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The use of nonhuman primates in research on seasonal, pandemic and avian influenza, 1893–2014

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Abstract

Attempts to reproduce the features of human influenza in laboratory animals date from the early 1890s, when Richard Pfeiffer inoculated apes with bacteria recovered from influenza patients and produced a mild respiratory illness. Numerous studies employing nonhuman primates (NHPs) were performed during the 1918 pandemic and the following decade. Most used bacterial preparations to infect animals, but some sought a filterable agent for the disease. Since the viral etiology of influenza was established in the early 1930s, studies in NHPs have been supplemented by a much larger number of experiments in mice, ferrets and human volunteers. However, the emergence of a novel swine-origin H1N1 influenza virus in 1976 and the highly pathogenic H5N1 avian influenza virus in 1997 stimulated an increase in NHP research, because these agents are difficult to study in naturally infected patients and cannot be administered to human volunteers. In this paper, we review the published literature on the use of NHPs in influenza research from 1893 through the end of 2014. The first section summarizes observational studies of naturally occurring influenza-like syndromes in wild and captive primates, including serologic investigations. The second provides a chronological account of experimental infections of NHPs, beginning with Pfeiffer's study and covering all published research on seasonal and pandemic influenza viruses, including vaccine and antiviral drug testing. The third section reviews experimental infections of NHPs with avian influenza viruses that have caused disease in humans since 1997. The paper concludes with suggestions for further studies to more clearly define and optimize the role of NHPs as experimental animals for influenza research.

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Keywords

influenza A virus; nonhuman primate; experimental infection; coinfection; pneumonia; pathogenesis

I. Introduction

Influenza viruses cause recurrent epidemics of respiratory illness in humans, ranging in severity from a mild, transient infection of the upper respiratory tract to severe pulmonary disease terminating in fatal bacterial pneumonia. Our current understanding of the etiology, transmission, prevention and treatment of pandemic and seasonal influenza is based on thousands of observational and experimental studies, dating back more than a century. Attempts to reproduce the features of human influenza in laboratory animals began in the 1890s, when bacteriology was a well-established science, but the existence of “filterable agents” was still a novel concept; reached solid ground with the identification of the influenza A viruses in the early 1930s; and continues through the present day.

In this article, we review the published medical literature to describe how nonhuman primates (NHPs) have been used to study human seasonal and pandemic influenza and the human disease caused by avian influenza viruses. To obtain the cited papers, we searched PubMed, using keywords such as “influenza,” “macaques”, “nonhuman primates” and related terms. We also made extensive use of the catalogue of the National Library of Medicine, which turned up a number of reports not cited in scientific papers. We carefully reviewed the reference list of each article to find earlier papers on the use of NHPs in influenza research, working our way back in time until we reached Pfeiffer’s 1893 study. Our search was not confined to the English-language literature, as we have included a number of French and German papers, beginning with Pfeiffer’s article. Although it’s possible that we missed a few isolated reports, we believe that we have found all published papers on the natural occurrence or experimental induction of influenza in NHPs that were of sufficient importance to be cited by other researchers.

We begin this article by examining reports that influenza viruses can spread naturally from infected humans to cause illness in NHPs in the wild, in research facilities, or in zoos, including both observational studies and serologic investigations. The scientific and common names of species discussed in this paper are listed in Table 1; common names are used in the text. We then present a chronological account of experimental infections of captive NHPs, from Pfeiffer’s inoculation of apes with material from influenza patients in 1893 through early 2014. Because most scientists are unfamiliar with work performed before the modern “molecular” era, our descriptions of research published in the period 1893–1980 are often more detailed than our summaries of more recent reports, to which readers have ready access on PubMed.

The chronological review of research on the use of NHPs to study human seasonal and pandemic influenza is followed by sections focusing on routes of exposure, vaccine and antiviral drug testing, and applications of pulmonary radiography. We then summarize research on neurologic complications of influenza, including its possible role in the

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occurrence of congenital anomalies. We then review how NHPs have been employed to study the novel avian influenza viruses that have caused severe disease in humans during the past two decades. Finally, we briefly summarize research on anatomic differences between the respiratory tracts of humans and NHPs that may be relevant to influenza research.

This review is accompanied by a supplemental file of 21 tables listing all published observational and experimental studies of seasonal, pandemic and avian influenza in NHPs. Each supplemental table is cited in the text the first time it is referenced. The tables are:

S1. Reports of naturally occurring influenza in wild or captive NHPs.

S2. Studies performed before the viral etiology of influenza was proven in 1933;

S3. Experimental infections of Old World monkeys other than rhesus and cynomolgus macaques with seasonal and pandemic influenza viruses;

S4. Experimental infections of rhesus macaques with seasonal or pandemic influenza viruses;

S5. Experimental infections of cynomolgus macaques with seasonal or pandemic influenza viruses;

S6. Assessment of the immunogenicity of inactivated or subunit vaccines, without virus challenge;

S7. Experimental infections of New World monkeys with seasonal or pandemic influenza A viruses;

S8. Experimental infections of New World monkeys with the 1976 New Jersey swine-origin H1N1 virus;

S9. Tests of the attenuation and immunogenicity of live, reassortant seasonal influenza vaccines in squirrel monkeys;

S10. Assessment of live, reassortant vaccines in chimpanzees;

S11. Assessment of vaccines in which immunized animals were challenged with a seasonal influenza virus;

S12. Evaluation of antiviral drugs for influenza;

S13. Experimental infection of macaques with the reconstructed 1918 H1N1 virus;

S14. Experimental infections of macaques with the 2009 pandemic H1N1 virus;

S15. Experimental infection with influenza B viruses;

S16. Studies of influenza and bacterial coinfection;

S17. Reports in which thoracic radiography was used to assess infected animals;

S18. Experimental studies of influenza-associated encephalitis;

S19. Experimental studies of the potential teratogenicity of influenza virus infection in pregnant women;

S20. Experimental infections with H5N1, H7N7 and H7N9 avian influenza viruses;

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S21. Evaluation of vaccines against avian influenza viruses;

S22. Evaluation of antiviral drugs against H5N1 avian influenza.

II. Investigations of naturally occurring influenza in NHPs

Because influenza viruses repeatedly infect all members of the human population, and humans occasionally come into contact with NHPs, either in the wild, in laboratories or in zoos, direct exposure of animals to infectious people must occasionally occur. However, only a few published articles provide evidence that influenza has been transmitted from humans to monkeys or apes. In this section, we review three types of reports: observations of a naturally occurring influenza-like illness in NHPs; recovery of influenza viruses from captive primates; and the detection of antibodies to human viruses in wild or captive animals. These reports are listed in Table S1. We then briefly summarize current approaches used by zoos to minimize exposure of their animals, especially endangered species, to human influenza.

A. Descriptions of naturally occurring influenza in NHPs

Only a few reports describe an influenza-like illness in wild or captive primates. In 1919, as the great pandemic was spreading through the human population of South Africa, articles appeared in the local press stating that wild monkeys and baboons were “dying in hundreds” from the disease (Anonymous, 1919a; Anonymous, 1919b; Anonymous, 1919c). However, there was no subsequent confirmation that these animals actually had influenza. In 1926, Mouquet described an acute respiratory illness that spread sequentially among 3 chimpanzees (*Pan troglodytes*) in a French zoo, at a time when influenza was present in the local population; after caring for one of the animals, he himself developed a flu-like illness (Mouquet, 1926). None of the monkeys housed nearby became ill.

