculosis (*Mycobacterium tuberculosis,* 1882). Joseph Lister (1827–1912), a professor of surgery in Glasgow, had heard about Pasteur's work, and he surmised that a sterile field should be maintained during surgery. Although many other scientists of that day contributed tools and concepts, it was principally Pasteur, Lister, and Koch who put together a new experimental approach for medical science.

These observations led Robert Koch to formalize some of Jacob Henle's original ideas for defining whether a microorganism is the causative agent of a disease. Koch's postulates state that (a) the organism must be regularly found in the lesions of the disease, (b) the organism must be isolated in pure culture, (c) inoculation of such a pure culture of organisms into a host should initiate the disease, and (d) the organism must be recovered once again from the lesions of the host. By the end of the 19th century, these concepts outlined an experimental method that became the dominant paradigm of medical microbiology. It was only when these rules broke down and failed to yield a causative agent that the concept of a virus was born.

THE BIRTH OF VIROLOGY

Pathogen Discovery, 1886–1903 (e-Table 1.1)

Adolf Mayer (1843–1942) was a German agricultural chemist and director of the Agricultural Experiment Station at Wageningen in The Netherlands when he was asked to investigate a disease of tobacco. He named the affliction tobacco mosaic disease after the dark and light spots that appeared on infected leaves (e-Fig. 1.1). To investigate the nature of the disease, Mayer inoculated healthy plants with the juice extracted from diseased plants by grinding up the infected leaves in water. Mayer reported that, "in nine cases out of ten (of inoculated plants), one will be successful in making the healthy plant…heavily diseased".[131] Although these studies established the infectious nature of the tobacco mosaic disease, neither a bacterial agent nor a fungal agent could be consistently cultured or detected in these extracts, so Koch's postulates could not be satisfied. In a preliminary communication in 1882,[130] Mayer speculated that the cause could be a "soluble, possibly enzyme-like contagium, although almost any analogy for such a supposition is failing in science." Later Mayer concluded that the mosaic disease "is bacterial, but that the infectious forms have not yet been isolated, nor are their forms and mode of life known".[131]

A few years later, Dimitri Ivanofsky (1864–1920), a Russian scientist working in St. Petersburg, was commissioned by the Russian Department of Agriculture to investigate the cause of a tobacco disease on plantations in Bessarabia, Ukraine, and the Crimea. Ivanofsky repeated Mayer's observations by showing that the sap of infected plants contained an agent that could transmit the disease to healthy plants. But he added an important step—before the inoculation step, he passed the infected sap through a Chamberland filter (e-Fig. 1.2). This device, made of unglazed porcelain and perfected by Charles Chamberland, one of Pasteur's collaborators, contained pores small enough to retard most bacteria. Ivanofsky reported to the Academy of Sciences of St. Petersburg on February 12, 1892, that "the sap of leaves infected with tobacco mosaic disease retains its infectious properties even after filtration through Chamberland filter candles".[94]

Ivanofsky, like Mayer before him, failed to culture an organism from the filtered sap and could not satisfy Koch's postulates. Consequently he suggested that a toxin (not a living, reproducing substance) might pass through the filter and cause the disease. As late as 1903, when Ivanofsky published his thesis,[95] he still believed that he had been unable to culture the bacteria that caused this disease. Bound by the dogma of Koch's postulates, Ivanofsky could not make a conceptual leap. It is therefore not surprising that Pasteur, who worked on the rabies vaccine[145] at the same time (1885), never investigated the unique nature of the infectious agent.

The conceptual leap was provided by Martinus Beijerinck (1851–1931), a Dutch soil microbiologist who collaborated with Adolf Mayer at Wageningen. Unaware of Ivanofsky's work, in 1898 Beijerinck independently found that the sap of infected tobacco plants could retain its infectivity after passage through a Chamberland filter. But he also showed that the filtered sap could be diluted and regain its "strength" after replication in living, growing tissue of the plant. This observation showed that the agent could reproduce (therefore, it was not a toxin) but only in living tissue, not in the cell-free sap of the plant. Suddenly it became clear why others could not culture the pathogen outside its host. Beijerinck called this agent a *contagium vivum fluidum*,[10] or a contagious living liquid. He sparked a 25-year debate about whether these novel agents were liquids or particles. This conflict was resolved when d'Herelle developed the plaque assay in 1917[36] and when the first electron micrographs were taken of tobacco mosaic virus (TMV) in 1939.[104]

Mayer, Ivanofsky, and Beijerinck each contributed to the development of a new concept: a novel organism smaller than bacteria—an agent defined by the pore size of the Chamberland filter—that could not be seen in the light microscope, and could multiply only in living cells or tissue. The term *virus,* from the Latin for slimy liquid or poison,[89] was at that time used interchangeably for any infectious agent, and so the agent of tobacco mosaic disease was called tobacco mosaic virus, or TMV. The literature of the first decades of the 20th century often referred to these infectious entities as filterable agents, and this was indeed the operational definition of viruses. Sometime later, the term *virus* became restricted in use to those agents that fulfilled the criteria developed by Mayer, Ivanofsky, and Beijerinck, and that were the first agents to cause a disease that could not be proven by using Koch's postulates.

Shortly after this pioneering work on TMV, the first filterable agent from animals was identified by Loeffler and Frosch—foot-and-mouth disease virus.[122] The first human virus discovered was yellow fever virus (1901), by Walter Reed and his team in Cuba.[154]

The years from 1930 to 1956 were replete with the discovery of a plethora of new viruses (e-Table 1.2). In fact, in this short time, virologists found most of the viruses we now know about. More fascinating perhaps is that these studies laid the groundwork for the birth of molecular virology.

Plant Viruses and the Chemical Period: 1929–1956

For the next 50 years, TMV played a central role in research that explored the nature and properties of viruses. With the development of techniques to purify proteins in the first decades of the 20th century came the appreciation that viruses were proteins and so could be purified in the same way. Working at the Boyce Thompson Institute in Philadelphia, Vinson and Petre (1927–1931) precipitated infectious TMV—using an infectivity assay developed by Holmes[88]—from the crude sap of infected plants using selected salts, acetone, or ethyl alcohol.[193] They showed that the infectious virus could move in an electric field, just as proteins did. At the same time, H. A. Purdy-Beale, also at the Boyce Thompson Institute, produced antibodies in rabbits that were directed against TMV and could neutralize the infectivity of this agent.[151] This observation was taken as further proof of the protein nature of viruses, although it was later realized that antibodies recognize chemicals other than proteins. With the advent of purification procedures for viruses, both physical and chemical measurements of the virus became possible. The strong flow birefringence of purified preparations of TMV was interpreted (correctly) to show an asymmetric particle or rod-shaped particle.[180] Max Schlesinger,[167] working on purified preparations of bacteriophages in Frankfurt, Germany, showed that the virions were composed of proteins and contained phosphorus and ribonucleic acid. This observation led to the first suggestion that viruses were composed of nucleoproteins. The crystallization of TMV in 1935 by Wendell Stanley,[173] working at the Rockefeller Institute branch in Princeton, New Jersey, brought this infectious agent into the world of the chemists. Within a year, Bawden and Pirie[8,9] had demonstrated that crystals of TMV contained 0.5% phosphorus and 5% RNA. The first "view" of a virus came from x-ray crystallography using these crystals to show rods of a constant diameter aligned in hexagonal arrays containing RNA and protein.[16] The first electron micrographs of any virus were of TMV, and they confirmed that the virus particle is shaped like a rod[105] (e-Fig. 1.3).

The x-ray diffraction patterns[16] suggested that TMV was built from repeating subunits. These data and other considerations led Crick and Watson[33] to realize that most simple viruses had to consist of one or a few species of identical protein subunits. By 1954–1955, techniques had been developed to dissociate TMV protein subunits, allowing reconstitution of infectious TMV from its RNA and protein subunits[64] and leading to an understanding of the principles of virus self-assembly.[25]

The concept that viruses contained genetic information emerged as early as 1926, when H. H. McKinney reported the isolation of "variants" of TMV with a different plaque morphology that bred true and could be isolated from several geographic locations.[132,133] Seven years later, Jensen confirmed McKinney's observations[101] and showed that the plaque morphology phenotype could revert. Avery's DNA transformation experiments with pneumococcus[5] and the Hershey-Chase experiment with bacteriophages,[83] both demonstrated that DNA was genetic material. TMV had been shown to contain RNA, not DNA, and this nucleic acid was shown to be infectious, and therefore comprise the genetic material of the virus, in 1956[64,72]—the first demonstration that RNA could be a genetic material. Studies on the nucleotide sequence of TMV RNA confirmed codon assignments for the genetic code, added clear evidence for the universality of the genetic code, and helped to elucidate the mechanisms of mutation by diverse agents.[63] Research on TMV and related plant viruses has contributed significantly to both the origins of virology and its development as a science.

BACTERIOPHAGES

Early Years: 1915–1940

Frederick W. Twort was superintendent of the Brown Institution in London when he discovered viruses of bacteria in 1915. In his research, Twort was searching for variants of vaccinia virus (the smallpox vaccine virus), which would replicate in simple defined media outside living cells. In one of his experiments, he inoculated nutrient agar with an aliquot of the smallpox vaccine. The virus failed to replicate, but bacterial contaminants flourished on the agar medium. Twort noticed that some of these bacterial colonies changed visibly with time and became "watery looking" (i.e., more transparent). The bacteria within these colonies were apparently dead, as they could no longer form new colonies on fresh agar plates. He called this phenomenon glassy transformation. Simply adding the glassy transforming principle could rapidly kill a colony of bacteria. It readily passed through a porcelain filter, could be diluted a million-fold, and when placed upon fresh bacteria would regain its strength, or titer.[188–190]

Twort published these observations in a short note[190] in which he suggested that a virus of bacteria could explain glassy transformation. He then went off to serve in World War I, and when he returned to London, he did not continue this research.

While Twort was puzzled by glassy transformation, Felix d'Herelle, a Canadian medical bacteriologist, was working at the Pasteur Institute in Paris. When a *Shigella* dysentery infection devastated a cavalry squadron of French soldiers just outside of Paris in August 1915, d'Herelle readily isolated and cultured the dysentery bacillus from filtered fecal emulsions. The bacteria multiplied and covered the surface of his agar plates, but occasionally d'Herelle observed clear circular spots devoid of growth. He called these areas *taches vierges,* or plaques. He followed the course of an infection in a single patient, noting when the bacteria were most plentiful and when the plaques appeared.[35,36] Plaques appeared on the fourth day after infection and killed the bacteria in the culture dish, after which the patient's condition began to improve.

d'Herelle found that a filterable agent, which he called a bacteriophage, was killing the *Shigella* bacillus. In the ensuing years he developed fundamental techniques in virology that are utilized to this day, such as the use of limiting dilutions to

determine the virus titer by plaque assay. He reasoned that the appearance of plaques showed that the virus was particulate, or "corpuscular," and not a liquid as Beijerinck had insisted. d'Herelle also found that if virus was mixed with a host cell and then subjected to centrifugation, the virus was no longer present in the supernatant fluid. He interpreted this to mean that the first step of a virus infection is attachment, or adsorption, of virus to the host cell. Furthermore, viral attachment occurred only when bacteria sensitive to the virus were used, demonstrating that host specificity can be conferred at a very early step in infection. Lysis of cells and the release of infectious virus were also described in startlingly modern terms. d'Herelle clearly established many of the principles of modern virology.[34,35]

Although d'Herelle's bacteriophages lysed their host cells, by 1921 it had become apparent that under certain situations the virus and cell existed peacefully—a condition called lysogeny. In some experiments it became impossible to separate the virus from its host. This conundrum led Jules Bordet of the Pasteur Institute in Brussels to suggest that the transmissible agent described by d'Herelle was nothing more than a bacterial enzyme that stimulates its own production.[22] Although incorrect, the hypothesis has remarkable similarities to modern ideas about prion structure and replication (see Chapter 77).

During the 1920s and 1930s, d'Herelle sought ways to use bacteriophages for medical applications, but he never succeeded. Furthermore, the basic research of the era was frequently dominated by the interpretations of scientists with the strongest personalities. Although it was clear that there were many diverse bacteriophages, and that some were lytic while some were lysogenic, their interrelationships remained ill defined. The highlight of this period was the demonstration by Max Schlesinger that purified phages had a maximum linear dimension of 0.1 micron and a mass of about 4×10^{-16} grams, and that they were composed of protein and DNA in roughly equal proportions.[166,167] In 1936, no one quite knew what to make of that observation, but over the next 20 years it would begin to make a great deal of sense.

Phages and the Birth of Molecular Biology: 1938–1970 (e-Table 1.3)

Max Delbrück was trained as a physicist at the University of Göttingen, and his first position was at the Kaiser Wilhelm Institute for Chemistry in Berlin. There he joined a diverse group of individuals who were actively discussing how quantum physics related to an understanding of heredity. Delbrück's interest in this area led him to develop a quantum mechanical model of the gene, and in 1937 he moved to the biology division at the California Institute of Technology to study genetics of *Drosophila.* Once there, he became interested in bacteria and their viruses, and teamed up with another research fellow, Emory Ellis,[51] who was working with the T-even group of bacteriophages, T2, T4, and T6. Delbrück soon appreciated that these viruses were ideal for the study of virus replication, because they allowed analysis of how genetic information could determine the structure and function of an organism. Bacteriophages were also viewed as model systems for understanding cancer viruses or even for understanding how a sperm fertilizes an egg and a new organism develops. Together with Ellis, Delbrück showed that viruses reproduced in one step, in contrast to the multiplication of other organisms by binary fission.[52] This conclusion was drawn from the elegant one-step growth curve experiment, in which an infected bacterium liberates hundreds of phages synchronously after a half-hour period during which viral infectivity was lost (e-Fig. 1.4). The one-step growth curve became the experimental paradigm of the phage group.

When World War II erupted, Delbrück remained in the United States (at Vanderbilt University) and met an Italian refugee, Salvador E. Luria, who had fled to America and was working at Columbia University in New York (on bacteriophages T1 and T2). After their encounter at a meeting in Philadelphia on December 28, 1940, they went to Luria's laboratory at Columbia where they spent 48 hours doing experiments with bacteriophages. These two scientists eventually established the "phage group," a community of researchers focused on using bacterial viruses as a model for understanding life processes. Luria and Delbrück were invited to spend the summer of 1941 at Cold Spring Harbor Laboratory, where they pursued research on phages. The result was that a German physicist and an Italian geneticist joined forces during the war years to travel throughout the United States and recruit a new generation of biologists (e-Fig. 1.5).

When Tom Anderson, an electron microscopist at the RCA Laboratories in Princeton, New Jersey, met Delbrück, the result was the first clear pictures of bacteriophages.[126] At the same time, the first phage mutants were isolated and characterized.[125] By 1946, the first phage course was being taught at Cold Spring Harbor, and in March 1947, the first phage meeting attracted eight people. From these humble beginnings grew the field of molecular biology, which focused on the bacterial host and its viruses.

Developing the Modern Concept of Virology (see e-Tables 1.3 to 1.5)

The next 25 years (1950–1975) was an intensely productive period of bacteriophage research. Hundreds of virologists produced thousands of publications that covered three major areas: (a) lytic infection of *Escherichia coli* with the T-even phages; (b) the nature of lysogeny, using lambda phage; and (c) the replication and properties of several unique phages such as ϕX174 (single-stranded circular DNA), the RNA phages, and T7. This work set the foundations for modern molecular virology and biology.

The idea of examining, at the biochemical level, the events occurring in phage-infected cells during the latent period had come into its own by 1947–1948. Impetus for this work came from Seymour Cohen, who had trained first with Erwin Chargaff at Columbia University, studying lipids and nucleic acids, and then with Wendell Stanley working on TMV RNA. His research direction was established when after taking Delbrück's 1946 phage course at Cold Spring Harbor, Cohen examined the effects of phage infection on DNA and RNA levels in infected cells using a colorimetric analysis. The results showed a dramatic alteration of macromolecular synthesis in infected cells. This included cessation of RNA accumulation, which later formed the basis for detecting a rapidly turning-over species of RNA and the first demonstration of messenger RNA (mRNA).[4] DNA synthesis also halted, but for 7 minutes, followed by resumption at a 5- to 10-fold increased rate. At the same time, Monod and Wollman showed that the synthesis of a cellular enzyme, the inducible β-galactosidase, was inhibited

after phage infection.[134] Based on these observations, the viral eclipse period was divided into an early phase, prior to DNA synthesis, and a late phase. More importantly, these results demonstrated that a virus could redirect cellular macromolecular synthetic processes in infected cells.[32]

By the end of 1952, two experiments had a critical effect on virology. First, Hershey and Chase asked whether viral genetic information is DNA or protein. They differentially labeled viral proteins ($^{35}SO_4$) and nucleic acids ($^{32}PO_4$), and allowed the "tagged" particles to attach to bacteria. When they sheared the viral protein coats from the bacteria using a Waring blender, only DNA was associated with the infected cells.[83] This result proved that DNA had all the information needed to reproduce new virus particles. A year later, the structure of DNA was elucidated by Watson and Crick, a discovery that permitted full appreciation of the Hershey-Chase experiment.[195] The results of these two experiments formed a cornerstone of the molecular biology revolution.[26]

While these blockbuster experiments were being carried out, G. R. Wyatt and S. S. Cohen were quietly making another seminal finding.[207] They identified a new base, hydroxymethylcytosine, in the DNA of T-even phages, which replaced cytosine. This began a 10-year study of how deoxyribonucleotides were synthesized in bacteria and phage-infected cells, and it led to the critical observation that the virus introduces genetic information for a new enzyme into the infected cell.[60] By 1964, Mathews and colleagues had proved that hydroxymethylase does not exist in uninfected cells and must be encoded by the virus.[32] These experiments introduced the concept of early enzymes, utilized in deoxypyrimidine biosynthesis and DNA replication,[109] and provided biochemical proof that viruses encode new information that is expressed as proteins in an infected cell. At the same time, phage genetics became extremely sophisticated, allowing mapping of the genes encoding these viral proteins. Perhaps the best example of genetic fine structure was done by Seymour Benzer, who carried out a genetic analysis of the rII A and B cistrons of T-even phages with a resolution of a single nucleotide (without doing any DNA sequencing!).[13] Studies on viral DNA synthesis, using phage mutants and cell extracts to complement and purify enzyme activities *in vitro,* contributed a great deal to our understanding of DNA replication.[1] A detailed genetic analysis of phage assembly, utilizing the complementation of phage assembly mutants *in vitro,* revealed how complex structures are built by living organisms using the principles of self-assembly.[47] The genetic and biochemical analysis of phage lysozyme helped to elucidate the molecular nature of mutations,[176] and the isolation of phage amber mutations (nonsense mutations) provided a clear way to study second-site suppressor mutations at the molecular level.[14] The circular genetic map of the T-even phages[176] was explained by the circularly permuted, terminally redundant (giving rise to phage heterozygotes) conformation of these DNAs.[186]

The remarkable reprogramming of viral and cellular protein synthesis in phage-infected cells was dramatically revealed by an early use of sodium dodecyl sulfate (SDS)–polyacrylamide gels,[112] showing that viral proteins are made in a specific sequence of events. The underlying mechanism of this temporal regulation led to the discovery of sigma factors modifying RNA polymerase and conferring gene specificity.[75] The study of gene regulation at almost every level (transcription, RNA stability, protein synthesis, protein processing) was revealed from a set of original contributions derived from an analysis of phage infections.