In a 1930 article describing the experimental transmission of the common cold to chimpanzees, Dochez et al. noted that several animals that had recovered from their colds later developed a flu-like illness; at the same time, several employees were suffering from influenza (Dochez et al., 1930). In 1942, an outbreak that had “…some of the characteristics of acute epidemic influenza” occurred at the Penrose Research Laboratory in Philadelphia (Ratcliffe, 1942). Four of 15 tufted capuchins (*Cebus apella)* in an outdoor cage died of an acute respiratory disease. The animals did not display a progressive development of illness; instead, they “seemed well until about a quarter of an hour before death,” when they appeared apprehensive, then collapsed. Necropsies showed extensive pulmonary hemorrhage, but no virus was recovered and no specific diagnosis was made.

B. Isolation of influenza virus from captive animals

Four papers have reported the isolation of human seasonal influenza viruses from captive primates. In the first, researchers studying the microbial flora of common marmosets (*Callithrix jacchus*) imported from South America recovered an H2N2 virus from the lungs of an animal that had died of bronchopneumonia, at a time when the same virus was circulating among laboratory personnel (Deinhardt et al., 1967). Five years later, the same team isolated an H3N2 virus from 9 of 25 recently imported white-lipped and white-

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moustached tamarins (*Saguinus nigricollis* and *S. mystax*), none of which showed signs of respiratory illness (Murphy et al., 1972). In 1975, Malherbe et al. reported finding a virus with the electron-microscopic features of an influenza virus in tissues of recently imported yellow baboons (*Papio cynocephalus*), but they did not make a definitive identification (Malherbe et al., 1975).

In their investigation of the possible occurrence of influenza in lemurs in Madagascar, Clerc and colleagues reported in 1979 that they had recovered a virus apparently identical to the then-circulating 1977 H1N1 strain from throat swabs of 14 captive animals of 8 different species (Clerc et al., 1981). None of the animals displayed cough, respiratory distress or other signs of illness.

C. Attempts to obtain serologic evidence of influenza virus infection

In the first report indicating that human influenza viruses might infect wild or captive primates in settings other than the research laboratory, Indian scientists detected hemagglutination-inhibiting (HI) antibodies to influenza A viruses in serum samples from some free-ranging Hanuman langurs (*Semnopithecus entellus*) and bonnet and rhesus macaques (*Macaca radiata and M. mulatta*) (Bhatt et al., 1966). The following year, Kalter, Heberling and colleagues at the Southwest Research Institute in San Antonio published the first of a series of studies examining whether influenza and other human viral diseases occurred in captive animals (Kalter et al., 1967). They identified HI antibodies to influenza A H1 and H2 viruses and to influenza B virus in small percentages of the chimpanzees, Bornean orangutans (*Pongo pygmaeus*), black-handed gibbons (*Hylobates agilis*), baboons (*Papio sp.)* and rhesus macaques held in their facility. A study of wild-caught cynomolgus macaques (*M. fascicularis*) imported into Japan found no HI antibodies against the recently emerged H3N2 influenza A virus, but half of animals had antibodies to influenza B (Kawai et al., 1968).

In 1969, Atoynatan and Hsiung tested paired serum samples collected from NHPs on arrival at a primate center in Connecticut and several months later for antibodies to influenza soluble antigen (Atoynatan and Hsiung, 1969). They found that many rhesus macaques and African green monkeys (AGMs) (*Cercopithecus aethiops*) were seropositive on arrival; in contrast, most baboons were initially seronegative, but became positive within a few months, suggesting exposure to infected humans. In 1969, Kalter *et al*. reported that captive gorillas lacked antibodies to influenza, but the next year a study in Japan detected antibodies to H1N1 viruses in AGMs (Kalter et al., 1969b; Owada et al., 1970).

A few years later, researchers at the National Institutes of Health in Bethesda tested serum samples from rhesus, cynomolgus and pig-tailed macaques (*Macaca nemestrina*), AGMs and patas monkeys (*Erythrocebus patas*) for antibodies to influenza viruses (O'Brien and Tauraso, 1973). They detected HI antibodies to H2N2 and H3N2 viruses in up to 100% of AGMs at some time points in 1968–71, and noted that some of the samples had been collected from animals captured in East Africa and Asia before the H3N2 virus was recognized in humans. The authors speculated about a natural source of exposure to influenza, but such results may simply indicate that the available serologic tests were somewhat nonspecific. Another study performed in Uganda found a high prevalence of anti-

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influenza antibodies in wild and captive red-tailed (*Cercopithecus ascanius*) and AGMs (Mutanda and Mufson, 1974). The above studies are reviewed in several articles (Dick and Dick, 1974;Kalter, 1969;Kalter and Heberling, 1971).

The unexpected emergence of the swine-origin H1N1 virus in New Jersey in 1976 gave Kalter and Heberling the opportunity to test the specificity of some of the serologic reactions they had observed in samples from captive primates. They assayed serum samples from chimpanzees and baboons for antibodies to the seasonal H3N2 virus that was currently circulating in San Antonio and to the swine-origin virus, which was not present in the area (Kalter and Heberling, 1978). They found that many NHPs and laboratory employees had HI antibodies to the H3N2, but not the H1N1 virus, suggesting that members of the laboratory staff had transmitted the seasonal virus to the captive primates. However, none of the animals had shown signs of respiratory tract infection.

Two decades later, the same authors published a summary of the results of more than 50,000 serologic tests performed in their facility, and reported a prevalence of antibodies to human influenza viruses of 1–10% in chimpanzees, gorillas, orangutans and gibbons, while all macaques were negative (Kalter et al., 1997). Additionally, in 1984, influenza A and B were considered as a possible etiologies for a predisposition to invasive pneumococcal illness in a group of chimpanzees. However, influenza serology was negative and there was evidence of an earlier outbreak of parainfluenza-3 (Jones et al., 1984).

A 2001 study in Indonesia found that 5 of 15 wild Tonkean macaques (*M. tonkeana*) and 2 of 11 pet macaques had antibodies against influenza A viruses, but all of them were negative for influenza B (Jones-Engel et al., 2001). A more recent study found no evidence that wild Barbary macaques (*M. sylvanus*) in Gibraltar with frequent, close interaction with humans had been exposed to influenza A viruses (Karlsson et al., 2012). However, the investigators detected antibodies to influenza A viruses in 14 of 48 free-ranging macaques, including both pig-tailed and cynomolgus species; lower percentages of troupes of macaques in Singapore, Bangladesh, and Sulawesi were also positive. Nearly all antibodies were to H3N2 and H1N1 seasonal strains. The researchers detected viral shedding in Cambodian macaques by PCR, but were unable to isolate infectious virus.

In 1995, in a retrospective study of fibrosing cardiomyopathy in captive Western lowland gorillas (*Gorilla gorilla gorilla*) Schulman *et al.,* tested paired stored sera from one animal for influenza antibodies as part of a wider panel of viruses, and found no evidence of infection (Schulman et al., 1995). In 2005, Whittier *et al.*, reported positive titers to influenza A and B viruses as part of a wider survey of seroprevalence of infectious agents in free-living mountain gorillas (*Gorilla beringei* ssp.) in Central Africa (Whittier et al., 2005). Three seroprevalence studies in wild lemur populations including the ring-tailed lemur (*Lemur catta*), red-fronted brown lemur (*Eulemur fulvus rufus*), Von der Decken's sifaka (*Propithecus verreauxi deckeni*), and diademed sifaka (*Propitehcus diadema*) did not detect antibodies against influenza viruses (Dutton et al., 2003;Irwin et al., 2010;Junge and Louis, 2005).

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In the most recent study of the prevalence of antibodies against human seasonal influenza viruses in captive primates, researchers tested a large number of serum specimens from captive chimpanzees, gorillas and orangutans in three different research colonies, and detected antibodies against H1N1 and H3N2 viruses in varying percentages of animals by a multiplex magnetic bead assay (Buitendijk et al., 2014). There was no description of respiratory tract disease, and samples collected over time revealed no evidence of the spread of infection within the colonies.

D. Protection of zoo primates against influenza

The most recent comprehensive textbook of zoo medicine briefly notes that influenza viruses have caused explosive outbreaks of respiratory and gastrointestinal illness in captive great apes, with high morbidity but low mortality, and recommends symptomatic treatment and vaccination (Loomis, 2003). However, no publications are cited. Another chapter in the same text states that cebus monkeys, baboons and marmosets have acquired influenza from humans, resulting in respiratory and gastrointestinal illness; deaths were attributed to secondary bacterial infection, but no references are cited (Joslin, 2003). The author recommends that human caretakers with influenza or other respiratory tract infections should wear masks. The author (ASD)'s personal experience in multiple zoo environments confirms that their policies recommend that employees with respiratory illnesses or other infections not work with animals or prepare food, because these infections may be transmissible to NHPs and other species (Baker, 2002).

In their Species Survival Plans, the American Association of Zoo Veterinarians (aazv.org) states that bonobos, an endangered relative of the chimpanzee, should be vaccinated each fall against influenza A and B, and that animal care staff also be vaccinated. The AAZV also recommends serologic testing of orangutans, bonobos and chimpanzees for evidence of past infection with these viruses. Although occasional news reports have described the vaccination of chimpanzees, orangutans and gorillas against influenza, our own discussion with zoo veterinarians (Michael Stoskopf, North Carolina State University College of Veterinary Medicine and Bobby Schopler, Duke Lemur Center, personal communications) suggests that human influenza viruses have not been documented to cause significant illness in populations of captive primates, including lemurs.

III. Experimental infections of NHPs with human influenza viruses

A. Chronological review of experimental studies of seasonal and pandemic influenza in NHPs

In the following summaries of efforts to reproduce the clinical features of seasonal and pandemic influenza in captive NHPs, we base our evaluation on widely accepted descriptions of the influenza syndrome in humans. For example, the US Centers for Disease Control and Prevention (CDC) states that the clinical signs and symptoms of influenza in humans typically include the sudden onset of illness and fever, with signs of upper and lower respiratory tract infection (runny nose, sore throat and cough), plus nonspecific signs and symptoms such as muscle aches, headache, fatigue, malaise, lethargy and diarrhea (more commonly seen in pediatric patients) (CDC, 2013). In some patients, especially the

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very young, the very old, and those with chronic underlying diseases, influenza virus infection of the respiratory tract may progress to bacterial pneumonia, which is the most common etiology of fatal cases (Morens et al., 2008; Taubenberger and Morens, 2008).

The history of efforts to experimentally recapitulate the syndromes of human influenza in NHPs can be divided into three phases. In the first, investigators focused on identifying the etiological agent of influenza. This effort began with the pandemic of 1890 and lasted through the isolation of influenza A viruses in the early 1930s. Experiments in NHPs typically consisted of the inoculation of a few animals either with bacteria cultured from respiratory secretions of influenza patients or with preparations of respiratory secretions that had been passed through fine-pore filters. Researchers then monitored the animals for fever and visible signs of respiratory illness, and sometimes examined their lungs at necropsy.

The second phase of research began with demonstration of the viral etiology of influenza in the 1930s, accompanied by the development of a model of the disease in ferrets and the discovery that the virus could be propagated in embryonated eggs and maintained through sequential passage in the lungs of suckling mice. The ability to prepare virus in eggs or mice freed researchers from the need to obtain virus directly from patients for use in animal studies. Research over the next few decades, performed principally in rhesus macaques, focused on observing infected animals for signs of illness and detecting pathologic changes in the respiratory tract at necropsy.

Beginning in the mid-1970s, a third phase of influenza research began when researchers acquired the ability to propagate and titrate viruses in cell culture, facilitating quantification of the magnitude and duration of viral shedding. The discovery that squirrel monkeys developed a disease similar to human influenza led to them becoming the species of choice for studies of live, attenuated vaccines in the early 1980s, as researchers hoped that squirrel monkeys would also respond to vaccination in a manner similar to humans, providing a shorter path to vaccine approval. However, significant differences between human volunteers and squirrel monkeys in the observed level of replication of candidate avian/human reassortant vaccines led to the abandonment of this approach by the early 1990s (Clements et al., 1992). Subsequent research on influenza in NHPs has principally employed rhesus and cynomolgus macaques, and has increasingly focused on molecular studies of host responses to infection, especially gene expression during the early post-infection period, with the cynomolgus macaque as the primary species utilized.

1. Attempts to establish the causative agent of influenza, 1893–1930s—In work that began during the pandemic of 1890, Richard Pfeiffer, working in Koch's laboratory in Berlin, isolated a microbe from influenza patients that he believed to be the cause of the disease. He tested his hypothesis by inoculating cultures of the bacterium, now known as *Haemophilus influenzae*, into a variety of experimental animals, including mice, guinea pigs, and rabbits, but he was only able to produce visible disease in NHPs (Pfeiffer, 1893) (Table S2). When he injected a small dose of bacteria directly into the lungs of apes (species not identified), he observed a mild illness, with fever and cough. In contrast, intratracheal (IT) inoculation of a large dose of bacteria was followed within hours by prostration,

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hypothermia, and death, which necropsy suggested was the result of intoxication by bacterial products.

At the onset of the 1918 pandemic, it was widely accepted that “Pfeiffer’s bacillus” played a role in severe influenza. However, many researchers soon came to doubt that it could be the etiological agent of the disease, because they were frequently unable to isolate the bacterium from patients with the typical clinical syndrome, and it was often recovered from persons with unrelated conditions. Clinicians familiar with influenza were also aware that most patients developed only a mild, nonspecific febrile illness, and that cough and signs of pulmonary involvement were often late, secondary developments. The inability of researchers to consistently isolate a bacterial species from the respiratory tract of patients early in the disease course suggested that the true cause of influenza might be a “filterable agent,” similar to those which had been recently been shown to cause foot-and-mouth disease in cattle and poliomyelitis in humans (Foster, 1917).

Identifying the causative agent of influenza became an urgent priority as the new pandemic began to exact a high death toll in the autumn of 1918. Describing patients at Cook County Hospital, Nuzum noted, “…we were impressed with the paucity of bacteria in the nasopharynx at the onset of disease, and the marked degree of prostration exhibited by the patients. [This suggested] the possibility of a filterable virus as the cause of the disease…” (Nuzum et al., 1918). He and others attempted to detect the presence of a virus by passing saline suspensions of their patients’ respiratory secretions through fine-pored filters and inoculating the filtrate into human volunteers or animals, including NHPs. Their reports placed great emphasis on the point in the disease course when samples were obtained from patients and the appearance of the respiratory secretions. For example, Gibson *et al*. state that, “The sputum used was as a rule collected as early as possible in the disease. As uncomplicated cases of influenza as a rule present with a pyrexial period of only a few days duration, we considered that it would be during those few days that we should have the greatest chance of recovering the virus.”(Gibson et al., 1919b).

In the first attempt to use NHPs to detect a filterable agent of influenza, Nicolle and Lebailla prepared a saline suspension of respiratory secretions from patients who “presented with classic symptoms of influenza” and produced “an abundant and clear expectoration” (Nicolle and Lebailla, 1919). When they inoculated filtrates of this material intranasally (IN) and onto the conjunctival membranes of two toque macaques (*Macaca sinica*), the animals developed a transient febrile illness, which lacked any visible signs of respiratory tract involvement, but still permitted the investigators to claim, “the agent of influenza is a filterable organism.”