Although this remarkable progress had begun with the lytic phages, no one knew quite what to make of the lysogenic phages. This situation changed in 1949 when André Lwoff began his studies with *Bacillus megaterium* and its lysogenic phages at the Pasteur Institute. By using a micromanipulator, Lwoff could show that single lysogenic bacteria divided up to 19 times without liberating a virus particle. No virions were detected when lysogenic bacteria were broken open by the investigator. But from time to time a lysogenic bacterium spontaneously lysed and produced many viruses.[128] Ultraviolet light was found to induce the release of these viruses, a key observation that began to outline this curious relationship between a virus and its host.[129] By 1954, Jacob and Wollman[97,98] at the Pasteur Institute had made the important observation that a genetic cross between a lysogenic bacterial strain and a nonlysogenic recipient resulted in the induction of the virus after conjugation, a process they called zygotic induction. In fact, the position of the lysogenic phage or prophage in the chromosome of its host *E. coli* could be mapped by interrupting mating between two strains.[98] This experiment was crucial for our understanding of lysogenic viruses, because it showed that a virus behaved like a bacterial gene on a chromosome in a bacterium. It was also one of the first experimental results to suggest that the viral genetic material was kept quiescent in bacteria by negative regulation, which was lost as the chromosome passed from the lysogenic donor bacteria to the nonlysogenic recipient host. This conclusion helped Jacob and Monod to realize as early as 1954 that the "induction of enzyme synthesis and of phage development are the expression of one and the same phenomenon".[128] These experiments laid the foundation for the operon model and the nature of coordinate gene regulation.

Although the structure of DNA was elucidated in 1953[195] and zygotic induction was described in 1954, the relationship between the bacterial chromosome and the viral chromosome in lysogeny was still referred to as the attachment site and literally thought of in those terms. The close relationship between a virus and its host was appreciated only when Campbell proposed the model for lambda integration of DNA into the bacterial chromosome,[27] based on the fact that the sequence of phage markers was different in the integrated state than in the replicative or vegetative state. This model led to the isolation of the negative regulator or repressor of lambda, a clear understanding of immunity in lysogens, and one of the early examples of how genes are regulated coordinately.[150] The genetic analysis of the lambda bacteriophage life cycle is one of the great intellectual adventures in microbial genetics.[82] It deserves to be reviewed in detail by all students of molecular virology and biology.

The lysogenic phages such as P22 of *Salmonella typhimurium* provided the first example of generalized transduction,[210] whereas lambda provided the first example of specialized transduction.[137] The finding that viruses could not only carry within them cellular genes, but transfer those genes from one cell to another, provided not only a method for fine genetic mapping but also a new concept in virology. As the genetic elements of bacteria were studied in more detail, it became clear that there was a remarkable continuum from lysogenic phages to episomes,

transposons and retrotransposons, insertion elements, retroviruses, hepadnaviruses, viroids, and prions. Genetic information moves between viruses and their hosts to the point where definitions and classifications begin to blur. The genetic and biochemical concepts that emerged from the study of bacteriophages made the next phase of virology possible. The lessons of the lytic and lysogenic phages were often relearned and modified as the animal viruses were studied.

ANIMAL VIRUSES

Cell Culture Technology and Discovery: 1898–1965 (see e-Tables 1.1 to 1.3)

Once the concept of viruses as filterable agents took hold, many diseased animal tissues were subjected to filtration to determine if a virus were involved. Filterable agents were found that were invisible in a light microscope, and replicated only in living animal tissue. There were some surprises, such as the transmission of yellow fever virus by a mosquito vector,[154] specific visible pathologic inclusion bodies (virions and subviral particles) in infected tissue,[95,142] and even viral agents that can "cause cancer".[50,159]

Throughout this early time period (1900–1930), a wide variety of viruses were found (see e-Tables 1.1 and 1.2) and characterized with regard to their size (using the different pore sizes of filters), resistance to chemical or physical agents (e.g., alcohol, ether), and pathogenic effects. Based on these properties alone, it became clear that viruses were a very diverse group of agents. Some were even observable in the light microscope (vaccinia in dark-field optics). Some were inactivated by ether, whereas others were not. Viruses were identified that affected every tissue type. They could cause chronic or acute disease; they were persistent agents or recurred in a periodic fashion. Some viruses caused cellular destruction or induced cellular proliferation. For the early virologists, unable to see their agents in a light microscope and often confused by this great diversity, their studies certainly required an element of faith. In 1912, S. B. Wolbach, an American pathologist, remarked, "It is quite possible that when our knowledge of filterable viruses is more complete, our conception of living matter will change considerably, and that we shall cease to attempt to classify the filterable viruses as animal or plant".[204]

The way out of this early confusion was led by the plant virologists and the development of techniques to purify viruses and characterize both the chemical and physical properties of these agents (see previous section, The Plant Viruses and the Chemical Period: 1929–1956). The second path out of this problem came from the studies with bacteriophages, where single cells infected with viruses in culture were much more amenable to experimental manipulation than were virus infections of whole animals. Whereas the plant virologists of that day were tethered to their greenhouses, and the animal virologists were bound to their animal facilities, the viruses of bacteria were studied in Petri dishes and test tubes. Nevertheless, progress was made in the study of animal viruses one step at a time: from studying animals in the wild, to laboratory animals, such as the mouse[66] or the embryonated chicken eggs,[205] to the culture of tissue, and then to single cells in culture. Between 1948 and 1955, a critical transition converting animal virology into a laboratory science came in four important steps: Sanford and colleagues at the National Institutes of Health (NIH) overcame the difficulty of culturing single cells[163]; George Gey at Johns Hopkins Medical School cultured and passaged human cells for the first time and developed a line of immortal cells (HeLa) from a cervical carcinoma[71]; and Harry Eagle at the NIH developed an optimal medium for the culture of single cells.[46] In a demonstration of the utility of all these advanced, Enders and his colleagues showed that poliovirus could replicate in a nonneuronal human explant of embryonic tissues.[54]

These ideas, technical achievements, and experimental advances had two immediate effects on the field virology. They led to the development of the polio vaccine, the first ever produced in cell culture. From 1798 to 1949, all the vaccines in use (smallpox, rabies, yellow fever, influenza) had been grown in animals or embryonated chicken eggs. Poliovirus was grown in monkey kidney cells that were propagated in flasks.[84,117] The exploitation of cell culture for the study of viruses began the modern era of molecular virology. The first plaque assay for an animal virus in culture was done with poliovirus,[43] and it led to an analysis of poliovirus every bit as detailed and important as the contemporary work with bacteriophages. The simplest way to document this statement is for the reader to compare the first edition of *General Virology* by S. E. Luria in 1953[124] to the second edition by Luria and J. E. Darnell in 1967,[127] and to examine the experimental descriptions of poliovirus infection of cells. The modern era of virology had arrived, and it would continue to be full of surprises.

The Molecular and Cell Biology Era of Virology (see e-Tables 1.4 to 1.6)

The history of virology has so far been presented chronologically or according to separate virus groups (plant viruses, bacteriophages, animal viruses), which reflects the historical separation of these fields. In this section, the format changes as the motivation for studying viruses began to change. Virologists began to use viruses to probe questions central to understanding all life processes. Because viruses replicate in and are dependent on their host cells, they must use the rules, signals, and regulatory pathways of the host. By using viruses to probe cells, virologists began to make contributions to all facets of biology. This approach began with the phage group and was continued by the animal virologists. The recombinant DNA revolution also took place during this period (1970 to the present), and both bacteriophages and animal viruses played a critical and central role in this revolution. For these reasons, the organization of this section focuses on the advances in cellular and molecular biology made possible by experiments with viruses. Some of the landmarks in virology since 1970 are listed in e-Tables 1.4 to 1.6.

The Role of Animal Viruses in Understanding Eukaryotic Gene Regulation

The closed circular and superhelical nature of polyomavirus DNA was first elucidated by Dulbecco and Vogt[42] and Weil and Vinograd.[197] This unusual DNA structure was intimately related to the structure of the genome packaged in virions of simian vacuolating virus 40 (SV40). The viral DNA is wound around nucleosomes[70]; when the histones are removed, a superhelix is produced. The structure of polyoma viral DNA served as an excellent model for the *E. coli* genome[206] and the mammalian

chromosome.[113] Viral genomes have unique configurations not found in other organisms, such as single-stranded DNA (ssDNA),[171] plus or minus strand RNA, or double-stranded RNA (dsRNA) as modes of information storage.

Many elements of the eukaryotic transcription machinery have been elucidated with viruses. The first transcriptional enhancer element (acts in an orientation- and distance-independent fashion) was described in the SV40 genome,[76] as was a distance- and orientation-dependent promoter element observed with the same virus. The transcription factors that bind to the promoter, SP-1,[44] or to the enhancer element, such as AP-1 and AP-2,[116] and which are essential to promote transcription along with the basal factors, were first described with SV40. AP-1 is composed of fos and jun family member proteins, demonstrating the role of transcription factors as oncogenes.[21] Indeed, the great majority of experimental data obtained for basal and accessory transcription factors come from *in vitro* transcription systems using the adenovirus major late promoter or the SV40 early enhancer–promoter.[196] Our present-day understanding of RNA polymerase III promoter recognition comes, in part, from an analysis of the adenovirus *VA* gene transcribed by this polymerase.[62]

Almost everything we know about the steps of messenger RNA (mRNA) processing began with observations made with viruses. RNA splicing of new transcripts was first described in adenovirus-infected cells.[15,31] Polyadenylation of mRNA was first observed with poxviruses,[102] the first viruses shown to have a DNA-dependent RNA polymerase in the virion.[103] The signal for polyadenylation in the mRNA was identified using SV40.[59] The methylated cap structure found at the 5′ end of most mRNAs was first discovered on reovirus mRNAs.[67] What little is known about the process of RNA transport out of the nucleus has shown a remarkable discrimination of viral and cellular mRNAs by the adenovirus E1B-55 Kd protein.[147]

Most of our understanding of translational regulation has come from studies of virus infected cells. Recruitment of ribosomes to mRNAs was shown to be directed by the 5′ cap structure first discovered on reovirus mRNAs. The nature of the protein complex that allows ribosomes to bind the 5′ cap was elucidated in poliovirus-infected cells, because viral infection leads to cleavage of one of the components, eIF4G. Internal initiation of translation was discovered in cells infected with picornaviruses (poliovirus and encephalomyocarditis virus).[99,146] Interferon, discovered as a set of proteins that inhibits viral replication, was subsequently found to induce the synthesis of many antiviral gene products that act on translational regulatory events.[92,93] Similarly, the viral defenses against interferon by the adenovirus VA RNA has provided unique insight into the role of eIF-2 phosphorylation events.[108] Mechanisms for producing more than one protein from a eukaryotic mRNA (there is no "one mRNA one protein" rule in bacteria) were discovered in virus-infected cells, including polyprotein synthesis, ribosomal frameshifting, and leaky scanning. Posttranslational processing of proteins by proteases, carbohydrate addition to proteins in the Golgi apparatus, phosphorylation by a wide variety of important cellular protein kinases, or the addition of fatty acids to membrane-associated proteins have all been profitably studied using viruses. Indeed, a good deal of our present-day knowledge of how protein trafficking occurs and is regulated in cells comes from the use of virus-infected cell systems. The field of gene regulation has derived many of its central tenets from the study of viruses.

Animal Viruses and the Recombinant DNA Revolution

The discovery of the enzyme reverse transcriptase,[6,185] not only elucidated the replication cycle of retroviruses, but also provided an essential tool to convert RNA molecules to DNA, which could then be cloned and manipulated. The first restriction enzyme map of a chromosome was done with SV40 DNA, using the restriction enzymes HindII plus HindIII DNA,[37,38] and the first demonstration of restriction enzyme specificity was carried out with the same viral DNA cleaved with EcoRI.[136,138] Some of the earliest DNA cloning experiments involved insertion of SV40 DNA into lambda DNA, or human β-hemoglobin genes into SV40 DNA, yielding the first mammalian expression vectors.[96] A debate about whether these very experiments were potentially dangerous led to a temporary moratorium on all such recombinant experiments following the scientist-organized Asilomar Conference. From the earliest experiments in the field of recombinant DNA, several animal viruses had been developed into expression vectors to carry foreign genes, including SV40,[74] the retroviruses,[198] the adenoviruses,[69,78] and adeno-associated virus.[162] which has the remarkable property of preferential integration into a specific genomic site.[110] Modern-day strategies of gene therapy rely on some of these recombinant viruses. Hemoglobin mRNA was first cloned using lambda vectors, and the elusive hepatitis virus C (non-A, non-B) viral genome was cloned from serum using recombinant DNA techniques, reverse transcriptase, and lambda phage vectors.[30]

Animal Viruses and Oncology

Much of our present understanding of the origins of human cancers is a consequence of work on two major groups of animal viruses: retroviruses and DNA tumor viruses. Oncogenes were first discovered in the genome of Rous sarcoma virus, and subsequently shown to exist in the host cell genome.[174] Since those seminal studies, virologists have identified a wide variety of oncogenes that have been captured by retroviruses (see Chapter 8). Additional oncogenes were identified when they were activated by insertion of the proviral DNA of retroviruses into the genomes of cells.[77] The second group of genes that contribute to the origins of human cancers, the tumor suppressor genes,[118] has been shown to be intimately associated with the DNA tumor viruses. Genetic alterations at the p53 locus are the single most common mutations known to occur in human cancers—they are found in 50% to 80% of all cancers.[119] The p53 protein was first discovered in association with the SV40 large T-antigen.[115,120] SV40, the human adenoviruses, and the human papillomaviruses all encode oncoproteins that interact with and inactivate the functions of two tumor suppressor gene products, the retinoblastoma susceptibility gene product (Rb) and p53.[40,44,115,120,164,200,201] Our understanding of the roles of cellular oncogenes and the tumor suppressor genes in human cancers would be far less significant without the insight provided by studies with these viruses. Curiously, none of the four human polyoma viruses central to these studies was associated with human cancers. However, in 2008, a new polyomavirus associated with Merkel cell carcinoma was discovered.[57]

Viruses that cause cancers have provided some of the most extraordinary episodes in modern animal virology.[135] The recognition of a new disease and the unique geographic distribution of Burkitt's lymphoma in Africa[20] set off a search for viral agents that cause cancers in humans. From D. Burkitt[24] to Epstein, Achong, and Barr[56] to W. Henle and G. Henle,[81] the story of the Epstein-Barr virus and its role in several cancers, as well as in infectious mononucleosis, is a science detective story without rival. Similarly, the identification of a new pathologic disease, adult T-cell leukemia, in Japan by K. Takatsuki[181,191] led to the isolation of a virus that causes the disease by I. Miyoshi and Y. Hinuma[208] and the realization that this virus (human T-cell leukemia virus type 1 [HTLV-1]) had been identified previously by Gallo and his colleagues.[149] Even with the virus in hand, there is still no satisfactory explanation of how this virus contributes to adult T-cell leukemia.

An equally interesting detective story concerns hepatitis B virus and hepatocellular carcinoma. By 1967, S. Krugman and his colleagues[111] had strong evidence indicating the existence of distinct hepatitis A and B viruses, and in the same year B. Blumberg[20] had identified the Australia antigen. Through a tortuous path, it eventually became clear that the Australia antigen was a diagnostic marker—the coat protein—for hepatitis B virus. Although this discovery freed the blood supply of this dangerous virus, Hilleman at Merck Sharp & Dohme and the Chiron Corporation (which later isolated the hepatitis C virus) went on to produce the first human vaccine that prevents hepatitis B infections and very likely hepatocellular carcinomas associated with chronic virus infections (see Chapter 69). The idea of a vaccine that can prevent cancer—first proven with the Marek's disease virus and T-cell lymphomas in chickens,[18,49]—comes some 82 to 85 years after the first discoveries of tumor viruses by Ellerman, Bang, and Rous. An experiment is under way in Taiwan, where 63,500 newborn infants have been inoculated to prevent hepatitis B infections. Based on the epidemiologic predictions, this vaccination program should result in 8,300 fewer cases of liver cancer in that population in 35 to 45 years.

Vaccines and Antivirals

Among the most remarkable achievements of our century is the complete eradication of smallpox, a disease with a greater than 2,000-year-old history.[79] In 1966, the World Health Organization began a program to immunize all individuals who had come into contact with an infected person. This strategy was adopted because it simply was not possible to immunize entire populations. In October 1977, Ali Maolin of Somalia was the last person in the world to have a naturally occurring case of smallpox (barring laboratory accidents). Because smallpox has no animal reservoir and requires person-to-person contact for its spread, most scientists agree that we are free of this disease, at least as a natural infection.[79] As a consequence, most populations have not maintained immunity to the virus and the world's populations are becoming susceptible to infection. Many governments now fear the use of smallpox virus as a weapon of bioterrorism, and the debate continues over whether to destroy the two known stocks of smallpox virus in the United States and Russia.[80] As a consequence, the development of new, more effective vaccines and safe anti-smallpox virus drugs has risen high on the list of priorities for some countries, and such vaccines have already been stockpiled in the United States. It is paradoxical that humankind's most triumphant medical accomplishment is now tarnished by the spectre of biowarfare.