A few months later, using the methods pioneered by Nicolle and Lebailly, Gibson *et al.* prepared saline suspensions of sputum from patients in the early phase of influenza, passed some of it through filters, and inoculated the filtered or unfiltered material into rhesus macaques (Gibson et al., 1919a, 1919b). The animals developed diarrhea and depression, without any reported signs of respiratory illness, but necropsies revealed pulmonary inflammation and consolidation. The investigators also attempted to characterize their

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filtered material by culturing it in “Noguchi tubes” containing fragments of rabbit kidney in bacteriologic medium, and observed increasing numbers of minute coccoid particles.

Other investigators obtained similar results. Bradford and his colleagues collected blood or sputum from patients, passed suspensions through fine-pored porcelain filters, and noted that the product contained “very minute rounded coccus-like bodies” which were anaerobic, Gram-positive and “resist heating to 56° C for 30 minutes” (Bradford et al., 1919a; Bradford et al., 1919b). When inoculated intravenously (IV) or subdurally into guinea pigs and rhesus macaques, the material produced an acute illness (not further described), with extensive lobular pneumonia seen at necropsy. The authors therefore claimed that a “filter-passing agent” caused influenza. In a study carried out in Germany in late 1918, Fejes *et al.* prepared a suspension of patient sputum in saline, filtered it and inoculated the bacteria-free product subcutaneously (SC) into a variety of animals (Fejes, 1919). After seeing no response in rabbits and guinea pigs, the investigators performed a series of experiments in rhesus macaques and baboons, obtaining a variety of results, ranging from the absence of illness through fatal hemorrhagic sepsis.

Studies in which human volunteers were challenged with filtered material from influenza patients via a variety of routes produced a range of symptoms, from mild headaches to influenza-like disease (Nuzum et al., 1918;Yamanouchi et al., 1919). However, when Nuzum, *et al.* injected a rhesus macaque IN and IV with a filtered suspension of bronchial mucosa from a deceased influenza patient, and subsequently with a filtered suspension of nose and throat washings from patients, it did not become ill.

In addition to efforts to reproduce the influenza syndrome with a filterable agent, further attempts were made to define the role of bacterial infection. In 1920, Blake and Cecil reported the results of using *H. influenzae* to infect NHPs (Blake and Cecil, 1920). Because a preliminary experiment had shown that IT inoculation of a monkey with their bacterial stock produced no signs of illness, they passaged it 11 times intraperitoneally (IP) in mice, then 11 more times IP in monkeys. When they took the final product and inoculated it IN in white-headed capuchins (*Cebus capucinus)* or *Macacus syrichtus*, likely the Philippine macaque (*Macaca fascicularis philippensis*), the animals became acutely ill in 3–5 hours, and soon developed signs of upper respiratory tract infection. IT inoculation of the same material produced bronchopneumonia in most animals. The authors concluded that *H. influenzae* could produce a disease that resembled influenza, but admitted that their experiments had not proven that it was the causative agent of the disease.

In a second study using the same NHP species, Cecil and Blake described the pathological findings at necropsy in great detail, and concluded that these changes “differ in no essential respect from those which occur in human influenza” (Cecil and Blake, 1920). Finally, Gordon outlined a small infection study in rabbits, guinea pigs and rhesus macaques using filtered samples of nasal secretions from infected nursing staff at St. Bartholomew’s hospital in London. One of two intracerebrally infected rhesus macaques appeared to have “a typical attack of influenza with other symptoms suggestive of encephalitis” (Gordon, 1933) (see further discussion in section III C 1).

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Because attempts to identify the causative agent of the 1918 pandemic could only be performed while it was in progress, using material recovered from patients who unquestionably had the disease, research largely ended with the passing of the outbreak. Despite the many studies that had been performed in human volunteers and laboratory animals, little light had been shed on the etiology of influenza. As regards Pfeiffer's bacillus, Shope later commented that "…the role of the organism was more controversial after the smoke of the 1918 pandemic studies had cleared than it was before." (Shope, 1958). However, even though the causative agent was still in doubt, it was evident that bacterial pneumonia played a major role in severe and fatal influenza cases. Zinsser summarized the thoughts of many investigators by stating that, "The serious respiratory infections of the bronchi and lungs we can set down with reasonable certainty as complications due, certainly in the overwhelming majority of cases, to secondary bacterial invaders." (Zinsser, 1922).

By the beginning of the 1930s, no further progress had been made in elucidating the cause of influenza, allowing Long *et al.* to state that there were still "four schools of thought regarding [its] etiology," only one of which attributed it to a "true filtrable virus" (Long et al., 1931). In their own attempt to determine its etiology, the authors collected nasopharyngeal washings from seasonal influenza patients early in the course of illness, passed them through fine-pored filters and inoculated the product IN and IT into 4 young chimpanzees. The animals developed fever, moderate to marked prostration and leukopenia on the day after infection, but no signs of respiratory tract involvement were observed. The researchers monitored the nasopharyngeal bacterial flora of the animals during the illness, and found it to be unchanged.

In parallel work that supported a multifactorial etiology of severe influenza, Shope carried out a series of studies in the late 1920s, focused on swine influenza, which was first recognized during the 1918 pandemic, and appeared to be closely related to the human disease. In the critical experiment, he inoculated pigs first with filtered respiratory secretions, then several days later with bacteria and produced a respiratory illness with the typical features of swine influenza (Shope, 1931a;Shope, 1931b). His results were soon followed by the discovery that ferrets, which some years earlier had been found to be susceptible to the virus of canine distemper (Dunkin and Laidlaw, 1926), were also highly sensitive to influenza, developing nasal congestion, sneezing, cough and fever when inoculated IN with filtered material from patients, providing proof of the viral etiology of human influenza (Smith et al., 1933). Other researchers found that lung suspensions from sick ferrets produced pulmonary disease when introduced intranasally in mice, in which the virus could be maintained through sequential passage (Andrewes et al., 1934).

The next year, Shope demonstrated that ferrets could also be infected with swine influenza virus, and that the disease could be transmitted between ferrets (Shope, 1934). Two more critical developments soon followed, when Burnet reported that influenza viruses could be cultivated in the chorioallantoic membrane of embryonated eggs (Burnet, 1935) and Smith obtained similar results using minced chick embryos (Smith, 1935). The ability to maintain and propagate virus in mice and in eggs finally freed researchers from the need to collect samples from patients during an outbreak, and enabled them to share samples and perform experiments using the same virus stock. During the next few years, success in producing

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influenza in humans was reported by researchers in New York City who challenged volunteers with filtered nasal washings from patients (Dochez et al., 1936) and by scientists in Leningrad who used a bacteria-free suspension of the homogenized lungs of infected mice (Smorodintseff et al., 1937).

2. Infections of Old World monkeys and lemurs from the 1930s-early 1980s— By the late 1930s, investigators were able to culture influenza viruses in embryonated eggs, passage the virus in mice and use it to experimentally infect animals and humans. For example, McIntosh and Selbie prepared a suspension of respiratory secretions from an influenza patient, passed it through a fine-pore filter, incubated the product in broth, serially passaged it in mice, then challenged a *Cercopithecus* (guenon, species not stated) monkey IN with the 6th- passage lung extract (McIntosh and Selbie, 1937) (Table S3). Although the animal eventually became febrile, it did not develop an influenza-like illness. In a 1939 experiment that more closely resembled modern research, Vieuchange and colleagues challenged a chimpanzee IN with a mouse-passaged virus (Vieuchange, 1939) (Table S4). When the animal developed a mild fever on day 4, a saliva sample was inoculated into mice, which developed pulmonary disease. Similar inoculation of two rhesus macaques produced no change in one, but the second developed pulmonary infiltrates visible by chest X-ray on day 2 and dyspnea on effort evident by day 7, before recovering.