The Salk and Sabin poliovirus vaccines were the first products to benefit from the cell culture revolution. In the early 1950s in the United States, just before the introduction of the Salk vaccine, about 21,000 cases of poliomyelitis were reported annually. Today, thanks to aggressive immunization programs, polio has been eradicated from the United States (see Chapters 18 and 19).[141] As of this writing, only three countries have seen interruption of wild-type poliovirus circulation: Nigeria, Afghanistan, and Pakistan. With the substantial financial support of the Gates Foundation, there is hope that global immunization campaigns can lead to eradication of poliomyelitis from the planet.

The first viral vaccines deployed included infectious vaccines, attenuated vaccines, inactivated virus vaccines, and subunit vaccines. Both the Salk inactivated virus vaccine and the recombinant hepatitis B virus subunit vaccine were products of the modern era of virology. Today many new vaccine technologies are either in use or are being tested for future deployment.[3,23,168] These include recombinant subunit vaccines, virus-like particle vaccines, viral antigens delivered in viral vectors comprising vaccinia virus or adenovirus, and DNA plasmids that express viral proteins from strong promoters. Therapeutic vaccines boost the immune system using specific cytokines or hormones in combination with new adjuvants to stimulate immunity at specific locations in the host or to tailor the production of immune effector cells and antibodies. Considering that the first vaccines for smallpox were reported in the Chinese literature of the 10th century,[58] vaccinology has clearly been practiced well before the beginning of the field of virology.

Although vaccines have been extraordinarily successful in preventing specific diseases, up until the 1960s, few natural products or chemotherapeutic agents that cured or reduced viral infections were known. That situation changed dramatically with the development of Symmetrel (amantadine) by Dupont in the 1960s as a specific influenza A virus drug. Soon after, acyclovir, an inhibitor of herpesviruses, was developed by Burroughs-Wellcome. Acyclovir achieves its remarkable specificity because to be active, it must be phosphorylated by the viral enzyme thymidine kinase before it can be incorporated into viral DNA by the viral DNA polymerase. This drug blocks herpes simplex virus type 2 (HSV-2) replication after reactivation from latency and stopped a growing epidemic in the 1970s and 1980s (Chapter 14). The development of other nucleoside analogs has led to many compounds effective against DNA viruses. Until the human immunodeficiency virus (HIV) epidemic, few drugs effective against RNA viruses other than the influenza A virus were known. As natural products, the interferons (Chapter 9) are used successfully in the clinic for hepatitis B and C infections, cancer therapy, and multiple sclerosis. The interferons, novel cytokines found in the course of studying virus interference,[23,92,93] modulate the immune response and continue to play an increasing role in the treatment of many clinical syndromes.

Virology and the Birth of Immunology

Edward Jenner was a British surgeon who is credited with making the first smallpox vaccine in 1796, and has also been called

the "father of immunology." Jenner began a long tradition of virology providing seminal discoveries about the immune response. Two examples will serve to illustrate this pattern.

Alick Issacs and Jean Lindenmann, while working at the National Institute for Medical Research in London, found that addition of heat-inactivated influenza virus to the chorioallantoic membrane of chicken eggs interfered with the replication of influenza virus. When they published this observation in 1957, they coined the term *interferon* (IFN).[92] In the 1970s the protein was purified from cells by Sidney Pestka and Alan Waldman,[161] and subsequently the genes encoding the proteins were cloned.[73] This allowed formal proof that IFN—by that time known to comprise a variety of different proteins—could interfere with viral replication. Extensive work with viruses showed that IFNs bind to cell-surface receptors, and through the JAK-STAT signal transduction pathway, induce the synthesis of more than 1,000 mRNAs that establish an antiviral state.[39] IFNs protect against both viral and bacterial infections, and also play a role in tumor clearance.

While working at the John Curtin School of Medical Research in Australia, Rolf Zinkernagel and Peter Doherty provided seminal insight into how cytotoxic T cells (CTLs) recognize virus-infected cells. They were studying infection of mice with lymphocytic choriomeningitis virus (LCMV). Because this virus is noncytopathic, they hypothesized that brain damage in infected mice was a consequence of CTLs attacking virus-infected cells. They made the observation that CTLs isolated from LCMV-infected mice lysed virus-infected target cells *in vitro* only if both cell types had the same major histocompatibility complex (MHC) haplotype. This requirement was termed MHC restriction.[211] In other words, a CTL must recognize two components on a virus-infected cell: one virus specific and one from the host. Subsequent research revealed that CTLs recognize a short viral peptide bound to MHC class I (MHC-I) proteins on the surface of target cells. These observations revolutionized our understanding of T-cell–mediated killing, thereby establishing a foundation for understanding the general mechanisms used by the immune system to recognize both foreign microorganisms and self-molecules. The results have had wide implications for clinical medicine, not only in infection but also in areas such as cancer and autoimmune reactions in inflammatory diseases.

Emerging Viruses

In general, emerging viruses cause human infections that have not been seen or reported before. They usually attract the public's attention, often by media sound bites like "killer viruses emerge from the jungle." The fact is that spread of infections through different hosts is well known in virology. Most so-called emerging infections represent zoonotic infections: infection of humans by a virus that normally exists in an animal population in nature.[187]

Perhaps the most infamous emerging virus infection of the 20th century is the human immunodeficiency virus type 1, HIV-1, a retrovirus.[85] Progenitor HIV viruses exist in primates, and we now believe they infected humans as a result of hunting and slaughter for food.[170] HIV was first recognized as a new disease entity by clinicians and epidemiologists in the early 1980s, and they rapidly tracked down the venereal mode of virus transmission. The virus was detected in blood products and transplant tissue. The immune system of HIV-infected individuals is severely compromised, which results in a variety of infections by usually benign microbes. The first published report of acquired immunodeficiency syndrome (AIDS) was in June 1981. Possible causative agents were first suggested in 1983.[7] and then 1984.[68] Had this pandemic occurred in 1961 instead of 1981, neither the nature of retroviruses nor the existence of its host cell (CD4 helper T cell) would have been understood. HIV is a lentivirus (*lenti* is Latin for slow) and despite its recent appearance in humans, lentiviruses have been around for a long time. In fact, one of the first animal viruses to be identified in 1904 was the lentivirus that causes infectious equine anemia.

Many other examples of emerging viruses have attracted global concern and an exceptional rapid response of scientists and health officials.[187] The severe acute respiratory syndrome (SARS) and West Nile virus epidemics revealed the presence of a new human coronavirus (SARS), identified with unprecedented speed, and the invasion of an Old World virus into the Western hemisphere (West Nile virus).[90,140] In 2006, chikungunya virus (an endemic virus infection in Africa) spread explosively to several countries where it was hitherto unknown.[169] On La Reunion Island, more than 40% of the population of 800,000 people was infected. The first appearance of avian influenza A (H5N1) virus in humans in 1997 produced fears of a pandemic of serious proportions because humans had no immunological history of infection by this avian strain.[182] Soon thereafter, the emergence of the pandemic H1N1 influenza virus in 2009 produced similar worries because of the relationship of the virus to the deadly 1918 influenza epidemic.[184] The mobilization of world health networks, public health officials, vaccine producers, veterinarians, clinicians, and molecular virologists marked a new chapter in dealing with emerging diseases.

Epidemiology of Viral Infections

The study of the incidence, distribution, and control of disease in a population is an integral part of virology. The technology advancements of the last 50 years have provided epidemiology with a terrific boost. The discovery of specific molecular reagents (e.g., recombinant DNA technology, antibodies, polymerase chain reaction [PCR], rapid diagnostic tests, high volume DNA and RNA sequencing) now enables detection of virions, proteins, and nucleic acids in body fluids, tissue samples, or in the environment. Moreover, we now can compare and classify viral isolates rapidly, determine the relationships between virus strains, and track the spread of infections around the world. The marriage of behavioral, geographic, and molecular epidemiology made this a most powerful science.[87]

The understanding of epidemics and pandemics of our most common viral infections such as influenza requires the perspectives of ecology, population biology, and molecular biology.[106,182] G. Hirst and his colleagues (1941–1950) developed the diagnostic tools that permitted both the typing of the hemagglutinin (HA protein) of influenza A strains and the monitoring of the antibody response to this antigen in patients (see Chapters 42 and 43). These observations have been expanded, with more and more sophisticated molecular approaches, to prove the existence of animal reservoirs for influenza viruses, the reassortment of viral genome segments between human and

animal virus strains (antigenic shift), and a high rate of mutation (antigenic drift) caused by RNA-dependent RNA synthesis with no known RNA editing or corrective mechanisms.[153,184] These molecular events that lead to episodic local epidemics and worldwide pandemics are understood in broad outline. Many viruses are now known to evolve at high rates following basic Darwinian principles in a time frame shorter than that of any other organism. Indeed, we now understand that RNA virus populations exist as a quasispecies or a swarm of individual viral genomes where every member is unique. Influenza viruses are successful because they have evolved to carry the very engines of evolution: mechanisms of mutation and recombination (reassortment), Influenza A virus has not been eliminated even with effective vaccines and antiviral drugs. Variants always arise that escape effective immune responses thorough high mutation (drift), and when co-infection occurs with viruses spreading from nonhuman hosts, new reassortants regularly arise. Expression of these new combinations of viral genes can change the pattern of infection from local to pandemic via an antigenic shift of its HA and NA subunit proteins. These studies (Chapters 42 and 43) have revealed an extraordinary lifestyle that reverberates around the planet in birds, farm animals, and humans. The study of the mechanisms of viral pathogenesis and modulation of the immune system have led to new insights in the virus–host relationship.

New technology discovered and developed over the last 35 years is changing the way viral infections are studied in the laboratory and in the field, and is changing our appreciation of epidemiology and virus ecology.[183] Amplification technologies such as PCR permit rapid sampling of viral nucleic acids without growth in culture or plaque purification. Microarray technology where discriminatory DNA sequences from all sequenced viral genomes are put on a single array enables rapid classification of PCR-amplified nucleic acids.[194] Rapid genome sequencing has revealed hitherto described viral genomes, relationships among viruses, and sequence heterogeneity within a virus population.[123] Mutations can be detected rapidly, documented, and localized in the viral genome. Importantly, the biological consequences can be monitored quickly. For example, in the late 1970s, viral epidemiologists were confronted with a highly transmissible, lethal infection of puppies.[144] In record time, scientists found that just two mutations in the capsid gene of feline parvovirus altered the host range such that the mutant could infect dogs. In less than a year, a completely new, highly pathogenic virus called canine parvovirus spread all around the world. Its evolution has continued to be monitored, and a highly effective vaccine was developed. A similar type of molecular archeology enabled scientists to analyze serum samples collected from patients in the 1950s in efforts to understand the origins of HIV.[85] Sequence analysis of the HIV genome from one sample (ZR 1959) suggested that the virus may have emerged in the 1940s to 1950s. Field studies in Africa of viruses present in primate feces indicated that HIV most likely derived from a chimpanzee lentivirus in Africa.[170] After the initial human infection, rapid mutation and selection established the first human variants of this lentivirus that replicated and continued to evolve as they spread through their new human hosts.

The advances in our understanding of the viral etiology of tumors pay tribute to the modern epidemiology strategy by D. Burkitt and K. Takatsuki, leading to the identification of Epstein-Barr virus (EBV) and HTLV-1. Similarly, the recombinant DNA revolution overcame the problems of propagating human papillomaviruses. The human papillomaviruses (see Chapter 56) differ in transmission, location on the body, their nature of pathogenesis, and persistence. New technology permitted the identification of new virus serotypes, triggering epidemiologic correlations for high- or low-risk cancer viruses.[212] The same technology enabled the development and use of an effective vaccine against cervical cancer. We cannot forget the considerable impact of veterinary virus epidemiology on our understanding of complicated human diseases. For example, careful epidemiologic work by Sigurdsson and colleagues on unusual diseases of sheep[175] provided the first understanding of slow infections in sheep (Visna-Maedi virus; a lentivirus) and infectious proteins (prions), which cause spongiform encephalopathies (Chapter 78).

As we describe in the next section, molecular epidemiology is reaching new levels of sophistication, not only in detecting new viruses, but also taking inventory of the viral ecosystem. Whether the next human epidemic will result from a novel variant of Ebola virus, coronavirus, or Norwalk virus, or the more likely possibility of a new pandemic variant of influenza virus, remains to be seen. The new technologies also enable analysis of virus populations in natural communities of nonhuman animals. For example, we can now monitor pandemic spread of avian influenza virus in wild birds and other nonhuman hosts.[153] These alternative hosts have never been sampled for virus populations in such molecular detail. New insights into the selection pressures and bottlenecks are emerging almost faster than the viruses. What is abundantly clear, however, is that the demographics of the human population on earth are changing at unprecedented rates (Table 1.1). Even as birth rates slow, our planet will house 8 to 10 billion people by 2050 to 2100. For the first time, there will be three to four times more people older than the age of 60 than younger than 3 to 4 years of age. Not only are we an aging population, we are moving to urban environments, with more than 20 to 30 cities containing more than 10 million people. Clearly, patterns of human behavior (increased population density, increased travel, increased ages of the population) will provide the environment for the selection of emerging viruses and the challenges to the new field of molecular epidemiology.

HOST–VIRUS INTERACTIONS AND VIRAL PATHOGENESIS

The technologies that contributed most to the modern era of virology (1960 to present), were advances in cell culture and molecular biology.[55] Virologists were able to describe the replicative cycles of viruses in great detail under well-defined conditions, and they demonstrated the elaborate interactions between viral genomes, viral proteins, and the cellular machinery of the host. As indicated previously, these advances resulted in an extraordinary inquiry into the functions of infected or uninfected host cells using the tools of both molecular biology and cell biology. As this approach matured, it became more reductionist in nature, and the questions became more detailed. However, some virologists used the new knowledge to move back to more complicated *in vivo* systems to study previously difficult problems in host–virus

TABLE 1.1 Advances and Challenges

Vaccines	Yellow fever virus vaccine, live attenuated Salk and Sabin vaccines for poliovirus, killed and live attenuated Recombinant hepatitis B vaccine, subunit Vaccinia virus vaccine to eradicate natural smallpox virus from the planet Influenza virus vaccines, inactivated and live attenuated Varicella-zoster virus vaccines, live attenuated Rotavirus vaccines, live attenuated Measles vaccines, live attenuated Recombinant human papillomavirus vaccine, subunit; prevents cancers and virus infections
Antiviral drugs	Acyclovir against herpes simplex type 1 and type 2 Combination therapy: Protease, reverse transcriptase, and integrase inhibitors against HIV Interferon therapy for hepatitis B and C Amantadine against influenza A virus Neuraminidase inhibitors against influenza virus
Epidemiologic advances	Understanding the molecular basis of antigenic shift and drift in influenza viruses Identification of the causes of AIDS and SARS Prion diseases recognized and mechanisms elucidated Deep sequencing, genome analysis; pathogen discovery, uncovering the molecular nature of epidemic and pandemic infections Recognition of the role of zoonotic infections in the emergence of new viral diseases Recognition of specific viruses as causative agents in human cancers Elucidation of the concept of viral quasispecies and the molecular biology of viral populations
Viral pathogenesis	Identification of viral virulence genes Identification of host genes affecting virus replication and spread Identification of the molecular bases for antiviral immune defenses (adaptive immunity) Identification of the molecular basis of front-line cellular defenses (intrinsic and innate immunity) including apoptosis and induction of defensive cytokines Understanding of the molecular basis for viral tropism Elucidation of the mechanisms involved in viral quiescence and persistence
The challenges (societal)	Population explosion: more people now live on the planet than at any time in our existence (predicted to be 8 to 10 billion in the next few decades) Population concentration: world populations are concentrating in large urban centers of 10 to 20 million people or more
The challenges (scientific)	Population demographics: for the first time there are more people older than the age of 60 than younger than the age of 4 Population interactions: world populations interact physically at rates and extents never before possible Pandemic viral diseases and bioterrorism provide continuing challenges for human survival Research costs money: how do we alleviate the pressures on funding and support of fundamental research Discoveries cannot be predicted: how to balance true discovery research with applied (translational) research Public support: how do we develop support and advocacy for virology research Policy makers need to understand virology: more engagement of scientists with lawmakers and the general public Public education about vaccination and other public health issues Discovering an effective vaccine against HIV Developing vaccines against persistent viruses Discovering and developing new antiviral drugs Development of rapid viral diagnostic and identification strategies Coupling new technology with established procedures Balancing risks and benefits of dangerous pathogen research Developing surrogates for Koch's postulates in modern pathogen discovery programs Defining and understanding the composition and interplay of microbial communities inside and outside hosts (natural versus unnatural flora)

AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; SARS, severe acute respiratory syndrome.

interactions involving the natural host or animal models of infection. Chief among these new questions was, how does a virus cause disease processes in the animal? How do we quantitate viral virulence and what is the genetic basis of an attenuated virus? These studies have identified, in selected viruses, a set of genes and functions that broadly influence our understanding of pathogenesis.

Despite an abundance of data, we have distilled six general categories relating to viral pathogenesis. Four of these involve viral gene products and two involve the hosts.

1. Mutations in genes that impair virus replication in the host, lower the threshold of pathogenesis by reducing the number of progeny produced. These mutations are found in essential genes (essential for life) *in vivo.*
2. A second class of mutations impairs virulence (reduces the degree of pathogenicity), but does not alter normal virus replication (at least in some cell or tissue types). Here, host- or tissue-range mutations are most common. Mutations can change the pattern of virion adsorption to a particular cell type and so prevent viral entry into a cell. Mutations in viral enhancer elements can alter viral transcription in selected cell types. In some viral genomes, mutations affect rates of translation such that virulence is reduced. A classic example comes from analysis of the attenuated strains of poliovirus in the Sabin vaccine. All three strains of the Sabin poliovirus vaccine contain mutations in the 5′ untranslated region of the viral RNA genome, which impair translation of these RNAs, and as a consequence virus yields are reduced. As a result, after infection, viral replication occurs, the host is immunized, but disease does not occur.
3. A third class of genes affecting virulence is involved in producing products that modify the host defenses. Intrinsic host defenses depend on receptors inside and on the surface of cells that detect viral gene products. When these receptors are activated, cytokines can be produced to alert more global innate immune defenses, the cell may die by apoptosis, or autophagy may be induced to engulf virus particles. It is likely that every successful virus can bypass or modulate these most fundamental cell-autonomous defenses. Mutations in these primary defense systems or viral proteins that block them affect virulence and spread. Some viruses encode genes that produce viral homologs of host cytokines (virokines). These proteins are secreted from infected cells and modify the immune response to infection. Other viruses encode decoy receptors that bind host-produced cytokines and reroute the immune response as a result. Many viral genomes encode genes whose products block infected cells from undergoing apoptosis in response to a virus infection. Some viruses, such as African swine fever virus, secrete a pro-apoptotic factor that kills lymphocytes and enhances its virulence. Many viruses produce proteins that alter the MHC proteins (MHC-I and MHC-II; also known as human leukocyte antigens or HLA proteins). These complex proteins display on the cell surface, short peptides derived from newly made or newly ingested proteins inside the cell. T cells detect these complexes and respond if non–self-peptides are detected. Many viral infections alter the expression or function of these MHC proteins. Other viruses encode superantigens that stimulate or eliminate lymphoid cells of a selected specificity or with a class of receptors. HIV infection kills CD4 T cells and disrupts the immune response.
4. A fourth class of viral virulence genes enhances the spread of a virus in the host. Some viruses are released from infected cells at the apical or basolateral surface, permitting selected spread *in vivo.* Some RNA viruses acquire infectivity (maturation) only after specific proteolytic cleavage of their structural proteins. In some cases, maturation is accomplished by a viral protease and in others by a cellular protease, each with a specific amino acid sequence required for proper cleavage and resulting spread of the virus. Altering this sequence will affect virulence and overall transmissibility of the infection in a host population.
5. A fifth class involves host gene products. A wide variety of polymorphisms or mutations in the host result in modulated resistance or virulence of a virus. These host mutations can even be selected during viral epidemics, changing the gene pool of the surviving host population. In humans, polymorphisms in a chemokine receptor gene (a co-receptor) impart resistance to HIV infection at the level of viral absorption. New antiviral drugs have been designed to target this viral–cytokine interaction. Variations in the immune responses of diverse hosts in a population will result in large variations in viral virulence. The host mechanisms that minimize viral diseases after infection are certainly major topics in viral pathogenesis.
6. The final class involves the society and interaction of hosts. Changes in population density, lifestyles, cultural traditions, and economic factors all play a major role in viral virulence. Poliovirus was a minor endemic virus infection for 3,000 years before the introduction of improved sanitation in the last century. As a result, human populations were infected for the first time at a later age and large poliovirus epidemics resulted. It may not have been a coincidence that the worst influenza epidemic in the century, killing 20 to 40 million people, started in about 1918 toward the end of World War I, with so many people dislocated and moving about the world in very crowded and poor conditions. If there is a general lesson from history it is that cultural and environmental changes will surely play a role in the virulence of viruses in the future.

THE FUTURE OF VIROLOGY? (E-TABLE 1.7 AND TABLE 1.1)

The future of virology is unpredictable, but it is guaranteed to be exciting. Who knows what discoveries remain? Certainly, the number of astounding and groundbreaking discoveries in biology over the last 50 years is remarkable.[55] Most could not have been predicted or even imagined, prior to their discovery. That virologists participated in making many of these discoveries is no accident: Viral gene products have evolved to engage all the key nodes of biology ranging from the atomic to the organismal. We only have to be smart enough to figure out how to identify these nodes. The forces that will drive our field are technology development, public health, information processing, and, of course, personal curiosity. Indeed new life science technologies invariably

will give rise to new, unexpected insights in virology to meet our current challenges. That has been, and continues to be, the future of virology (see Table 1.1).

Despite a cloudy crystal ball, three general trends are likely to rise to the forefront of virology research over the next 10 years.

1. **The detailed understanding of the systems biology inherent in virus–host interactions.** Although virus particles are inanimate, it is the living, infected cell that delivers the phenotype promoted by the viral genome. The change of state of a cell or tissue from uninfected to infected is fertile ground for modern systems biology. The constellation of new gene products (viral and host) and altered host pathways produced in an infected cell give rise to biological outputs that go far beyond the single cell in the laboratory. Viruses offer useful modalities for the systems biologist. One can synchronize an infection and go from the uninfected to infected state within minutes, or use the same virus to produce an acute or a quiescent infection. Regulatory circuits, modulation of host defenses, emergence of pathogenesis, and modes of efficient transmission in a hostile environment, are all inherent in the nanobiology of viruses. How can a viral genome with so few genes relative to the host, dominate a cell and the host so quickly and dynamically? How does it all work? How has evolution produced such diversity of infected cell phenotypes? Microarrays, PCR, mass spectroscopy, microfluidics, large-scale nucleic-acid sequencing, massive database assembly, and computer modeling are what toothpicks and Petri dishes were to the students of the Delbrück phage school 60 years ago.
2. **The understanding of viruses as integral participants in the ecosystem.** Such knowledge means uncovering the multiple interrelationships and interactions of all viruses and their hosts. This is ecology, but on a scale that has hitherto been unimaginable for virologists. Viruses exist wherever life is found, and they are the most abundant entities on the planet. Indeed their biomass rivals that of the prokaryotes. Estimates are that we know less than 1% of the viral genomes on the planet, but first principles inform us that there can be only a limited number of genome strategies for replication and expression of information. Therefore, despite what appears to be incredible diversity, we will be able to identify new viruses by the unique signatures of a viral genome. The viral ecology problem, therefore, is one of knowing what is out there and why. The powerful techniques of interrogating virus populations in the wild for their RNA, DNA, proteins, and unique small molecules have changed the worldview of ecologists and molecular biologists alike. The new biology will require the intellectual firepower of computer scientists, engineers, chemists, and physicists, as well as biologists. As part of this growing knowledge of the viral ecosystem, virologists will come to be more ecumenical in their studies and not balkanize the field into animal and plant virology or viruses of single cell hosts.
3. **Health of humans and the world.** The fundamental need for public health measures is unprecedented, as the human population is now greater than ever before. However, despite all attempts to prove otherwise, humans are not the top of the food chain. Every living thing ultimately engages every other entity directly or indirectly—and, as far as we know, every living thing is infected with viruses. These infections shape human existence on the planet. A human centric view of public health is short-sighted. First principles tell us that all successful viruses today carry a collection of genes that have survived the best defenses that hosts can muster. Our knowledge of the microbial world must be used to inform our national and international health policies. The bedrock of old-fashioned public health policies cannot be ignored: clean water, sewage treatment, proper nutrition, and management of epidemic childhood disease by vaccines. However, the continuing divide between rich and poor nations, the conflicts among ethnic and religious groups, the changing climate, and resulting calamities of drought and other natural disasters stress even these most basic attempts at maintaining public health. Certainly the high-tech approach to public health of developed counties will find no purchase in those countries where the basics of survival are lacking.

Intrinsic and Extrinsic Defenses Against Viral Infections

It is likely that considerable work in the future will be directed to the host defenses that meet viral infections in the first minutes to hours. All viral infections begin as individual, single-cell events that either are resolved or expand to produce the characteristic phenotypes of the persistent or acute infection. Ancient single-cell pathways of response to external stimuli have been honed over millions of years to provide cells and communities of cells, a repertoire of defensive actions that are now being revealed. Every cell is capable of responding to infection immediately (so-called intrinsic resistance) by processes whose nature and actions will fuel discovery research in the near future.[17,61] These processes act immediately upon infection, before the so-called innate and adaptive immune responses are called into action. We understand some of these processes, such as apoptosis in some detail, but others, including RNA interference (RNAi), autophagy, DNA repression, and the restriction factors first defined by retrovirologists, remain fertile ground for discovery.[28,29,41] The interaction between signals of early warning from single cells with the local multicellular innate immune response and the global adaptive immune response are likely to be key to recognizing and responding to the various patterns of viral infections that arise in nature. Primary questions concerning the molecular biology and cellular biology of persistent and latent infection cannot be answered without knowledge of early defense responses of single cells and local tissues.

DNA microarray technology has enabled the measurement of the whole genome responses of single cells exposed to a wide variety of viral infections.[100] The systematic profiling of gene-expression changes has provided an exceptionally rich database from which we now are learning of cell-common and cell-specific responses to infection. The differences and similarities are proving to be the proverbial gold mine of information on the definition of evolutionarily conserved host-defense components and viral gene products that counter them. Understanding the relationship of common cell-stress responses and

pathogen-specific responses and counter-responses will certainly provide insights into potential diagnostic and therapeutic targets for viral infections.[100]

Viruses and Cancer

Since the 1960s, seven different human viruses have been isolated, identified, and shown to be associated with the etiology of human cancer.[135] Surprisingly, even after 50 years, we have only a rudimentary understanding of the oncogenic pathogenesis of these infectious agents.[135] The first cancer-associated virus was discovered in 1964 when Epstein, Achong, and Barr[56] detected herpesvirus particles in cells obtained from a Burkitt's lymphoma.[24] The DNA episomes of the Epstein-Barr virus (or EBV) have been consistently found to be associated with some types of B-cell lymphomas. Despite this 40-year period, it remains unclear how or even if this virus actually causes this lymphoma. Although it is certain that the EBV genome contains one or more oncogenes (latent membrane protein 1, LMP-1), they are not expressed in the lymphoma cells. The only viral gene product expressed in these lymphoma cells is Epstein-Barr nuclear antigen 1 (EBNA-1), and its possible role of contributing to lymphomas is still controversial. Similarly the HTLV-1 viral genome does not contain a cellular oncogene, and it does not integrate into the host-cell DNA near a cellular proto-oncogene in a consistent fashion. Therefore, HTLV-1 does not employ the two most common mechanisms for tumor formation observed with the retroviruses. There is no clear association of any hepatitis B or C gene products in the causation of liver cancers. Rather it appears that immune destruction of liver cells followed by the regeneration of this tissue activates several growth factors made by the surrounding tissue resulting in fibrosis. The local milieu of inflammation and the positive feedback loop for growth drives the division of liver cells and hepatocellular carcinoma. This complex mix of infection, immune-mediated cell death, and chronic inflammation in a tissue with regenerative capacity is challenging to analyze. Although Kaposi's sarcoma herpesvirus also encodes potential oncogenes, no clear mechanism of how it initiates or propagates cancer is available. On the other hand, studies of the human papillomaviruses[45] have provided a mechanistic understanding of how these viruses transform cells. The viral E7 protein binds to the cellular retinoblastoma protein and inactivates its function, thereby initiating entry of the cell into the cell cycle and division. The viral E6 protein binds the cellular p53 protein and promotes its ubiquitylation and proteolytic degradation, thereby preventing cellular apoptosis.[165] More research is needed to fully understand the mechanisms that lead to cancers after infection by these viruses.[199]

A Role for Systems Biology in Virology

Not too long ago, molecular virology was limited to studies of one virus and one gene or gene product at a time. More complex studies often were seen as "descriptive." Times have changed! New technology enables virologists to interrogate simultaneously many viruses and large groups of genes or gene products in ever-expanding environments and biological networks. In this context, a network is defined as the interconnected intracellular processes that control everything within a cell, for example, DNA replication, processes of gene expression, organelle biogenesis, and metabolism to name a few.[139] The definition also encompasses networks of intercellular communication at the tissue, organ, and whole-organism level. Virologists are beginning to embrace a tenet of systems biology where information flows through these networks and disease arises when these networks are perturbed. Viral gene products cause changes in network architecture and thereby alter the dynamics of information flow. Future studies of viral pathogenesis are likely to involve identification and understanding of specific viral signatures of network imbalance that do not affect just one pathway but alter the fundamental homeostatic balance.[19,55,152,179]

Genomics and the Predictive Power of Sequence Analysis

The development of technologic advances in biology often drives new approaches and permits one to ask novel questions that could not even be framed in the past. In the last decade of the 20th century, rapid and inexpensive DNA-sequencing methods paved the way to sequence the genomes of many viruses and their hosts. This created large databases containing information about the variation of DNA or RNA sequences within a single virus (e.g., HIV, influenza) and permitted predictions about the nature of the mutations that were driving selective changes, mutation frequencies of different viruses, and evolutionary changes from isolates around the world. The correlations of these sequence variations with drug resistance, changes in the genetic background of the host, and virulence have been informative. By combining this information with the three-dimensional structure of the influenza A hemagglutinin (HA) protein, J. Plotkin and colleagues have examined codon use in this gene and suggested that the degeneracy of codon use was being optimized to permit changes in amino acids at critical positions in this protein, so as to reduce the impact of the immune response to this virus.[148] Although this concept has been controversial, it has permitted a set of predictions of the direction of future changes in these codons as the host develops its immune response and immunity of the population. Predicting the future changes in influenza strains provides a testable hypothesis and might then impact how we prepare for genetic drift in virus populations by designing vaccines.[184]

The degeneracy of the genetic code means that there are different codons that encode the same amino acid. As a result, many sequences can encode the same protein. This choice of sequences is constricted by several selective forces such as restrictions on transfer RNA (tRNA) availability in a host, giving rise to preferential codon use, the overall G-C content of a genome, the frequency in which two or three amino acids appear next to each other in proteins encoded by the virus, or the avoidance of some sequence contexts due to a high mutational load.[158] The low level of CpG dinucleotides in some genomes may result because a C-residue can be methylated. This change is mutagenic because methyl-C will pair with a T residue, causing a C to T transition in the genome. Once these restrictions on the frequency of certain dinucleotide to septanucleotide sequences are appreciated, they can be factored into a calculation of whether certain nucleotide sequences are over-represented or under-represented in a genome despite these selected pressures observed in a particular genome.

Algorithms have been designed to accomplish this, and it is clear from an analysis of 209 prokaryotic genomes and 90 bacteriophages that replicate in these hosts, that selected sequences of di-septanucleotides are over-represented and others are under-represented in these viral and bacterial genomes.[157] Having factored out the genetic codon preferences in this algorithm, these preferences represent a second code of under- or over-represented frequencies of nucleotide sequences, and the available data indicate that these sequences are functional and are selected for over evolutionary time scales. First, coding regions of a genome have been shown to have different over- or under-represented sequences in a genome. Second, if these coding regions sequences are employed to assemble a phylogenetic tree, these sequences do an excellent job in reconstructing the known evolutionary relationships of these 209 prokaryotic genome sequences (done originally by aligning the ribosomal gene sequences). Third, about 80% of the viruses in these databases can be correctly assigned to their hosts by matching the over- and under-represented sequences in their viral and host genomes. The same selection pressure acting upon this second code in a host genome also acts upon the genomes of their parasites. We now await the application of this algorithm to the more complex genomes and viruses of eukaryotes. Host genomes contain an amazing number of viral or viral-related sequences. More than 50% of the DNA sequences found in the human genome were derived from retroviruses, retrotransposons, DNA transposons and randomly amplified sequences of genes (short interspersed nuclear element [SINES] and the 7S RNA gene), pseudogenes, and repetitive DNA sequences.[114,192] Viruses certainly have left a major mark upon the evolution of their host's genomes in addition to the selective pressures they exert via virus infections and deaths. During the evolution of humans from their ancestral line, retroviruses and retro-transposons (the long interspersed nuclear element [LINE-1]) have entered the germ line, amplified their copy numbers, and integrated at various sites in the genome. This process introduces mutations, alters patterns of gene expression, and creates new interactions of viruses with their hosts. This is clearly one of the drivers of host evolution. Over time these retroviruses (human endogenous retroviruses, or HERVs) accumulate mutations in their genes, and some recombine out of the genome leaving only the long terminal repeats (LTRs) as a remnant marking their past insertion. Although humans no longer contain viable HERVs, the multiple copies of HERV–H or HERV-K viruses when transcribed in cells, produce functional viral proteins from different copies of these viruses, and the viral particles that are produced are defective and very poorly transmitted. Cellular transcription factors regulate the expression of the HERVs, and the p53 transcription factor (activated by stress and DNA damage) transcribes the HERV-H genome and produces particles in response to such stress.[209] Similarly the LINE-1 retrotransposons, which have about 300 viable and movable elements in the human genome today, are responsible for about 1% of the mutations found in each generation. LINE-1 transposons also contain p53 DNA response elements[86] and thus are also regulated by stress responses recorded by the host. Although it is clear that retroviruses and transposons can shape the host genome, it is equally clear that the host genome is a place for new viral genomes to evolve, recombine with exogenous viral genomes, and possibly produce a new agent optimized for replication in its host. Understanding of the dynamics of these vestiges of viruses that reside in our genome is a challenge for the future.

With many host-genome sequences representing all kingdoms of life in the databases, it has been possible to do some rather eye-opening analyses. For example, the resurrection of endogenous retroviruses from inactive sequences in host DNA has allowed the investigation of interactions between extinct pathogens called paleoviruses and their hosts that occurred millions of years ago.[53] By cloning these sequences, it has been possible to identify the cellular receptor of these extinct retroviruses.[172] Perhaps more amazing is that similar "viral genome fossils" representing DNA copies of filoviruses and bornaviruses as well as parvoviruses and circoviruses have been found in a variety of host genomes.[11,12] When the evolutionary history of various host genomes harboring these viral sequences were compared, it was possible to deduce that ancestors of modern viruses were in existence millions of years ago. What is even more curious is that these genome-insertion events seemed to happen around the same time in a wide variety of mammals. What global event could have stimulated such activities?

The Virome: How Many Viruses Are There? Where Are They? Why Are They There?