In 1941, Burnet published the results of an ambitious study, in which he challenged cynomolgus macaques with several strains of egg-propagated virus (Burnet, 1941) (Table S5). None of the animals inoculated by the IN route developed fever or signs of illness, but IT infection was more successful. Several macaques developed a fever, became inactive and refused to eat. After IT inoculation of the “W.S.” strain of egg-passaged virus (presumably A/WS/1933 H1N1), one animal developed progressive weakness and died 8 days postinfection. Although it showed no visible signs of respiratory illness, extensive bronchopneumonia was observed at necropsy.

In the same year, Woolpert and colleagues published a series of papers describing the infection of rhesus macaques with the mouse-passaged influenza A/Puerto Rico/8/1934 H1N1 (PR8) virus strain, with or without subsequent infection with *Streptococcus hemolyticus*. In the first study, 7 IN-infected monkeys showed a significant leukopenia the day after challenge, but no other signs of illness (Woolpert, 1941). When the authors used the same approach to infect animals with *S. hemolyticus*, they observed an acute illness characterized by fever, anorexia, weight loss and leukocytosis, but without signs of respiratory tract disease (Schwab et al., 1941). In a third set of experiments, the authors challenged macaques IN with either virus plus bacteria, bacteria followed four days later by virus, or virus followed four days or two weeks later by bacteria (Merino et al., 1941). Of the 16 animals in the study, all but one developed minimal signs of illness. In the fourth study, they explored the impact of re-exposure to *S. hemolyticus* at later time-points in monkeys employed in the earlier studies; 2 of 8 animals developed an illness attributed to renal damage (Doan et al., 1941).

Five years later, the same investigators reported the results of inoculating rhesus macaques with the PR8 virus, either alone or followed by *S. hemolyticus*. In the first experiment, four

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monkeys given virus IN displayed no visible signs of illness, while two of four injected IT became listless and developed injected conjunctiva, but no visible signs of respiratory infection (Saslaw et al., 1946). In an attempt to increase the macaques' sensitivity to influenza, the researchers held some in a room chilled to 4–6°C, then challenged them with virus. The animals became lethargic and weak, developed respiratory distress and died over the course of 2 weeks, showing peribronchial consolidation at necropsy. IN infection with the PR8 virus also proved lethal for animals rendered nutritionally deficient through a special diet.

In a later report, the same researchers inoculated rhesus macaques IN either with a mixture of virus and *S. hemolyticus*, or with virus followed 4 or 16 days later by the same bacteria (Wilson et al., 1947). As in the earlier experiments, virus alone produced no visible signs of illness, but some macaques inoculated with bacteria four days after virus challenge became listless and irritable. Two of three that received the bacteria on day 16 developed a more severe illness, but without any visible signs referable to the respiratory tract.

In 1954, Verlinde and Makstenieks performed a similar study of bacterial coinfection, in which they focused on pathologic changes in the lining of the respiratory tract at necropsy (Verlinde and Makstenieks, 1954). Rhesus macaques did not become visibly ill when inoculated IN with virus, and only developed a fever after IT challenge; in contrast, IN inoculation produced a fever in almost all cynomolgus macaques. The latter animals developed a slight nasal discharge, but no other apparent illness; the same pattern was seen in a few animals treated with cortisone. Inoculation of *S. aureus* alone or following virus infection also produced only a limited fever. However, microscopic study of lung tissues obtained at necropsy revealed extensive inflammation of the bronchial epithelium in virus-infected macaques and more severe bronchiolitis and bronchopneumonia in co-infected animals.

NHPs were also employed to a limited extent in vaccine research. Following the isolation of influenza virus in the 1930s, researchers moved quickly to develop and assess egg-grown, inactivated virus vaccines, using the PR8 and WS strains, with testing performed in mice, ferrets and human volunteers, but not in NHPs. By 1943, a vaccine had been demonstrated to be at least partially protective in Army troops during an epidemic in the USA, and two years later a bivalent A and B vaccine also proved beneficial. However, the failure of the same vaccine to have any impact on the 1947 epidemic led both to the recognition of antigenic variation, implying that new vaccines would be needed each flu season, and to efforts to increase vaccine potency. In the first study of influenza vaccines employing NHPs, Jonas Salk and his colleagues reported in 1951 and 1952 that the standard vaccine prepared in a water-in-oil emulsion and delivered intramuscularly to rhesus macaques elicited a much stronger antibody response than the vaccine without adjuvant (Salk and Laurent, 1952;Salk et al., 1951) (Table S6). The animals were not subsequently challenged with influenza virus.

By 1960, it had been concluded that macaques typically did not develop cough, respiratory distress or other visible signs of respiratory tract infection when inoculated with influenza virus by the IN or IT route; a remaining alternative was to deliver it in a small-particle aerosol. In a study published in 1965, Saslaw and Carlisle exposed rhesus and cynomolgus

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macaques to aerosols of the PR8 virus or a seasonal H1N1 or H2N2 virus (Saslaw and Carlisle, 1965). A few animals of each species developed transient listlessness, weakness and chills, but most remained healthy. In 1974, Berendt *et al.* challenged rhesus macaques with an aerosolized H3N2 virus in a small-particle aerosol, and detected leukopenia, but no visible signs of illness (Berendt, 1974).

The researchers then performed a second study, in which animals were first exposed to aerosolized influenza virus, then to *S. pneumoniae*, but again saw no signs of illness (Berendt et al., 1974). The only effect observed was that bacteria could be recovered for a longer time from the respiratory tracts of animals that had been previously exposed to virus. During this time period, Marois *et al*. performed another study in which NHPs were challenged with influenza virus only by the IN route (Marois et al., 1971). They inoculated rhesus macaques either with a seasonal H3N2 virus or with an avian or an equine influenza virus. None produced any visible signs of illness.

In the late 1960s, a number of researchers began to evaluate influenza virus infection of species other than rhesus or cynomolgus macaques. Kalter et al. performed a study in yellow baboons (*Papio cynocephalus*), in which they challenged three animals IN with the recently emerged H3N2 virus, while three control animals were kept in adjoining cages (Kalter et al., 1969a). Two of the inoculated baboons developed low-grade fevers, and one appeared anorexic, but no other signs of disease were observed. Virus was isolated from the respiratory tracts of the co-housed animals, demonstrating successful transmission.

In a further study, the authors employed baboons to determine if the response to influenza virus infection could be modified by treatment with the recently developed polyribosinic acid complex, polyIC, which appeared to act as an interferon inducer (Heberling and Kalter, 1970). Untreated animals remained well or showed a slight nasal discharge, and all developed antibodies to the virus, while animals given polyIC at the time of virus challenge remained well and showed no antibody response, indicating suppression of viral replication. Further studies of polyIC showed that it was rapidly broken down in the plasma of humans and nonhuman primates, but it was much more stable when complexed with polylysine, as polyICLC. Toxicity thresholds for rhesus macaques were investigated in three studies (Levy et al., 1975;Sammons et al., 1977;Stephen et al., 1977a).

In a unique report in 1971, Johnsen et al. described an outbreak of respiratory illness in a laboratory colony of white-handed gibbons (*Hylobates lar lar*) following the IN inoculation of some animals with seasonal influenza viruses (Johnsen et al., 1971). Following a small preliminary experiment in which no signs of illness were observed, the researchers inoculated 10 gibbons IN with an H3N2 virus and 12 with an H2N2 virus, and again observed no disease. After 2–3 weeks, however, an outbreak of respiratory tract illness, characterized by rhinitis, cough, anorexia, gastrointestinal disturbances and fever, occurred throughout the colony, affecting 36 gibbons and resulting in four deaths. H3N2 influenza virus was recovered from five sick animals that had not been experimentally inoculated. The fatal cases showed widespread purulent pneumonia at necropsy. Two species of *Staphylococcus* were isolated from one animal and a *Streptococcus* and *Proteus sp.* were isolated from a second. Despite the apparent susceptibility of these animals to a seasonal

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influenza virus, no further reports have described research using this now endangered species.