Virus ecology, as a result of modern virus discovery technology, is posing many questions (see 106,183). In 1977, when Fred Sanger sequenced the DNA genome of coliphage phiX174, many virologists were impressed with the wealth of information contained in a "simple" DNA sequence and the congruence of genetic and biochemical data with the genome structure. In fewer than 25 years, sampling, sequencing, and computer technology now provide the wherewithal to identify and sequence entire viral communities from their natural environment without the intervention of time-held techniques of isolation and characterization of individual viruses.[48,178,194] In early 2003, a novel viral DNA microarray was used to reveal and partially sequence a previously uncharacterized coronavirus in a viral isolate cultured from a patient with SARS. This chip technology has advanced to the point that essentially all the known viral genomes can be represented on a single microarray. New techniques for discovery and analysis of viral populations are certain to be found. As can be expected in this "omics" era, the identification and study of an entire community of viruses in their natural habitat has been called metagenomics.[2,156,202] The diversity of viruses in the environment is essentially unknown, as we have been limited to studying only those viruses that are easy to work with in the laboratory or those that have major impact on human health. The first metagenomic studies on viruses have revealed stunning diversity of genes and gene products that remain to be understood even in principle.[178,203] The combination of host and bacteriophage genome sequencing in the bacteria has proved to be an exceptional window on genome evolution and gene transfer. The practical value of identifying new gene products with novel functions cannot be overestimated. The repertoire of tactics for gene control and regulation is far more extensive than any of us imagined before the era of metagenomics. We can only expect that as the metagenomics of animal and plant viruses advances,

the effect of knowing everything that is out there and the resulting knowledge of the dynamics of host–parasite interactions will be mind-boggling.[177]

Pathogen Discovery

Historically, discovery of new viral pathogens followed identification of diseases of consequence to humans, animals, and plants. Field biologists, clinicians, veterinarians, and the lay public noted syndromes, unusual behaviors, or drastic changes of animal and plant populations, which motivated scientists to discover the cause. The early days of virology were all "translational research." Koch's postulates were developed to identify the causative agent for a given disease. Advances in virus identification were driven in large part by technology developments such as porcelain filters, animal models, tissue and cell culture, microscopic visualization of cytopathic effect, serology, immunoassays, hybridization, western blotting, PCR, sequencing, microarrays, and imaging technology. These advances paved the way to our current understanding of viral pathogens and provided the data to advance our current understanding of mechanisms of pathogenesis. Modern pathogen discovery has entered a new phase where via sequencing technology, virologists can detect and identify viral nucleic acids with unprecedented sensitivity in essentially any sample.[123] We no longer need to be able to grow a virus stock to be able to identify it and develop diagnostic reagents, vaccines, or antiviral drugs.

The discovery of new viral genomes is proceeding at an amazing pace.[143] Although the discovery process is straightforward, understanding what these viruses are doing is a serious challenge.[91,155] If one finds novel viral genomes in samples from patients with disease, are these viruses the cause of the disease? Is it possible that they may be part of the normal flora of an individual (the microbiome;[107])? There are many populations of microbes in and on various parts of the body. Just identifying the microbiome differences in body sites of a single individual is challenging enough; cataloging the microbiome variation from individual to individual is even more difficult.[156] What functions does the microbiome have? There is evidence that our normal microbial flora stimulates local and systemic immune responses that protect against or suppress responses that contribute to pathogenesis by more-virulent microbes. Future virologists will have to unravel these heretofore unknown microbial relationships, and to do so we will need new technology. Whatever we find will undoubtedly reveal unanticipated insights about viruses and their hosts. Modern pathogen discovery will require the interaction of infectious disease specialists, epidemiologists, and bioinformatics specialists; virologists will have to be professionally "multilingual".[121]

Perhaps of fundamental importance is that proof of causation can no longer rely on the time-honored Koch's postulates.[91] This assertion is made not only because it may be difficult to propagate new viruses and find models to test their pathogenicity, it also is likely that many diseases will involve the interaction of multiple microbial communities (viruses, bacteria, fungi) that will be difficult to reproduce in the laboratory. Pathogen discovery will require new biomarkers of health and disease, methods to improve sampling and stability of samples, technology to record relevant data, and capacity to associate all this data with the sample. In the past, pathogen identification methods were slow and tedious, and working with multiple samples was difficult if not impossible. It is now possible to collect and analyze serial samples over time as patients move from health to disease. Assembling data, maintaining databases, and providing access for analysis will also involve advances in software and bioinformatics. In the end, the fundamental challenge will be how one moves from correlation of the presence of an agent or agents in disease to proof of causation.

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CHAPTER 2

Richard C. Condit

Principles of Virology

Viruses are unique in nature. They are the smallest of all self-replicating organisms, historically characterized by their ability to pass through filters that retain even the smallest bacteria. In their most basic form, viruses consist solely of a small segment of nucleic acid encased in a simple protein shell. Viruses have no metabolism of their own but rather are obliged to invade cells and parasitize subcellular machinery, subverting it to their own purposes. Many have argued that viruses are not even living,[128] although to a seasoned virologist, they exhibit a life as robust as any other creature.

The apparent simplicity of viruses is deceptive. The truth is that as a group, viruses infect virtually every organism in nature, they display a dizzying diversity of structures and lifestyles, and they embody a profound complexity of function.

The study of viruses—virology—must accommodate both the uniqueness and the complexity of these organisms. The singular nature of viruses has spawned novel methods of classification and experimentation entirely peculiar to the discipline of virology. The complexity of viruses is constantly challenging scientists to adjust their thinking and their research to describe and understand some new twist in the central dogma revealed in a *simple* virus infection.

This chapter explores several concepts fundamental to virology as a whole, including virus taxonomy, virus cultivation and assay, and virus genetics. The chapter is not intended as a comprehensive or encyclopedic treatment of these topics, but rather as a relatively concise overview with sufficient documentation for more in-depth study. In addition to primary resources and practical experience, the presentation draws heavily on previous editions of *Fields Virology*[35–37] for the taxonomy and genetics material, plus several excellent texts for material on virus cultivation and assay.[20,34,41,59,70,76,81] It is hoped that this chapter will be of value to anyone learning virology at any stage: a novice trying to understand basic principles for the first time, an intermediate student of virology trying to understand the technical subtleties of virological protocols in the literature, or a bewildered scientist in the laboratory wondering why the host-range virus mutant received from a colleague does not seem to manifest the described host range.

VIRUS TAXONOMY

A coherent and workable system of classification—a taxonomy—is a critical component of the discipline of virology. However, the unique nature of viruses has defied the strict application of many of the traditional tools of taxonomy used in other disciplines of biology. Thus, scientists who concern themselves with global taxonomy of organisms have traditionally either ignored viruses completely as nonliving entities or left them scattered throughout the major kingdoms, reasoning that viruses have more in common with their individual hosts than they do with each other.[82,90] By contrast, for practical reasons at least, virologists agree that viruses should be considered together as a separate group of organisms regardless of host, be it plant, animal, fungus, protist, or bacterium, a philosophy borne out by the observation that in several cases viruses now classified in the same family—for example, family *Reoviridae*—infect hosts from different kingdoms. Interestingly, the discipline of virus taxonomy brings out the most erudite and thought-provoking, virtually philosophical discussions about the nature of viruses, probably because the decisions that must be made to distinguish one virus from another require the deepest thought about the nature of viruses and virus evolution. In the end, all of nature is a continuum, and the business of taxonomy has the unfortunate obligation of drawing boundaries within this continuum, an artificial and illogical task but necessary nevertheless. The execution of this obligation results today in a free-standing virus taxonomy, overseen by the International Committee on Taxonomy of Viruses (ICTV), with rules and tools unique to the discipline of virology. The process of virus taxonomy that has evolved

uses some of the hierarchical nomenclature of traditional taxonomy, identifying virus species and grouping these into genera, genera into families, and families into orders, but at the same time, to cope with both the uniqueness and diversity of viruses as a group, the classification process has been deliberately nonsystematic and thus is "based upon the opinionated usage of data".[92]

Most importantly, the virus taxonomy that has been developed works well. For the trained virologist, the mention of a virus family or genus name, such as "family *Herpesviridae*" or "genus *Rotavirus*" immediately conjures forth a set of characteristics that form the basis for further discussion or description. Virus taxonomy serves an important practical purpose as well, in that the identification of a limited number of biological characteristics, such as virion morphology, genome structure, or antigenic properties, quickly provides a focus for identification of an unknown agent for the clinician or epidemiologist and can significantly impact further investigation into treatment or prevention of a virus disease. Virus taxonomy is an evolving field, and what follows is a summary of the state of the art, including important historical landmarks that influenced the present system of virus taxonomy, a description of the system used for virus taxonomy and the means for implementation of that system, and a very brief overview of the taxonomy of viruses that infect humans and animals.

History and Rationale

Virology as a discipline is scarcely 100 years old, and thus the discipline of virus taxonomy is relatively young. In the early 1900s, viruses were initially classified as distinct from other organisms simply by virtue of their ability to pass through unglazed porcelain filters known to retain the smallest of bacteria. As increasing numbers of filterable agents became recognized, they were distinguished from each other by the only measurable properties available, namely the disease or symptoms caused in an infected organism. Therefore, animal viruses that caused liver pathology were grouped together as hepatitis viruses, and viruses that caused mottling in plants were grouped together as mosaic viruses. In the 1930s, an explosion of technology spawned a description of the physical properties of many viruses, providing numerous new characteristics for distinguishing viruses one from another. The technologies included procedures for purification of viruses, biochemical characterization of purified virions, serology, and perhaps most importantly, electron microscopy, in particular negative staining, which permitted detailed descriptions of virion morphology, even in relatively crude preparations of infected tissue. In the 1950s, these characterizations led to the distinction of three major animal virus groups, the myxoviruses, the herpesviruses, and the poxviruses. By the 1960s, because of the profusion of data describing numerous different viruses, it became clear that an organized effort was required to classify and name viruses, and thus the ICTV (originally the International Committee on Nomenclature of Viruses [ICNV]) was established in 1966. The ICTV functions today as a large, international group of virologists organized into appropriate study groups, whose charge it is to develop rules for the classification and naming of viruses and to coordinate the activities of study groups in the implementation of these rules.

Early in its history, the ICTV wrestled with the fundamental problem of developing a taxonomic system for classification and naming of viruses that would accommodate the unique properties of viruses as a group and that could anticipate advancements in the identification and characterization of viruses. Perhaps the most critical issue was whether the classification of viruses should consider virus properties in a monothetical, hierarchical fashion or a polythetical, hierarchical fashion. A *monothetic* system of classification is defined as a system based on a single characteristic or a series of single characteristics. *Polythetic* is defined as sharing several common characteristics without any one of these characteristics being essential for membership in the group or class in question. Thus, a monothetical, hierarchical classification, modeled after the Linnaean system used for classification of plants and animals, would effectively rank individual virus properties, such as genome structure or virion symmetry, as being more or less important relative to each other and use these individual characteristics to sort viruses into subphyla, classes, orders, suborders, and families.[79] Although the hierarchical ordering of viruses into groups and subgroups is desirable, a strictly monothetical approach to using virus properties in making assignments to groups was problematic because both the identification of individual properties to be used in the hierarchy and the assignment of a hierarchy to individual properties seemed too arbitrary. A polythetic approach to classification would group viruses by comparing simultaneously numerous properties of individual viruses without assigning a universal priority to any one property. Thus, using the polythetic approach, a given virus grouping is defined by a collection of properties rather than a single property, and virus groups in different branches of the taxonomy may be characterized by different collections of properties. One argument against the polythetic approach is that a truly systematic and comprehensive comparison of dozens of individual properties would be at least forbidding if not impossible. However, this problem could be avoided by the adoption of a nonsystematic approach, namely, using study groups of virologists within the ICTV to consider together numerous characteristics of a virus and make as rational an assignment to a group as possible. Therefore, the system that is currently being used is a nonsystematic, polythetical, hierarchical system. This system differs from any other taxonomic system in use for bacteria or other organisms; however, it is effective, useful, and has withstood the test of time.[91] As our understanding of viruses increases, and as new techniques for characterization are developed, notably comparison of gene and genome sequences, the methods used for taxonomy will undoubtedly continue to evolve.

As a consequence of the polythetic approach to classification, the virus taxonomy that exists today has been filled initially from the middle of the hierarchy by assigning viruses to genera, and then elaborating the taxonomy upward by grouping genera into families and, to a limited extent, families into orders. By 1970, the ICTV had established two virus families each containing 2 genera, 24 floating genera, and 16 plant *groups*.[133] A rigorous species definition,[126] discussed later, was not approved by the ICTV until 1991 but has now been applied to the entire taxonomy and has become the primary level of classification for viruses. As of this writing, the currently accepted taxonomy recognizes 6 orders, 87 families, 19 subfamilies, 348 genera,

TABLE 2.1 Summary Characteristics of Vertebrate Virus Families

Family	Nucleocapsid morphology	Envelope	Virion morphology	Genome[a]	Host[b]
dsDNA viruses					
Adenoviridae	Icosahedral	No	Icosahedral	1 ds linear, 26–48 kb	V
Alloherpesviridae	Icosahedral	Yes	Spherical, tegument	2 ds linear, 135–294 kb	V
Asfaviridae	Icosahedral	Yes[c]	Icosahedral	1 ds linear, 165–190 kb	V, I
Herpesviridae	Icosahedral	Yes	Spherical, tegument	1 ds linear, 125–240 kb	V
Iridoviridae	Icosahedral	No[d]	Icosahedral	1 ds linear, 140–303	V, I
Papillomaviridae	Icosahedral	No	Icosahedral	1 ds circular, 7–8 kb	V
Polyomaviridae	Icosahedral	No	Icosahedral	1 ds circular, 5 kb	V
Poxviridae	Ovoid	Yes	Ovoid	1 ds linear, 130–375 kb	V, I
ssDNA viruses					
Anellovirus	Icosahedral	No	Icosahedral	1 – circular, 2–4 kb	V
Circoviridae	Icosahedral	No	Icosahedral	1 – or ± circular, 2 kb	V
Parvoviridae	Icosahedral	No	Icosahedral	1 +, – or ± linear, 4–6 kb	V, I
dsDNA reverse transcribing viruses					
Hepadnaviridae	Icosahedral	Yes	Spherical	1 ds circular, 3–4 kb	V
ssRNA reverse transcribing viruses					
Metaviridae	Spherical	Yes	Spherical	1 + linear, 4–10 kb	F, I, P, V
Retroviridae	Spherical, rod or cone shaped	Yes	Spherical	1 + linear dimer, 7–13 kb	V
dsRNA viruses					
Birnaviridae	Icosahedral	No	Icosahedral	2 ds linear, 5–6 kb	V, I
Picobirnaviridae	Icosahedral	No	Icosahedral	3 ds linear, 4 kb	V
Reoviridae	Icosahedral	No	Icosahedral, layered	10–12 ds linear, 19–32 kb	V, I, P, F
Negative sense ssRNA viruses					
Bornaviridae	ND[e]	Yes	Spherical	1 – linear, 9 kb	V
Deltavirus[f]	Isometric	Yes	Spherical	1 – circular, 2 kb	V
Filoviridae	Helical filaments	Yes	Bacilliform, filamentous	1 – linear, 19 kb	V
Orthomyxoviridae	Helical filaments	Yes	Pleomorphic, spherical	6–8 – linear, 10–15 kb	V
Paramyxoviridae	Helical filaments	Yes	Pleomorphic, spherical, filamentous	1 – linear, 13–18 kb	V
Rhabdoviridae	Coiled helical filaments	Yes	Bullet shaped	1 – linear, 11–15 kb	V, I, P
Positive sense ssRNA viruses					
Arteriviridae	Linear, asymmetric	Yes	Spherical	1 + linear, 13–16 kb	V
Astroviridae	Icosahedral	No	Icosahedral	1 + linear, 6–8 kb	V
Caliciviridae	Icosahedral	No	Icosahedral	1 + linear, 7–8 kb	V
Coronaviridae	Helical	Yes	Spherical	1 + linear, 26–32 kb	V
Flaviviridae	Spherical	Yes	Spherical	1 + linear, 9–13 kb	V, I
Hepevirus[e]	Icosahedral	No	Icosahedral	1 + linear, 7 kb	V
Nodaviridae	Icosahedral	No	Icosahedral	2 + linear, 4–5 kb	V, I
Picornaviridae	Icosahedral	No	Icosahedral	1 + linear, 7–9 kb	V
Togaviridae	Icosahedral	Yes	Spherical	1 + linear, 10–12 kb	V, I
Ambisense ssRNA viruses					
Arenaviridae	Filamentous	Yes	Spherical	2 ± linear, 11 kb	V
Bunyaviridae	Filamentous	Yes	Spherical	3 – or ± linear, 11–19 kb	V, I, P
Subviral agents: prions					
Prions	—	—	—	—	V, F

[a]Number of segments, polarity (ds, double stranded; +, mRNA like; −, cRNA like; ±, ambisense), conformation, size.
[b]V, vertebrate; P, plant; I, insect; F, fungus.
[c]Contains both an outer envelope plus a lipid membrane internal to the capsid.
[d]Contains a membrane internal to the capsid.
[e]ND, not determined.
[f]*Deltavirus* represents an unassigned genus.

and 2,290 species. The complete virus taxonomy is far too extensive to relate here; however, examples of the results of the taxonomy are offered in Tables 2.1 and 2.2. Table 2.1 lists the distinguishing characteristics of the vertebrate animal virus families, whereas Table 2.2 provides an example of the entire taxonomic classification of one virus order, namely order *Mononegavirales*.

The International Committee on Taxonomy of Viruses Universal System of Virus Taxonomy

Structure and Function

The ICTV is a committee of the Virology Division of the International Union of Microbiological Societies. The objectives of the ICTV are to develop an internationally agreed taxonomy

TABLE 2.2 Taxonomy of the Order *Mononegavirales*

Order	Family	Subfamily	Genus	Type species	Host
Mononegavirales	*Bornaviridae*		*Bornavirus*	*Borna disease virus*	V
	Rhabdoviridae		*Vesiculovirus*	*Vesicular stomatitis Indiana virus*	V, I
			Lyssavirus	*Rabies virus*	V
			Ephemerovirus	*Bovine ephemeral fever virus*	V, I
			Novirhabdovirus	*Infectious hematopoietic necrosis virus*	V
			Cytorhabdovirus	*Lettuce necrotic yellows virus*	P, I
			Nucleorhabdovirus	*Potato yellow dwarf virus*	P, I
	Filoviridae		*Marburgvirus*	*Lake Victoria marburgvirus*	V
			Ebolavirus	*Zaire ebolavirus*	V
	Paramyxoviridae	*Paramyxovirinae*	*Rubulavirus*	*Mumps virus*	V
			Avulavirus	*Newcastle disease virus*	V
			Respirovirus	*Sendai virus*	V
			Henipavirus	*Hendra virus*	V
			Morbillivirus	*Measles virus*	V
		Pneumovirinae	*Pneumovirus*	*Human respiratory syncytial virus*	V
			Metapneumovirus	*Avian metapneumovirus*	V

V, vertebrate; I, insect; P, plant.

and nomenclature for viruses, to maintain an index of virus names, and to communicate the proceedings of the committee to the international community of virologists. The ICTV publishes an update of the taxonomy at approximately 3-year intervals.[32,33,39,85,86,92,133] At the time of this writing, the ninth report is being completed. The official taxonomy is also available on line at the ICTV website: http://www.ictvonline.org.

Virus Properties and Their Use in Taxonomy

As introduced previously, the taxonomic method adopted for use in virology is polythetic, meaning that any given virus group is described using a collection of individual properties. The description of a virus group is nonsystematic in that there exists no fixed list of properties that must be considered for all viruses and no strict formula for the ordered consideration of properties. Instead, a set of properties describing a given virus is simply compared with other viruses described in a similar fashion to formulate rational groupings. Characters such as virion morphology, genome organization, method of replication, and the number and size of structural and nonstructural viral proteins are used for distinguishing different virus families and genera. Characters such as genome sequence relatedness, natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, physicochemical properties of virions, and antigenic properties of viral proteins are used for distinguishing virus species within the same genus.[127]

The Hierarchy

The ICTV has adopted a universal classification scheme that employs the hierarchical taxonomic levels of order, family, subfamily, genus, and species. Because the polythetic approach to classification introduces viruses into the middle of the hierarchy, and because the ICTV has taken a relatively conservative approach to grouping taxa, levels higher than order are not currently used. Interestingly, groupings above the level of order may prove to be inappropriate: Higher taxons imply a common ancestry for viruses, whereas multiple independent lineages for viruses now seems the more likely evolutionary scenario.[32] Taxonomic levels lower than species, such as clades, strains, and variants, are not officially considered by the ICTV but are left to specialty groups.

A virus species is defined as "a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche".[126] The formal definition of a polythetic class is "a class whose members always have several properties in common although no single common attribute is present in all of its members".[127] Thus, no single property can be used to define a given species, and application of this formal definition of a polythetic class to species accounts nicely for the inherent variability found among members of a species. The qualification of a replicating lineage implies that members of a species experience evolution over time with consequent variation, but that members share a common ancestor. The qualification of occupation of an ecological niche acknowledges that the biology of a virus, including such properties as host range, pathogenesis, transmission, and habitat, are fundamental components of the characterization of a virus. A *type species* has been identified for each genus. The type species is not necessarily the best characterized or most representative species in a genus; rather, it is usually the virus that initially necessitated the creation of the genus and therefore best defines or identifies the genus.

Taxonomic levels higher than species are formally defined by the ICTV only in a relative sense, namely a genus is a group of species sharing certain common characters, a subfamily is a group of genera sharing certain common characters, a family is a group of genera or subfamilies

sharing certain common characters, and an order is a group of families sharing certain common characters. As the virus taxonomy has evolved, these higher taxa have acquired some monothetic character. They remain polythetic in that they may be characterized by more than one virus property; however, they violate the formal definition of a polythetic class in that one or more defining properties may be required of all candidate viruses for membership in the taxon. Not all taxonomic levels need be used for a given grouping of viruses, thus whereas most species are grouped into genera and genera into families, not all families contain subfamilies, and only a few families have been grouped into orders. Consequently, the family is the highest consistently used taxonomic grouping, it therefore carries the most generalized description of a given virus group, and as a result has become the benchmark of the taxonomic system. Most families have distinct virion morphology, genome structure, and/or replication strategy (see Table 2.1).

Nomenclature

The ICTV has adopted a formal nomenclature for viruses, specifying suffixes for the various taxa, and rules for written descriptions of viruses. Names for genera, subfamilies, families, and orders must all be single words, ending with the suffixes -virus, -virinae, -viridae, and -virales, respectively. Species names may contain more than one word and have no specific ending. In written usage, the formal virus taxonomic names are capitalized and written in italics, and preceded by the name of the taxon, which is neither capitalized nor italicized. For species names that contain more than one word, the first word plus any proper nouns are capitalized. As an example, the full formal written description of human respiratory syncytial virus is as follows: order *Mononegavirales,* family *Paramyxoviridae,* subfamily *Pneumovirinae,* genus *Pneumovirus,* species *Human respiratory syncytial virus.* The ICTV acknowledges that vernacular (informal) taxonomic names are widely used; however, they should not be italicized or capitalized. For example, the vernacular name "herpesvirus" refers to a member of the family *Herpesviridae.*

Informal Groupings and Alternate Classification Schemes

For convenience in presenting or tabulating the virus taxonomy, informal categorical groupings of taxa are often used. The criteria applied for such groupings typically include nature of the viral genome (DNA or RNA), strandedness of the viral genome (single stranded or double stranded), polarity of the genome (positive sense, negative sense, or ambisense), and reverse transcription. Separate categories accommodate subviral agents (including viroids, satellites, and prions) and unassigned viruses. The Baltimore classification system, named after its creator David Baltimore, is a widely used scheme based on the nature of the genome packaged in virions and the pathway of nucleic acid synthesis that each group takes to accomplish messenger RNA (mRNA) synthesis.[1] This classification divides viruses into seven categories as depicted in Figure 2.1. Most usages of this system group ambisense virus families (family *Arenaviridae*

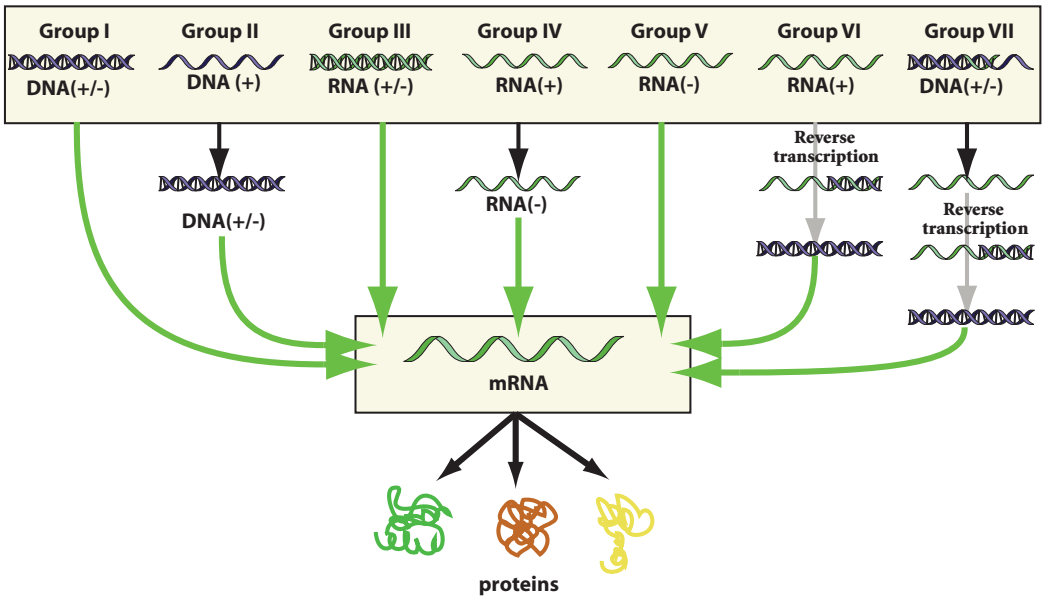

FIGURE 2.1. The Baltimore classification, a virus classification scheme based on the form of nucleic acid present in virion particles and the pathway for expression of the genetic material as messenger RNA.[1] The original scheme contained groups I through VI and has been expanded to accommodate DNA-containing, reverse transcribing viruses. Viruses containing ambisense single-stranded RNA genomes are grouped under negative sense single-stranded RNA viruses. (Reprinted from Hulo C, de Castro E, Masson P, et al. ViralZone: a knowledge resource to understand virus diversity. *Nucleic Acids Res* 2011;39 (Database issue):D576–D582; ViralZone, Swiss Institute of Bioinformatics, http://www.expasy.ch/viralzone/, with permission.)

and family *Bunyaviridae*) along with negative sense, single-stranded RNA (ssRNA) viruses. The families of vertebrate viruses listed in Table 2.1 have been grouped according to the Baltimore classification, with ambisense viruses split into an eighth genome category.

Universal Virus Database

To facilitate the management and distribution of virological data, the ICTV has established the universal virus database of the ICTV (ICTVdB). The ICTVdB is accessible on the Internet at http://www.ictvdb.org. Constructed from virus descriptions in the published reports of the ICTV, the database comprises searchable descriptions of all virus families, genera, and type species, including microscopic images of many viruses. The ICTVdB is a powerful resource for management of and access to virological data, and promises to considerably extend the reach and capability of the ICTV.

VIRUS CULTIVATION AND ASSAY

Different branches of science are defined in large part by their techniques, and virology is no exception. Whereas the study of viruses uses some general methods that are common to other disciplines, the unique nature of viruses and virus infections requires a unique set of technical tools designed specifically for their investigation. Conversely, what we know and *can* know about viruses is delimited by the techniques used; therefore, a genuine understanding of virology requires a clear understanding of virological methods. What follows is a summary of the major techniques essential and unique to all of virology, presented as fundamental background for understanding the discipline.

Initial Detection and Isolation

The presence of a virus is evidenced initially by effects on a host organism or, in the case of a few animal viruses, by effects on cultured cells. Effects on animal hosts obviously include a broad spectrum of symptoms, including skin and mucous membrane lesions; digestive, respiratory, or neurological disorders; immune dysfunction; specific organ failure such as hepatitis or myocarditis; and death. Effects on cultured cells include a variety of morphological changes in infected cells, termed *cytopathic effects* and described in detail later in this chapter and in Chapter 15. Both adenovirus[108] and the polyomavirus SV40[121] were discovered as cell culture contaminants before they were detected in their natural hosts.

Viruses can be isolated from an infected host by harvesting excreted or secreted material, blood, or tissue and testing for induction of the original symptoms in the identical host, or induction of some abnormal pathology in a substitute host or in cell culture. Historically, dogs, cats, rabbits, rats, guinea pigs, hamsters, mice, and chickens have all been found to be useful in laboratory investigations,[70] although most animal methods have now been replaced by cell culture methods.[81] Once the presence of a virus has been established, it is often desirable to prepare a genetically pure clone, either by limiting serial dilution or by plaque purification.

Viruses that are cultivated in anything other than the natural host may adapt to the novel situation through acquisition of genetic alterations that provide a replication advantage in the new host. Such adaptive changes may be accompanied by a loss of fitness in the original host, most notably by a loss of virulence or pathogenicity. Whereas this adaptation and attenuation may present problems to the basic scientist interested in understanding the replication of the virus in its natural state, it also forms the basis of construction of attenuated viral vaccines.

Hosts for Virus Cultivation

Laboratory Animals and Embryonated Chicken Eggs

Prior to the advent of cell culture, animal viruses could be propagated only on whole animals or embryonated chicken eggs. Whole animals could include the natural host or laboratory animals such as rabbits, mice, rats, and hamsters. In the case of laboratory animals, newborn or suckling rodents often provide the best hosts. Today, laboratory animals are seldom used for routine cultivation of virus; however, they still play an essential role in studies of viral pathogenesis.

The use of embryonated chicken eggs was introduced to virology by Goodpasture et al[44] in 1932 and developed subsequently by Beveridge and Burnet.[4] The developing chick embryo, 10 to 14 days after fertilization, provides a variety of differentiated tissues, including the amnion, allantois, chorion, and yolk sac, which serve as substrates for growth of a wide variety of viruses, including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, herpesviruses, and poxviruses.[70] Members of each of these virus families may replicate in several tissues of the developing egg, or replication may be confined to a single tissue. Several viruses from each of the previously mentioned groups cause discrete and characteristic foci when introduced onto the chorioallantoic membrane of embryonated eggs, thus providing a method for identification of virus types, or for quantifying virus stocks or assessing virus pathogenicity (Fig. 2.2). Although embryonated eggs have been almost wholly replaced by cell culture techniques, they are still the most convenient method for growing high titer stocks of some viruses and thus continue to be used both in research laboratories and for vaccine production.

Cell Culture

The growth and maintenance of animal cells *in vitro,* described generally (albeit incorrectly) as tissue culture, can be formally divided into three different techniques: organ culture, primary explant culture, and cell culture. In *organ culture,* the original three-dimensional architecture of a tissue is preserved under culture conditions that provide a gas–liquid interface. In *primary explant culture,* minced pieces of tissue placed in liquid medium in a culture vessel provide a source for outgrowth of individual cells. In *cell culture,* tissue is disaggregated into individual cells prior to culturing. Only cell culture will be discussed in detail here, because it is the most commonly used tissue culture technique in virology.

Cultured cells currently provide the most widely used and most powerful hosts for cultivation and assay of viruses. Cell cultures are of three basic types—primary cell cultures, cell strains, and cell lines—that may be derived from many animal species and that differ substantially in their characteristics. Viruses often behave differently on different types of cultured cells; in addition, each of the culture types possess technical

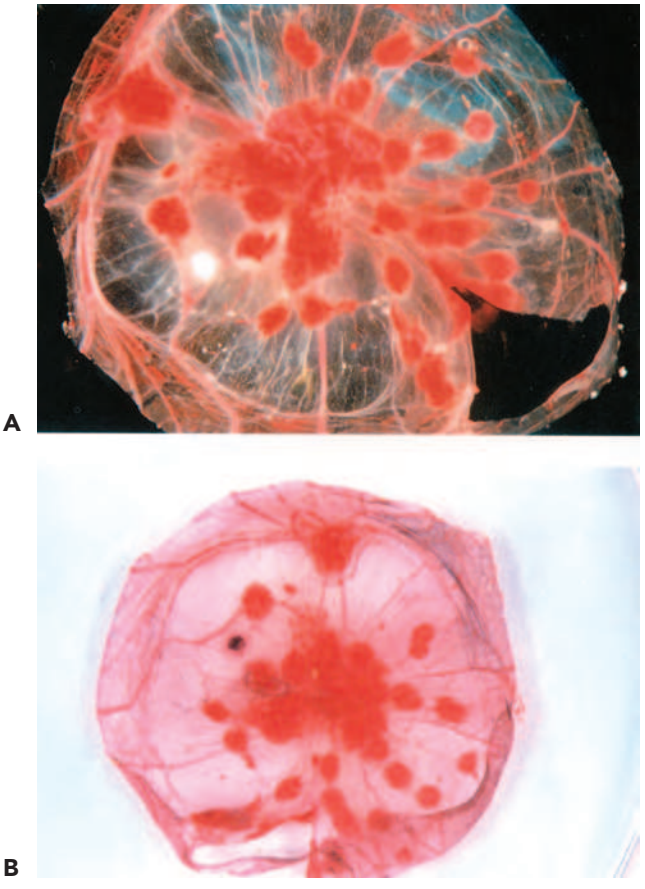

FIGURE 2.2. Cowpox-induced pock formation on the chorioallantoic membrane of chick embryos. The chorioallantoic membrane of intact chicken embryos, 11 days old, were inoculated with cowpox, and the eggs were incubated for an additional 3 days at 37.5°C. Chorioallantoic membranes were then dissected from the eggs and photographed. The membrane shown in **A** was untreated, whereas the membrane in **B** was stained with NBT, an indicator of activated heterophils.[40] Wild-type cowpox forms red hemorrhagic pocks on the membrane (**A** and **B**). Spontaneous deletion mutants of cowpox virulence genes occur at a high frequency, resulting in infiltration of inflammatory cells into the pock. The infiltration of inflammatory cells causes the pocks to appear white in unstained membrane preparations or dark blue on NBT-stained membranes. The unstained membrane preparation **(A)** contains a single white pock, whereas the NBT-stained preparation **(B)** contains a single blue pock. NBT, nitroblue tetrazolium. (Courtesy of Dr. R. Moyer.)

advantages and disadvantages. For these reasons, an appreciation of the use of cultured cells in animal virology requires an understanding of several fundamentals of cell culture itself. A detailed description of the theory and practice of cell and tissue culture is provided by Freshney,[41] and several additional texts provide excellent summaries of cell culture as it specifically applies to virology.[20,34,59]

Primary Cell Culture

A primary cell culture is defined as a culture of cells obtained from the original tissue that have been cultivated *in vitro* for the first time and that have not been subcultured. Primary cell cultures can be established from whole animal embryos or from selected tissues from embryos, newborn animals, or adult animals of almost any species. The most commonly used cell cultures in virology derive from primates, including humans and monkeys; rodents, including hamsters, rats, and mice; and birds, most notably chickens. Cells to be cultured are obtained by mincing tissue and dispersing individual cells by treatment with proteases and/or collagenase to disrupt cell–cell interactions and interactions of cells with the extracellular matrix. With the exception of cells from the hemopoietic system, normal vertebrate cells will grow and divide only when attached to a solid surface. Dispersed cells are therefore placed in a plastic flask or dish, the surface of which has been treated to promote cell attachment. The cells are incubated in a buffered nutrient medium in the presence of blood serum, which contains a complex mixture of hormones and factors required for the growth of normal cells. The blood serum may come from a variety of sources, although bovine serum is most commonly used. Under these conditions, cells will attach to the surface of the dish, and they will divide and migrate until the surface of the dish is covered with a single layer of cells, a monolayer, whereupon they will remain viable but cease to divide. If the cell monolayer is "wounded" by scraping cells from an isolated area, cells on the border of the wound will resume division and migration until the monolayer is reformed, whereupon cell division again ceases. These and other observations lead to the conclusion that the arrest of division observed when cells reach confluency results from cell–cell contact and therefore is called *contact inhibition.* Primary cultures may contain a mixture of cell types and retain the closest resemblance to the tissue of origin.