In the only published study to examine experimental influenza virus infection in lemurs, the same research group on Madagascar which in 1979 had reported isolating a circulating human H1N1 virus from several species of captive lemurs administered the same virus to three common brown lemurs (*Eulemur fulvus*) by IN inoculation (Clerc et al., 1982). They were able to recover the agent from the pharynx of most animals 24 hours later, and recorded an increase in body temperature, compared to a control, but observed no other signs of illness.

In a study focused on both influenza A and B virus infection in NHPs, bonnet macaques that had been locally trapped in India were infected IN with an H3N2 influenza A virus or an influenza B virus (Paniker and Nair, 1972). In both cases, the animals shed virus for several days, but did not become ill. The H3N2 virus was transmitted to co-housed macaques, but the influenza B virus was not.

3. Early use of New World monkeys, mid-1970s—In 1975, Berendt and colleagues began to use squirrel monkeys (*Saimiri sciureus*) in their research, inoculating them with the same H3N2 virus and *S. pneumoniae* preparation used in their previous studies (Berendt et al., 1975) (Table S7). In contrast to macaques, which did not become ill, the squirrel monkeys developed clinical signs of influenza, including fever, sneezing, coughing, tachypnea and dyspnea. Necropsies performed on day 6 postinfection revealed tracheitis and bronchopneumonia. When the monkeys were inoculated IT with a small dose of bacteria, they developed only mild illness, while a large dose produced severe pneumonia. However, infection with virus followed by a small dose of bacteria produced severe disease that was lethal in three of four animals. A subsequent study found that squirrel monkeys developed a similar illness when the H3N2 virus was delivered IT or by small-particle aerosol (Stephen et al., 1977b).

During the next two years, Berendt and his colleagues used squirrel monkeys to characterize the swine-origin H1N1 virus that had recently caused an outbreak at Fort Dix, New Jersey (Top and Russell, 1977) (Table S8). Although British investigators had shown that the novel “swine flu” agent caused only mild illness in human volunteers (Beare and Craig, 1976), American researchers were reluctant to perform such studies. In tests performed in the high-containment laboratories at Fort Detrick, Berendt and Hall found that the swine-origin virus caused an illness in squirrel monkeys similar to that produced by a seasonal H3N2 virus (Berendt and Hall, 1977). In subsequent experiments, they showed that treatment with the antiviral drug amantadine, begun before or after virus challenge, reduced signs of illness and prevented viral shedding (Scott et al., 1978). Monkeys immunized with a commercial vaccine were partially protected (Berendt and Scott, 1977).

The 1976 H1N1 New Jersey virus and a seasonal H3N2 virus were also evaluated in tufted and white-fronted capuchins via IN or IT inoculation (Grizzard et al., 1978). All 4 monkeys exposed to the H3N2 virus by the IN route developed rhinorrhea and conjunctivitis, while all 8 infected IT became inactive and showed evidence of respiratory distress; all showed

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radiographic evidence of pulmonary disease. Two of 4 monkeys inoculated IN with the New Jersey virus developed an upper respiratory tract illness, while 8 of 10 exposed IT showed signs of disease similar to, but milder than that caused by the H3N2 virus. Necropsies revealed areas of pulmonary consolidation in animals infected IT with the H3N2, but not the novel H1N1 virus; abnormalities in monkeys inoculated IN were limited to pathologic changes in the mucosal lining of the turbinates.

4. Vaccine studies principally employing squirrel monkeys, 1980–1995—In 1980, Murphy and colleagues, seeking a way to rapidly test new live, attenuated vaccines without performing studies in human volunteers, evaluated New World primates as experimental animals, administering virus by the IT route. In an initial study, they inoculated squirrel monkeys, northern owl monkeys (*Aotus trivirgatus*), tufted capuchins, white-fronted capuchins (*Cebus albifrons*) and a group of human volunteers with three different seasonal viruses (Murphy et al., 1980). All three viruses caused a febrile respiratory tract illness in the volunteers and a milder illness in the squirrel monkeys, accompanied by virus shedding. Unlike the capuchins in the study cited above (Grizzard et al., 1978), the capuchins and owl monkeys showed no signs of illness, despite the use of comparable $TCID_{50}$ virus doses of H3N2 and H1N1 viruses. The researchers concluded that squirrel monkeys were “moderately permissive primate hosts in which to investigate the genetic basis of virulence of influenza A viruses.” This susceptibility was confirmed in a follow-up study, in which squirrel monkeys were inoculated with an influenza A virus recovered from seals, which had caused only conjunctivitis in a human (Murphy et al., 1983). The animals developed a respiratory illness similar to that induced by an H3N2 seasonal virus, which was fatal in one case.

In a follow-up study, Murphy’s group exposed squirrel monkeys to 10 different avian influenza viruses, and found that, although some agents replicated well and produced disease, others were restricted in their replication in that species (Murphy et al., 1982a) (Table S9). This discovery suggested that reassortant viruses incorporating HA and NA genes from human seasonal viruses and other genes from an avian source might succeed as live, attenuated vaccines (Murphy et al., 1982b). Over the following decade, the researchers examined the replication of several such human-avian reassortant viruses in squirrel monkeys and observed significant restriction of replication for several viruses (Clements et al., 1986;Murphy et al., 1984;Murphy et al., 1989;Snyder et al., 1987;Snyder et al., 1986a;Snyder et al., 1986c;Tian et al., 1985;Treanor et al., 1989).

In a parallel effort, Murphy’s group also used chimpanzees to evaluate cold-adapted seasonal viruses as candidate vaccines. Because the higher core body temperature (38.8–39.8° C) of squirrel monkeys precluded their use in such experiments, the researchers employed chimpanzees, whose core temperature (37° C) is the same as humans (Snyder et al., 1986b) (Table S10). In the first experiment, IT inoculation of a wild-type H3N2 virus produced rhinorrhea in one of two chimps, but the other showed no signs of illness. Replication of the cold-adapted virus was markedly restricted in the lower respiratory tract of the other two animals. The researchers subsequently evaluated avian reassortant viruses in tandem with a squirrel monkey study and a candidate cold-adapted H3N2 virus whose loss of virulence resulted from a deletion in the NS1 gene, and found that its replication was not

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significantly restricted in the upper or lower respiratory tract of chimpanzees (Snyder et al., 1986a;Snyder et al., 1990). The same researchers reported that a cold-adapted influenza B virus that was attenuated in ferrets and had been found to be safe in humans was also restricted in replication in chimpanzees (Snyder et al., 1989).

The same reassortant viruses tested in squirrel monkeys were also evaluated in ferrets and in human volunteers, and their level of replication was found to differ significantly in humans and monkeys. In 1992, the investigators therefore concluded that "…it is not valid to extrapolate the results of studies of influenza virus single-gene substitution reassortant viruses with squirrel monkeys to humans." (Clements et al., 1992). The effort to use squirrel monkeys as a "short-cut" to approval of live recombinant vaccines was therefore abandoned. With the exception of a report in 1993, in which the role of a mutation in the PB2 gene in determining virus host range was first documented, and a 1995 study of reassortant viruses, in which chimpanzees were also employed, (Subbarao et al., 1993;Subbarao et al., 1995), there have been no further reports of influenza research employing squirrel monkeys.

5. Evaluation of novel influenza vaccines and adjuvants in NHPs—In addition to the reassortant vaccine research described in the preceding section, which principally used squirrel monkeys, investigators have evaluated the immunogenicity and efficacy of novel vaccines and adjuvants in at least six different NHP species. Since 2000, however, most researchers have used rhesus macaques, perhaps because they are more widely available. Reports that examine only immune responses to vaccination are listed in Table S6, and those that include the response of immunized animals to subsequent virus challenge are listed in Table S11.