Subcultivation

Cells from a primary culture may be subcultured to obtain larger numbers of cells. Cells are removed from the culture dish and disaggregated by treating the primary cell monolayer with a chelating agent, usually EDTA, or a protease, usually trypsin, or both, giving rise to a single cell suspension. This suspension is then diluted to a fraction of the original monolayer cell density and placed in a culture dish with fresh growth medium, whereupon the cells attach to the surface of the dish and resume cell division until once again a monolayer is formed and cell division ceases. Cultures established in this fashion from primary cell cultures may be called *secondary cultures.* Subsequently, cells may be repeatedly subcultured in the same fashion. Each subculturing event is called a *passage,* and each passage may comprise several cell generations, depending on the dilution used during the passage. Most vertebrate cells divide at the rate of approximately one doubling every 24 hours at 37°C. Thus, a passage performed with an eightfold dilution will require three cell doublings over 3 days before the cells regain confluency.

Cell Strains

Normal vertebrate cells cannot be passaged indefinitely in culture. Instead, after a limited number of cell generations, usually 20 to 100 depending on the age and species of the original animal, cultured normal cells cease to divide, then degenerate and die, a phenomenon called *crisis* or *senescence*[51] (Fig. 2.3). Starting with the establishment of a secondary culture and until cells either senesce or become transformed as described later, the culture is termed a *cell strain* to distinguish it from a primary culture

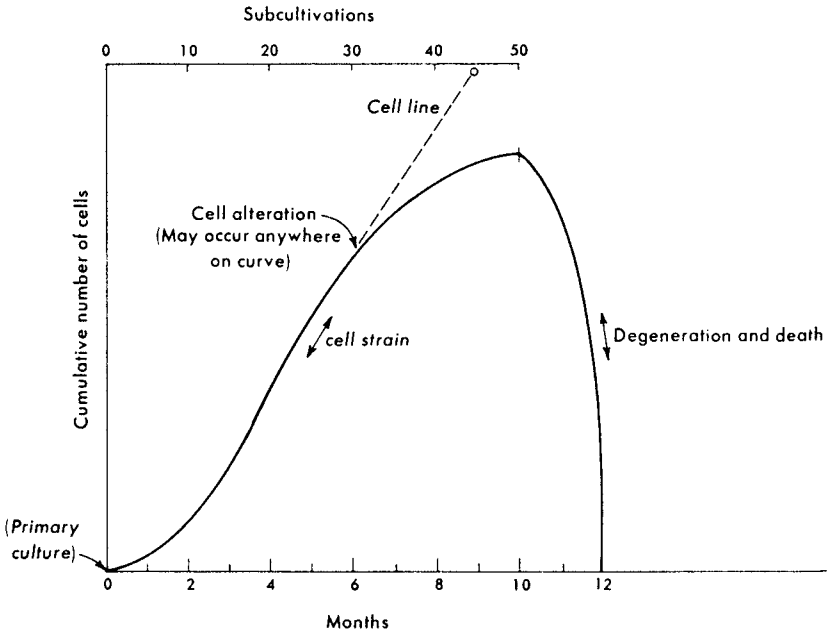

FIGURE 2.3. Growth of cells in culture. A primary culture is defined as the original plating of cells from a tissue, grown to a confluent monolayer, without subculturing. A cell strain (*solid line*) is defined as a euploid population of cells subcultivated once or more *in vitro,* lacking the property of indefinite serial passage. Cell strains ultimately undergo degeneration and death, also called *crisis* or *senescence.* A cell line (*dashed line*) is an aneuploid population of cells that can be grown in culture indefinitely. Spontaneous transformation or alteration of a cell strain to an immortal cell line can occur at any time during cultivation of the cell strain. The time in culture and corresponding number of subcultivations or passages are shown on the abscissas. The ordinate shows the total number of cells that would accumulate if all were retained in culture. (Reprinted from Animal cells: cultivation, growth regulation, transformation. In: Davis BD, Dulbecco R, Eisen HN, et al, eds. *Microbiology.* 4th ed. Philadelphia: J. B. Lippincott Company.)

on the one hand, or a transformed, immortal cell line on the other hand. During culture, cells in a strain retain their original karyotype and are thus called *euploid;* however, culturing induces profound changes in the composition and characteristics of the cell strain, which are manifested early during the passage history and may continue during passage. Whereas primary cell cultures may contain a mixture of cell types that survive the original plating of cells, only a few cell types survive subculturing; thus, by the second or third passage, typically only one cell type remains in the cell strain. Cell strains are usually composed of one of two basic cell types—fibroblast-like or epithelial-like—characterized based on their morphology and growth characteristics (Fig. 2.4). Fibroblasts have an elongated, spindle shape, whereas epithelial cells have a polygonal shape. Although after only a few passages only one cell type may remain in a cell strain, continued passage may select for faster-growing variants, such that the

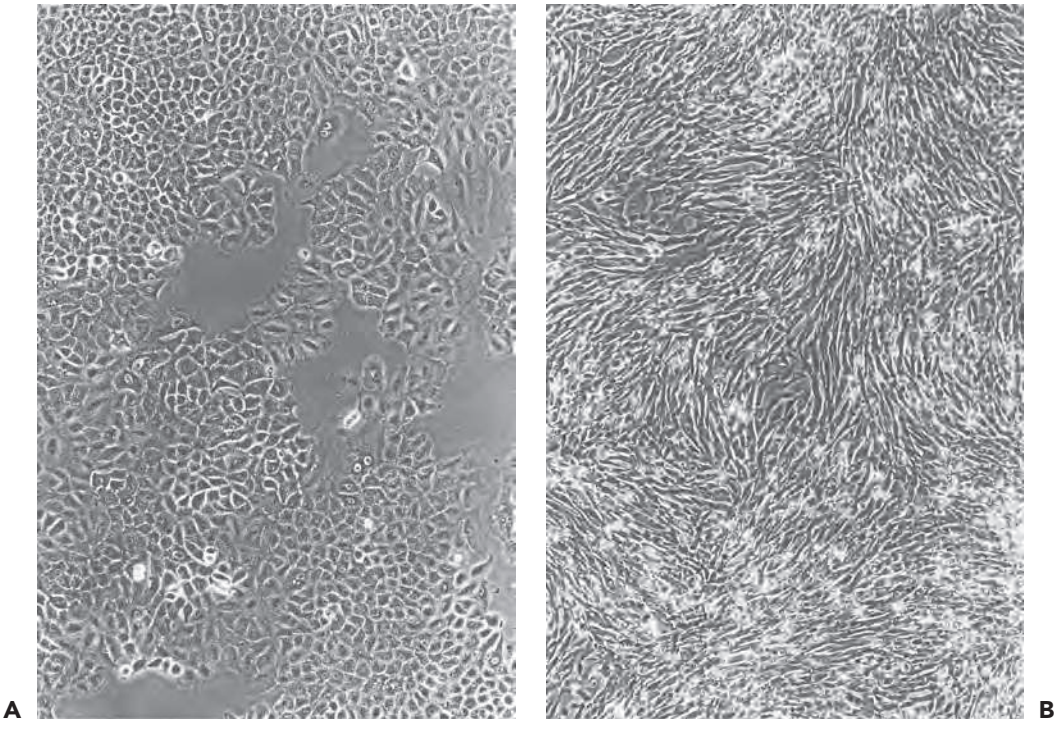

FIGURE 2.4. Cultured cell types. Phase contrast photomicrographs are shown. **A:** Epithelial-like cells, A549, a human lung carcinoma cell line, a slightly subconfluent monolayer. **B:** Fibroblast-like cells, BHK, a baby hamster kidney cell line. (A549 cell culture courtesy of J. I. Lewis. BHK cell culture courtesy of D. Holmes and Dr. S. Moyer.)

characteristics of a cell strain may change with increasing passage number. Despite the fact that normal cell strains experience senescence in culture, they may be maintained for many years by expanding the culture to a large number of cells early during the passage history and storing numerous small samples of low passage cells by freezing. Therefore, as a given strain approaches high passage number and senescence, low passage cells of the same strain may be thawed and cultured.

Cell Lines

At any time during the culture of a cell strain, cells in the culture may become *transformed* such that they are no longer subject to crisis and senescence but can be passaged indefinitely. Transformation is a complex phenomenon, discussed in more detail later and in Chapter 7; however, in the context of cell culture, the most important characteristic of transformation is that the transformed cells become immortalized. Immortal cell cultures are called *cell lines,* or sometimes *continuous cell lines,* to distinguish them from primary cultures and cell strains. Immortalization can occur spontaneously during passage of a cell strain, or it can be induced by treatment with chemical mutagens, infection with tumorigenic viruses, or transfection with oncogenes. In addition, cells cultured from tumor tissue frequently readily establish immortal cell lines in culture. Spontaneous immortalization does not occur in cultured cells from all animal species. Thus, immortalization occurs frequently during culture of rodent cells (e.g., in mouse and hamster cell strains), and it has been observed in monkey kidney cells, although it occurs rarely, if at all, during the culture of chicken or human cells. Immortalization is typically accompanied by genetic changes such that cells become aneuploid, containing abnormalities in the number and structure of chromosomes relative to the parent species, and not all cells in a culture of a continuous cell line necessarily display the same karyotype. Like cell strains, cell lines are usually composed of cells that are either fibroblast-like or epithelial-like in morphology.

As with the propagation of cell strains, continued culture of a cell line may result in selection of specific variants that outgrow other cells in the culture over time, and thus with passage the character of a cell line may change substantially, and cell lines of the same origin cultured in different laboratories over a period of years may have significantly different characteristics. It is prudent, therefore, to freeze stocks of cell lines having specific desirable properties so that these cells can be recovered if the properties disappear during culture. Likewise, it makes sense to obtain a cell line showing certain desired characteristics directly from the laboratory that described those characteristics, because cells from alternate sources may differ in character.

Transformation

Transformed cells are distinguished from normal cells by myriad properties that can be grouped into three fundamental types of changes: immortalization, aberrant growth control, and malignancy. *Immortalization* refers simply to the ability to be cultured indefinitely, as described previously. *Aberrant growth control* comprises a number of properties, several of which have relevance to experimental virology, including loss of contact inhibition, anchorage independence, and tumorigenicity. Loss of contact inhibition means that cells no longer cease to grow as soon as a monolayer is formed, and cells will now grow on top of one another. Anchorage independence means that the cells no longer need to attach to a solid surface to grow. Anchorage independence is often assayed as the ability to form colonies suspended in a semisolid medium such as agar, and a practical consequence of anchorage independence is the ability to grow in liquid suspension. Tumorigenicity refers to the ability of cells to form a tumor in an experimental animal, and *malignancy* refers to the ability to form an invasive tumor *in vivo.* While malignancy is obviously of vital importance as a phenomenon in its own right, it has limited application in virology except within the specific discipline of tumor virology (Chapter 7). Importantly, the many properties of transformed cells are not necessarily interdependent, and no one property is an absolute prerequisite for another. Thus, transformation is thought to be a multistep genetic phenomenon, and varying degrees of transformation are measurable. Tumorigenicity is often regarded as the most stringent assay for a fully transformed cell and is most closely correlated with anchorage independence.

The fact that the various characteristics of transformed cells are not interdependent has important consequences for experimental virology, especially in the assay of tumor viruses. Specifically, a transformed cell line that is immortalized but still contact inhibited may be used in a viral transformation assay that measures the further transformation to loss of contact inhibition. When cells in a monolayer are transformed by a tumor virus and lose contact inhibition, they grow on top of a confluent monolayer, forming a *focus,* literally a pile of cells, which is readily distinguishable from the rest of the monolayer. This property forms the basis for quantitative biological assay of tumor viruses,[129] described in more detail later.

Advantages and Disadvantages of Different Cultured Cell Types

The various types of cultured cells described previously have specific application to different problems encountered in experimental virology. For most applications, an adherent cell line provides the most useful host cell. Cell lines are relatively easy to maintain because they can be passaged indefinitely, and adherence is a prerequisite for a plaque assay, described later. A distinct technical advantage of adherent cells is that the culture medium can easily be changed for the purposes of infection or metabolic labeling by simply aspirating and replacing fluid from a monolayer, a process that requires repeated centrifugations with suspension cells. By contrast, relative to adherent cell lines, suspension cell lines are easier to sample than adherent cells, and they produce large numbers of cells from a relatively small volume of medium in a single culture vessel, which has significant advantages for some high-volume applications in virology. Unfortunately, not all viruses will grow on a cell line, and often under these circumstances, a primary cell culture will suffice. This may reflect a requirement for a particular cell type found only under conditions of primary cell culture, or it may reflect a requirement for a state of metabolism or differentiation closely resembling the *in vivo* situation, which is more likely to exist in a primary culture than it is in a cell line.

Lastly, some viruses do not grow in cell culture at all. In such cases, investigators are reliant either on the old expedients of natural hosts, laboratory animals, or embryonated eggs, or on some more modern advances in tissue culture and recombinant DNA technology. The papillomaviruses, which cause warts, provide an enlightening example of this situation (Chapter 54). Although the viral nature of papillomatosis was

demonstrated more than 90 years ago, progress on the study of papillomaviruses was seriously hampered in the virology heyday of the mid 20th century because the viruses grow well only on the natural host; they do not grow in culture. The inability to grow in culture is now reasonably well understood, and results from a tight coupling of the regulation of viral gene expression with the differentiation state of the target epithelial cell, which in turn is tightly coupled to the three-dimensional architecture of the epidermis, which is lost in culture. Specialized tissue culture techniques have now been developed that result in the faithful reconstruction of an epidermis by seeding primary keratinocytes on a "feeder" layer composed of an appropriate cell line and incubating these cells on a "raft" or grid at a liquid–air interface. On these raft cultures, the entire replication cycle of a papillomavirus can be reproduced *in vitro,* albeit with difficulty.[7] In the meantime, it is significant that a large fraction of the genetics and biology of papillomaviruses was determined primarily through the use of recombinant DNA technology, without ever growing virus in culture. Thus, the genetic structure of both the model bovine papillomavirus and many human papillomaviruses has been determined by cloning genomic DNA from natural infections, and regulation and function of many genes can be gleaned from sequence alone, from *in vitro* assays on individual gene products expressed *in vitro,* and from cell transformation assays that use all or parts of a papillomavirus genome. In summary, the inability to grow a virus in culture, although it increases the challenge, no longer presents an insurmountable impediment to understanding a virus.

Recognition of Viral Growth in Culture

Two principal methods exist for the recognition of a virus infection in culture: cytopathic effect and hemadsorption. *Cytopathic effect* comprises two different phenomena: (a) morphological changes induced in individual cells or groups of cells by virus infection that are easily recognizable under a light microscope, and (b) inclusion bodies, which are more subtle alterations to the intracellular architecture of individual cells. *Hemadsorption* refers to indirect measurement of viral protein synthesis in infected cells, detected by adsorption of erythrocytes to the surface of infected cells. Cytopathic effect is the simplest and most widely used criterion for infection; however, not all viruses cause a cytopathic effect, and in these cases, other methods must suffice.

Morphological changes induced by virus infection comprise a number of cell phenomena, including rounding, shrinkage, increased refractility, fusion, aggregation, loss of adherence or lysis. Morphological changes caused by a given virus may include several of these phenomena in various combinations, and the character of the cytopathic effect may change reproducibly during the course of infection. Morphological changes caused by a given virus are very reproducible and can be so precisely characteristic of the virus type that significant clues to the identity of a virus can be gleaned from the cytopathic effect alone (Chapter 15). Figure 2.5 depicts different cytopathic effects caused by two viruses—measles and vaccinia. Most important to the trained virologist, a simple microscopic examination of a cell culture can reveal whether an infection is present, what fraction of cells are infected, and how advanced the infection is. In addition, because cytopathology results directly from the action of virus gene products, virus mutants can be obtained that are altered in cytopathology, yielding either a conveniently marked virus or a tool to study cytopathology *per se.*

The term *inclusion bodies* refers generally to the observation of intracellular structures specific to an infected cell and discernible by light microscopy. The effects are highly specific for a particular virus type so that, as with morphological alterations, the presence of a specific type of inclusion body can be diagnostic of a specific virus infection. Electron microscopy, combined with a more detailed understanding of the biology of many viruses, reveals that inclusion bodies usually represent focal points of virus replication and assembly, which differ in appearance depending on the virus. For example, Negri bodies formed during a rabies virus infection represent collections of virus nucleocapsids[84] (Chapter 31).

Hemadsorption refers to the ability of red blood cells to attach specifically to virus-infected cells.[111] Many viruses synthesize cell attachment proteins, which carry out their function wholly or in part by binding substituents such as sialic acid that are abundant on a wide variety of cell types, including erythrocytes. Often, these viral proteins are expressed on the surface of the infected cell—for example, in preparation for maturation of an enveloped virus through a budding process. Thus, a cluster of infected cells may be easily detectable to the naked eye as areas that stain red after exposure to an appropriate preparation of red blood cells. Hemadsorption can be a particularly useful assay for detecting infections by viruses that cause little or no cytopathic effect.

Virus Cultivation

From the discussion presented previously, it may be obvious that ultimately the exact method chosen for growing virus on any particular occasion will depend on a variety of factors, including (a) the goals of the experiment, namely whether large amounts of one virus variant or small amounts of several variants are to be grown; (b) limitations in the *in vitro* host range of the virus, namely whether it will grow on embryonated eggs, primary cell cultures, continuous adherent cell lines, or suspension cell lines; and (c) the relative technical ease of alternative possible procedures. Furthermore, the precise method for harvesting a virus culture will depend on the biology of the virus—for example, whether it buds from the infected cell, lyses the infected cell, or leaves the cell intact and stays tightly cell associated. As a simple example, consider cultivation of a budding, cytopathic virus on an adherent cell line. Confluent monolayers of an appropriate cell line are exposed to virus diluted to infect a fraction of the cells, and progress of the infection is monitored by observing the development of the cytopathic effect until the infection is judged complete based on experience with the relationship between cytopathic effect and maximum virus yield. A crude preparation of virus can be harvested simply by collecting the culture fluid; it may not even be necessary to remove cells or cell debris. Most viruses can be stored frozen indefinitely either as crude or purified, concentrated preparations.