NHPs have been employed in efforts to simulate the immune response of elderly humans to vaccination. In 1988, Ershler and colleagues used a commercial trivalent HA subunit vaccine to examine antibody responses in young or old rhesus macaques, and found that responses were not influenced by age (Ershler et al., 1988). In contrast, a recent study observed a significant reduction in antibody responses to a trivalent influenza vaccine in old and very old rhesus macaques (Coe et al., 2012). Interestingly, Aspinall *et al.* have shown that treatment of aged rhesus macaques with IL-7 markedly improved their immune response to inoculation with an inactivated H1N1 vaccine (Aspinall et al., 2007).

A study of mucosal immune responses in rhesus macaques found that delivery of a killed influenza A vaccine by intraesophageal tubing elicited antibodies in the upper respiratory tract (Bergmann et al., 1986). A later report focused on the induction of mucosal immune responses to killed influenza A virus and *Streptococcus mutans* vaccines in the same species and found that, compared to humans, the animals mounted a weak IgA and strong IgM response to intestinal immune stimulation, suggesting that they were not a good model for researching mucosal IgA responses (Michalek et al., 1995). In an unusual study in 1983, Ishihara et al. vaccinated Japanese macaques (M*acaca fuscata*) with an HA vaccine, then tested their bronchial responses to inhaled methacholine and histamine, and demonstrated an increase in sensitivity after vaccination (Ishihara et al., 1983).

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After the pandemic H3N2 influenza A virus had emerged in 1968, a number of influenza researchers considered the development and production of vaccines to have been too slow, and they therefore attempted to determine how the immunogenicity of inactivated vaccines could be increased, so that less antigen would be required per dose. A 1969 study showed that antibody responses of AGMs to a quadrivalent killed vaccine were markedly enhanced if the vaccine was emulsified in peanut oil, or if the oligonucleotide polyIC was added to the inoculum (Woodhour et al., 1969). Similarly, when a swine-origin H1N1 virus emerged in 1976, it was found that adding polyICLC markedly enhanced antibody responses of rhesus macaques to a subunit vaccine (Stephen et al., 1977a).

In the late 1990s, cynomolgus macaques were used to test a novel immune-stimulating complex (ISCOM) vaccine, which employs an adjuvant related to saponins extracted from tree bark. Vaccinated animals were protected against a homologous IT challenge (Rimmelzwaan et al., 1997). Two subsequent studies showed that antibody responses to the ISCOM vaccine in ferrets and cynomolgus macaques were strongly cross-reactive with post-1992 H3N2 strains, but protection did not extend to a “drifted” descendant of the vaccine virus (Rimmelzwaan et al., 2002;Rimmelzwaan et al., 1999).

In a different approach, Chen *et al.* found that epidermal injection of a commercial trivalent influenza vaccine prepared as a powder elicited a HI titer in rhesus macaques equivalent to IM injection, but addition of an experimental adjuvant tripled the response (Chen et al., 2003). Another report showed that treatment with interleukin-15 at intervals following influenza vaccination resulted in a significant enhancement of antigen-specific CD8 memory T cells (Villinger et al., 2004). In a recent study using the commercial inactivated Fluzone® vaccine administered intradermally, Carroll and colleagues showed that an adjuvant consisting of alphavirus replicon particles enhanced the immune response (Carroll et al., 2011a). The same authors have since shown that the immune response of elderly rhesus macaques to the same inactivated vaccine could be markedly enhanced by a cationic lipid/DNA adjuvant (Carroll et al., 2014). Another recent study in cynomolgus macaques showed that the antigenicity of a H1N1 pdm09 split vaccine was preserved through spray drying and electron-beam sterilization (Scherliess et al., 2014).

Beginning in the early 1990s, a number of studies evaluated the efficacy of DNA vaccines against influenza in NHPs. A series of three reports showed that vaccination of rhesus macaques and AGMs with even a low dose of a DNA vaccine encoding an influenza HA elicited a strong antibody response, which was equivalent to or better than that induced by contemporary commercial whole and subunit vaccines, and that the DNA vaccine performed best in a prime/boost strategy (Donnelly et al., 1995;Liu et al., 1997;Ulmer et al., 1994). Bot *et al.* later demonstrated the efficacy of an influenza HA and NP DNA vaccine in infant baboons and found that humoral and cell-mediated immune responses were present after more than a year (Bot et al., 2001;Bot et al., 1999). The authors concluded that DNA vaccination was less subject to interference from maternal antibodies than conventional vaccines.

Two studies showed that ID (“gene gun”) and IM inoculation of a DNA vaccine were equally efficacious in rhesus macaques (Haensler et al., 1999;Loudon et al., 2010). Use of

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GMCSF as an adjuvant further enhanced mucosal and systemic responses to a particle-mediated HA DNA vaccine (Loudon et al., 2010). Laddy *et al.* also used rhesus macaques when they tested then further optimized a consensus HA + NA + NP DNA vaccine (Laddy et al., 2009;Laddy et al., 2008). They demonstrated the potential utility of electroporation and the value of vaccines that induce subtype cross-reactive humoral and cellular immunity.

Further work from the same group showed that the immunogenicity of the three-antigen DNA vaccine was increased if accompanied by plasmid expression of a low dose of IL-15 (Yin et al., 2009). In contrast, a high dose reduced immunogenicity, and decreased IFN-γ-producing cells and T cell proliferation. The authors provide no explanation of this unexpected result, noting that IL-15 administration had previously been associated with enhanced immune responses. In a different approach, the response of rhesus macaques to immunization with a plasmid encoding an H1N1 HA protein was markedly enhanced when the animals were boosted with an adenovirus vector encoding the same HA or with an inactivated seasonal vaccine (Wei et al., 2010).

Because the sequence of the influenza A M2 protein is highly conserved, it is a potential target for a universal vaccine (Frace et al., 1999;Neirynck et al., 1999;Slepushkin et al., 1995). Fan et al. developed an M2-peptide conjugate vaccine and evaluated it in multiple experimental animals, including rhesus macaques (Fan et al., 2004). The vaccine protected mice and ferrets and was immunogenic after a single dose in macaques, with an enhanced response on boosting.

The same group later evaluated the immunogenicity of the M2 peptide expressed on a bacterial carrier protein or on the surface of hepatitis B core antigen virus-like particles (VLPs) (Fu et al., 2009). In another recent study, an enveloped virus-like particle vaccine expressing the H3N2 HA protein was produced in an alphavirus replicon vector system and evaluated in mice, rabbits and rhesus macaques (Hubby et al., 2007). It induced high HI titers and cellular immune responses in all three species.

Because the influenza NS1 protein inhibits host interferon responses, live attenuated vaccines lacking a functional NS1 have been an area of active research (Falcon et al., 2005;Ferko et al., 2004). In 2007, Baskin and colleagues evaluated a recombinant virus with a truncated NS1 gene in pig-tailed macaques, using functional genomics in addition to serologic evaluation (Baskin et al., 2007). The live, NS1-truncated vaccine elicited a stronger IgG response than a formalin-fixed whole virus vaccine. Transcriptional analysis of infected tracheobronchial cells attributed enhanced humoral immunity to a stronger type I interferon response.

In a novel approach, Kasturi et al. produced nanoparticles expressing both an H1 HA molecule and ligands for toll-like receptors 4 and 7 on their surfaces, and showed that immunization with nanoparticles containing antigens plus ligands elicited robust antibody responses in rhesus macaques (Kasturi et al., 2011). In another report, Gabitzsch and colleagues tested the ability of a recombinant adenovirus Ad5 vaccine encoding an H1N1 HA to overcome pre-existing immunity to Ad5 in rhesus macaques (Gabitzsch et al., 2012). They measured a strong antibody response that increased further on boosting.