Quantitative Assay of Viruses

Two major types of quantitative assays for viruses exist: physical and biological. *Physical* assays, such as hemagglutination, electron microscopic particle counts, optical density measurements, or immunological methods, quantify only the presence of virus particles whether or not the particles are infectious. *Biological* assays, such as the plaque assay or various endpoint

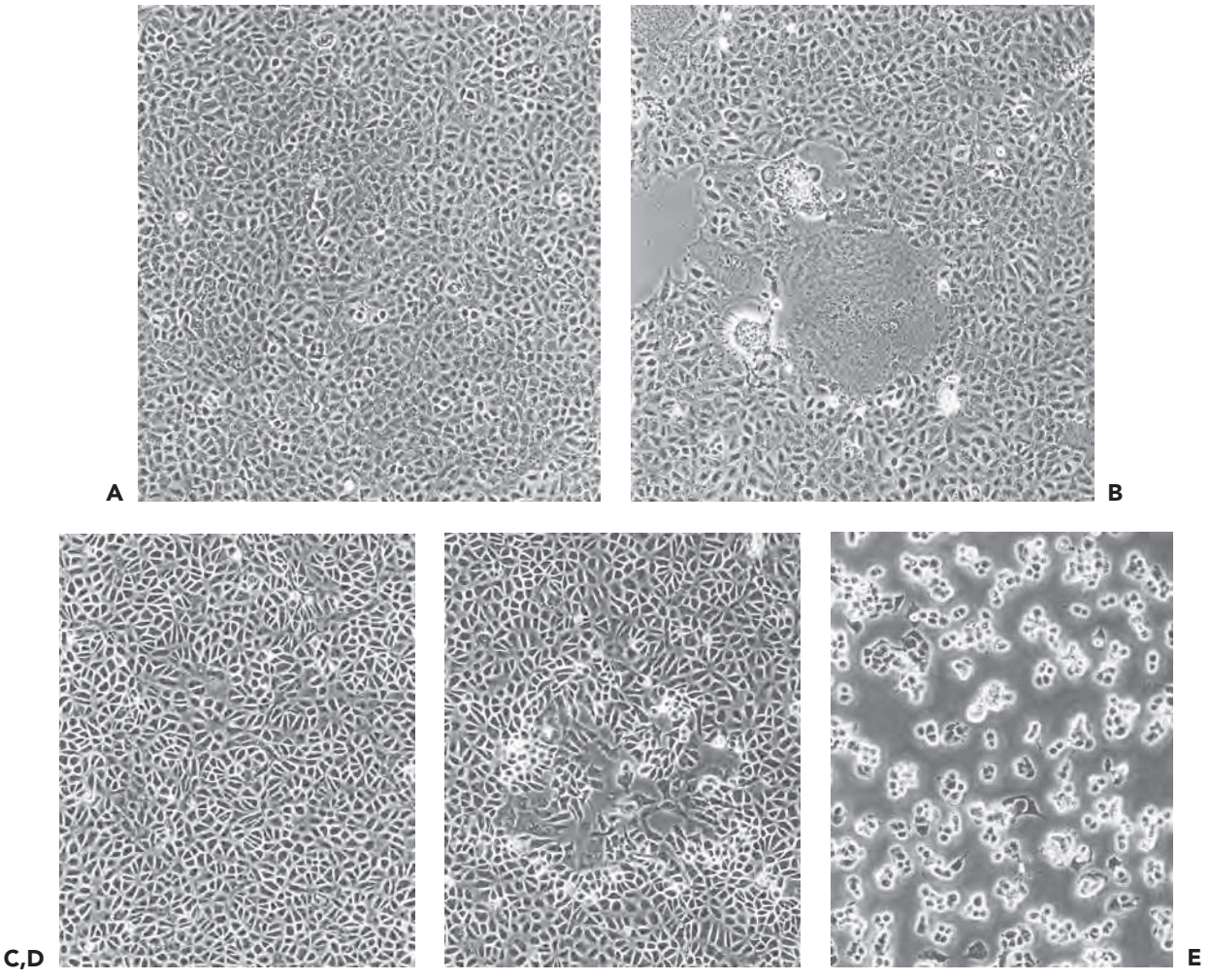

FIGURE 2.5. Virus-induced cytopathic effects. Phase contrast photomicrographs are shown. **A:** Uninfected A549 cells, a human lung carcinoma cell line. **B:** A549 cells infected with measles virus at a moi of less than 0.01 pfu/cell. Individual plaques can be discerned. Measles fuses cells, causing formation of syncytia. In mid field is a large syncytium containing multiple nuclei. Surrounding this area are additional syncytia, including two that have rounded and are separating from the dish. **C:** Uninfected BSC40 cells, an African green monkey cell line. **D:** BSC40 cells infected with vaccinia virus at a moi of less than 0.01 pfu/cell. A single plaque is shown in the middle of the field. **E:** BSC40 cells infected with vaccinia virus at a moi of 10 pfu/cell, 48 hours after infection. All cells are infected and display complete cytopathic effect. (Cultures of vaccinia infections courtesy of J. I. Lewis. Cultures of measles infections courtesy of S. Smallwood and Dr. S. Moyer.)

methods that have in common the assay of infectivity in cultured cells or *in vivo,* measure only the presence of infectivity and may not count all particles present in a preparation, even many that are in fact infectious. Thus, a clear understanding of the nature and efficiency of both physical and biological quantitative virus assays is required to make effective use of the data obtained from any assay.

Biological Assays

The Plaque Assay

The plaque assay is the most elegant, the most quantitative, and the most useful biological assay for viruses. Developed originally for the study of bacteriophage by d'Herelle[18] in the early 1900s, the plaque assay was adapted to animal viruses by Dulbecco and Vogt[28] in 1953, an advance that revolutionized animal virology by introducing a methodology that was relatively simple and precisely quantitative, which enabled the cloning of individual genetic variants of a virus, and which permitted a qualitative assay for individual virus variants that differ in growth properties or cytopathology.

The plaque assay is based simply on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathology on an otherwise normal monolayer of cultured cells. Specifically, if a single cell in a monolayer is infected with a single virus particle, new virus resulting from the initial infection can infect surrounding cells, which in turn produce virus that infects additional surrounding cells. Over a period of days (the exact length of time depending on the particular virus), the initial infection thus gives rise through multiple rounds of infection to an area of infection, called a *plaque.* Photomicrographs of plaques are shown in Figure 2.5, and stained monolayers containing plaques are shown in Figure 2.6.

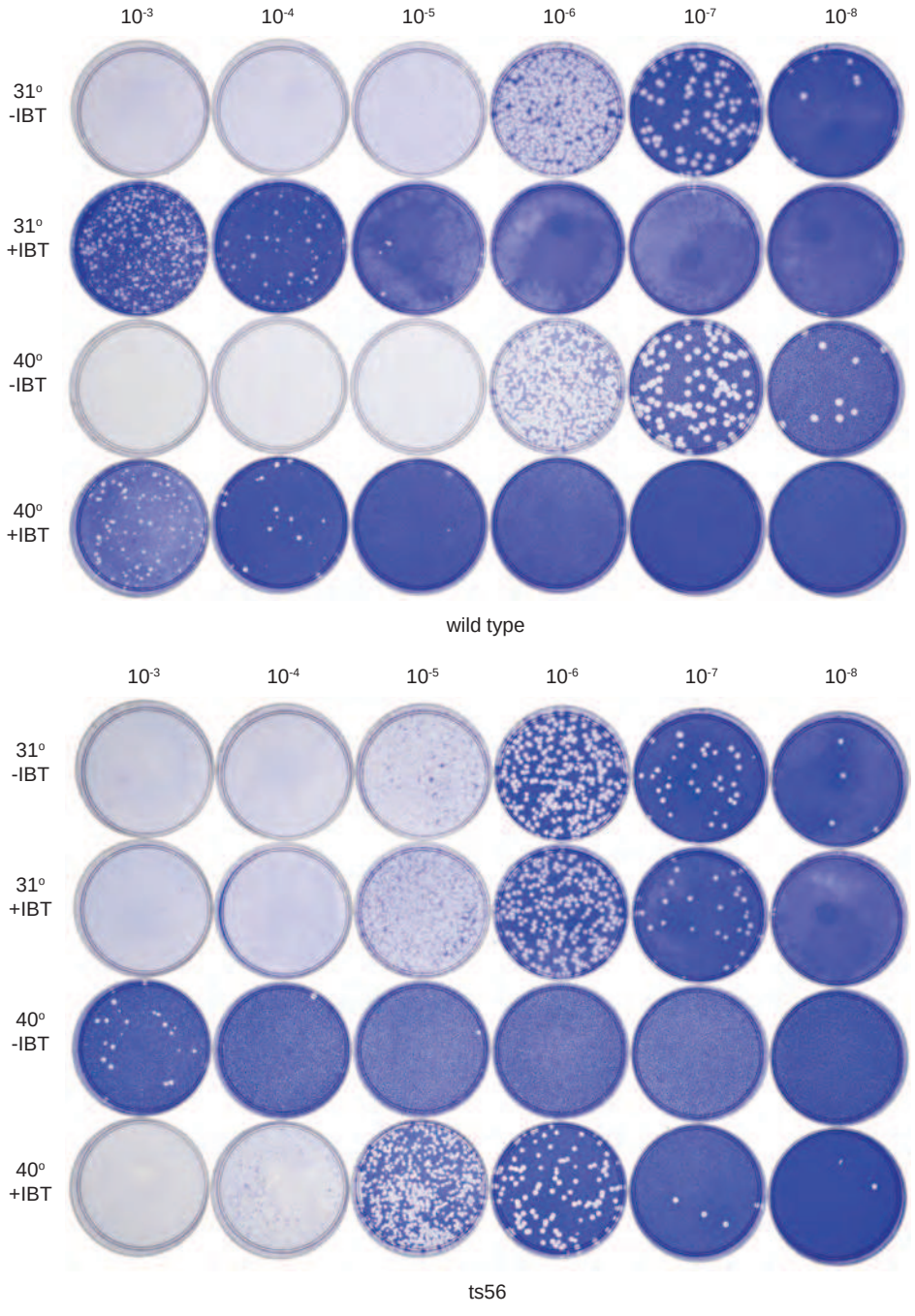

FIGURE 2.6. Plaque assay. Monolayers of the African green monkey kidney cell line BSC40 were infected with 0.5-mL portions of 10-fold serial dilutions of wild-type vaccinia virus or the temperature-sensitive vaccinia mutant, ts56, as indicated. Infected monolayers were overlayed with semisolid medium and incubated at 31°C or 40°C, the permissive and nonpermissive temperatures for *ts*56, in the presence of 45 μM isatin-β-thiosemicarbazone (IBT) or in the absence of drug as indicated, for 1 week. Overlays were removed, and monolayers were stained with crystal violet. Wild-type vaccinia virus forms plaques at both 31°C and 40°C; however, plaque formation is inhibited by IBT. Spontaneous IBT-resistant mutants in the wild-type virus stock are revealed as plaques forming at 10^{-3}, 10^{-4}, and 10^{-5} dilutions in the presence of IBT. *ts*56 carries a single-base missense mutation in the vaccinia gene *G2R*.[87] *G2R* is an essential gene that when completely inactivated renders virus dependent on IBT; hence, *ts*56 is not only temperature sensitive, forming plaques at 31°C but not at 40°C in the absence of IBT, but it is also IBT dependent at 40°C, forming plaques in the presence but not the absence of IBT. *ts*56 is slightly defective at 31°C; it forms smaller than wild-type plaques and is IBT resistant, forming plaques both in the presence and absence of drug, a phenotype intermediate between the wild-type IBT-sensitive phenotype and the null *G2R* mutant IBT-dependent phenotype. Wild-type, temperature-insensitive revertants present in the *ts*56 stock are revealed as plaques growing on the 10^{-3} plate at 40°C. Based on this assay, the titer of the wild-type stock is 2.0×10^9 pfu/mL, and the titer of the *ts*56 stock is 6.0×10^8 pfu/mL. IBT, isatin-β-thiosemicarbazone.

The plaque assay can be used to quantify virus in the following manner (see Fig. 2.6). A sample of virus of unknown concentration is serially diluted in an appropriate medium, and measured aliquots of each dilution are seeded onto confluent monolayers of cultured cells. Infected cells are overlayed with a semisolid nutrient medium usually consisting of growth medium and agar. The semisolid medium prevents formation of secondary plaques through diffusion of virus from the original site of infection to new sites, ensuring that each plaque that develops in the assay originated from a single infectious particle in the starting inoculum. After an appropriate period of incubation to allow development of plaques, the monolayer is stained so that the plaques can be visualized. The precise staining technique depends on the cytopathology; however, vital dyes such as neutral red are common. Neutral red is taken up by living cells but not by dead cells; thus, plaques become visible as clear areas on a red monolayer of cells. In cases where the virus cytopathology results in cell lysis or detachment of cells from the dish, plaques exist literally as holes in the monolayer, and a permanent record of the assay can be made by staining the monolayer with a general stain such as crystal violet, prepared in a fixative such as formalin. The goal of the assay is to identify a dilution of virus that yields 20 to 100 plaques on a single dish—that is, a number large enough to be statistically significant yet small enough such that individual plaques can be readily discerned and counted. Usually, a series of four to six 10-fold dilutions is tested, which are estimated to bracket the target dilution. Dishes inoculated with low dilutions of virus will contain only dead cells or too many plaques to count, whereas dishes inoculated with high dilutions of virus will contain very few, if any, plaques (see Fig. 2.6). Dishes containing an appropriate number of plaques are counted, and the concentration of infectious virus in the original sample can then be calculated taking into account the serial dilution. The resulting value is called a *titer* and is expressed in plaque-forming units per milliliter (pfu/mL) to emphasize specifically that only viruses capable of forming plaques have been quantified. Titers derived by serial dilution are unavoidably error prone, owing simply to the additive error inherent in multiple serial pipetting steps. Errors of up to 100% are normal; however, titers that approximate the real titer to within a factor of two are satisfactory for most purposes.

A critical benefit of the plaque assay is that it measures infectivity, although it is important to understand that infectivity does not necessarily correspond exactly to the number of virus particles in a preparation. In fact, for most animal viruses, only a fraction of the particles—as few as 1 in 10 to 1 in 10,000—may be infections as judged by comparison of a direct particle count, described later, with a plaque assay. This low *efficiency of plating*, or high particle to infectivity ratio, may have several causes. First, to determine a particle to infectivity ratio, virus must be purified to determine the concentration of physical particles and then subjected to plaque assay. If the purification itself damages particles, the particle to infectivity ratio will be increased. Second, some viruses produce empty particles, or particles that are for other reasons defective during infection, resulting in a high particle to infectivity ratio. Lastly, it is possible that not all infectious particles will form plaques in a given plaque assay. For example, infectious virus may require that cells exist in a specific metabolic state or in a specific stage of the cell cycle; thus, if not all cells in a culture are identical in this regard, only a fraction of the potentially infectious virions may be able to successfully launch an infection and form a plaque.

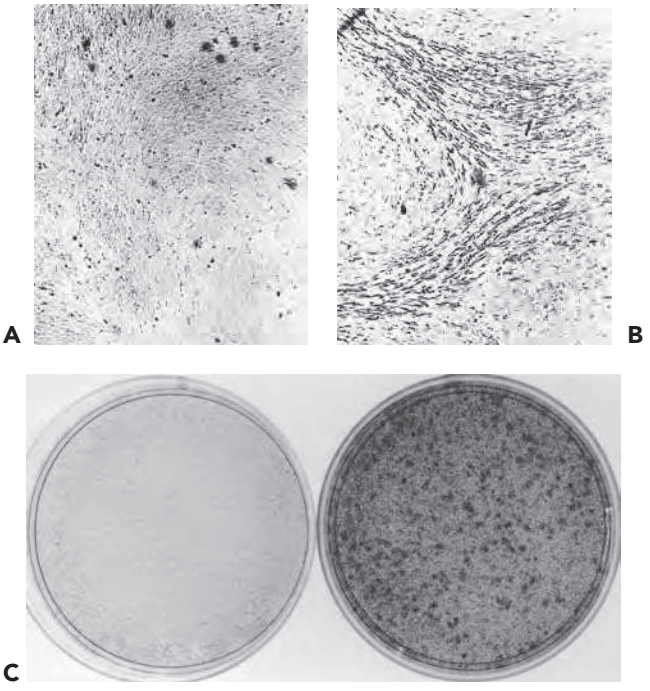

FIGURE 2.7. Focus assay. Monolayers of the NIH3T3 mouse fibroblast cell line were infected with Maloney murine sarcoma virus. **A, B:** Photomicrographs of uninfected cells **(A)** and a single virus-induced focus **(B). C:** Stained dishes of uninfected **(left)** and infected **(right)** cells. Foci are clearly visible as darker areas on the infected dish. (Courtesy of Dr. D. Blair.)

In addition to its utility as a quantitative assay, the plaque assay also provides a way to detect genetic variants of a virus that possess altered growth properties, and it provides a very convenient method to clone genetically unique variants of a virus (see Fig. 2.6). Genetic variants are considered in detail in the Virus Genetics section; in brief, they may comprise viruses that plaque only under certain conditions of temperature or drug treatment, or form plaques of altered size or shape. Because each plaque results from infection with a single infectious virus particle, unique genetic variants of a virus can be cloned simply by picking plaques—that is, literally excising a small plug of semisolid medium and infected cells from a plaque using a Pasteur pipette.

The Focus Assay

Some tumor viruses, most notably retroviruses, normally transform cells rather than killing them but can nevertheless be quantified by taking advantage of the transformation cytopathology.[116,129] For example, retrovirus transformed cells may lose contact inhibition and therefore grow as foci, literally piles of transformed cells, on top of a contact-inhibited cell monolayer. Dense foci of transformed cells stain more darkly than cells in a monolayer and thus can be quantified on treatment of an infected monolayer with an appropriate stain. Otherwise, the focus assay is similar to the plaque assay in both technique and function. Photomicrographs of foci and stained monolayers containing foci are shown in Figure 2.7.