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In two recent studies, Jegaskanda and colleagues characterized responses to vaccination and influenza virus infection in pigtail macaques (Jegaskanda et al., 2013a, 2013b). In the first, the authors immunized animals twice with the standard trivalent vaccine, then challenged them sequentially with H1N1 and H3N2 viruses. Vaccination did not elicit detectable type-specific ADCC or CTL responses, and although subsequent H1N1 challenge elicited type-specific ADCC, no cross-reactive response was observed; H3N2 challenge had little effect on ADCC, but boosted CTL. In the second study, the authors used a chimeric influenza/SIV vaccine to characterize the maintenance of immunity in the presence of SIV infection.

6. Evaluation of antiviral therapies for seasonal and pandemic influenza from 1970 through the present—Perhaps because only a handful of anti-influenza drugs have been developed, and researchers have been able to test them in mice and ferrets before proceeding to clinical evaluation in human volunteers, only a few papers describe the evaluation of medications in NHPs (Table S12). In the first such report, Heberling and Kalter showed that treatment of baboons with the novel interferon inducer, polyIC, at the time of challenge with an H3N2 virus increased their resistance to infection and prevented transmission of the virus to co-housed animals (Heberling and Kalter, 1970).

The discovery of amantadine as a specific therapy for influenza in the early 1960s and of the broad-spectrum antiviral ribavirin in 1970 led to two studies in squirrel monkeys. In the first, Stephen and colleagues showed that treatment with aerosolized ribavirin reduced the severity of illness of monkeys infected with an aerosolized H3N2 virus (Stephen et al., 1977b). In 1978, Scott and colleagues showed that amantadine therapy reduced the severity of illness and prevented virus shedding in squirrel monkeys infected with the 1976 H1N1 virus (Scott et al., 1978).

No reports of antiviral therapy of influenza in NHPs were published for the next 30 years. In 2008, as part of a study of the mechanism of the interferon-induced MxA protein, Carroll and coworkers showed that treatment with oseltamivir suppressed the replication of a seasonal H1N1 virus in the trachea of rhesus macaques; MxA expression was much lower in untreated animals (Carroll et al., 2008). Oseltamivir treatment prevented the development of fever; no other signs of illness were described. Three years later, the same group reported that prophylactic administration of pegylated IFN-alpha 2a reduced weight loss and fever in rhesus macaques infected with a seasonal H1N1 virus (Matzinger et al., 2011).

In a recent study of antibody therapy, Song et al. generated mab Z3G1 against the M2 matrix protein, which cross-reacted against M2 of a wide range of influenza A viruses (Song et al., 2014). When the investigators challenged cynomolgus macaques with an isolate of the 2009 pandemic H1N1 virus and gave them IV Z3G1 the day before or the day after infection, they observed less weight loss, a maintenance in blood oxygen saturation and less lung damage at necropsy, compared to controls; however, treatment did not prevent fever or reduce virus shedding.

In the only study of antiviral therapy of influenza B in NHPs, a single IV injection of the novel neuraminidase inhibitor peramivir immediately following virus infection was shown to significantly reduce nasal viral titers and fever in cynomolgus macaques (Kitano et al.,

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2011). The single dose of peramivir was more effective than five daily doses of oseltamivir. The evaluation of antivirals against avian influenza viruses is reviewed below.

7. Recent studies of infection of NHPs other than squirrel monkeys with seasonal viruses—In recent decades, a number of investigators have used NHPs other than squirrel monkeys for research on seasonal influenza. Pathogenesis studies performed in various species of Old World monkeys are reviewed below and listed in Tables S3–S5. Additionally, in a series of three studies focused on studying the major histocompatibility complex, Evans et al. infected cottontop tamarins (*Saguinus oedipus*), a New World monkey, IN with an H3N2 virus, and described shedding of virus, but no clinical signs of illness (Evans et al., 1999; Evans et al., 1997; Evans et al., 1998) (Table S7). Three reports describing influenza B virus infection of macaques are reviewed in section III B 1.

A major trend in influenza research over the past decade has been the effort to measure gene expression changes in infected NHPs and correlate them with clinical and pathologic findings. In the first such study, Baskin *et al.* inoculated pig-tailed macaques with a seasonal H1N1 virus and humanely killed animals on days 2, 4 and 7 for pathology, gene expression and proteomics analysis (Baskin et al., 2004). The infected macaques developed fever, anorexia, weight loss, nasal discharge and throat inflammation, and showed histologic changes consistent with viral pneumonia, without evidence of bacterial superinfection. The authors concluded that their experimental infections effectively simulated human seasonal influenza. A follow-up study identified marked differences in patterns of gene expression in tissue samples from various regions of the lung, depending on levels of viral replication (Baas et al., 2006).

More recently, Zinman and colleagues obtained alveolar macrophages from mice and cynomolgus macaques and infected them with two different seasonal influenza viruses (Zinman et al., 2011). The authors identified a core set of genes related to type I interferon responses that were expressed in both species, and other responses that were species- or virus-specific. In another study of host responses to infection, Jie et al. studied the response of dendritic cells in the lungs and associated lymph nodes of cynomolgus macaques infected bronchoscopically with an H3N2 virus (Jie et al., 2014). The authors note that humans and macaques possess myeloid and plasmacytoid DCs. In the infected animals, both cell types increased rapidly in the days following challenge, but only myeloid cells remained elevated at day 30.

Carroll and co-workers used rhesus macaques inoculated with a seasonal H1N1 virus in three separate studies. In the first, they showed that cellular expression of the MxA protein was higher in animals treated with oseltamivir than in untreated animals, indicating that influenza viral replication suppresses interferon-related gene expression (Carroll et al., 2008). In the second, they depleted B and CD8+ T cells in vaccinated macaques and showed that antibodies were sufficient to protect against virus challenge (Carroll et al., 2011b). The third study, assessing an experimental vaccine adjuvant, has been described (Carroll et al., 2011a).

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In the most recent study to examine viral-bacterial coinfection, researchers examined the consequences of inoculating cynomolgus macaques with a seasonal H3N2 virus and with *Staphylococcus aureus* (Kobayashi et al., 2013). Although the authors had anticipated that infection with both virus and bacteria would produce enhanced disease, no worsening of illness was observed. This study is discussed below in the context of other coinfection experiments.

Another report examined the virulence in cynomolgus macaques of an H2N3 virus recovered in 2006 from sick pigs in the USA (Richt et al., 2012). The investigators found that the swine virus caused more severe pulmonary disease and a more intense inflammatory response in the macaques than a human H2N2 virus, suggesting that it poses a significant public health threat.

In an effort to understand the susceptibility of human infants to severe influenza, Holbrook et al. infected infant and adult AGMs with a seasonal H1N1 virus by the IN and IT routes and euthanized the animals at day 14 postinfection (Holbrook et al., 2014). The infant macaques had higher viral loads early in the course of illness and more severe pulmonary damage at necropsy; while systemic IgG responses were similar in adults and infants, the latter developed much lower influenza-specific IgG levels in the respiratory tract.

8. Infection of NHPs with the reconstructed 1918 influenza virus—Two reports have described the infection of NHPs with the reconstructed 1918 pandemic H1N1 virus (Cilloniz et al., 2009;Kobasa et al., 2007), while a third employed a reassortant H1N1 seasonal strain in which the HA and NA genes came from the 1918 virus (Baskin et al., 2009). Tissues collected during the third study were used for further analyses, which were reported in three further articles (Table S13).

In the first study, cynomolgus macaques were inoculated simultaneously by the IN, IT, oral and conjunctival routes with either the 1918 virus or a seasonal H1N1 virus (Kobasa et al., 2007). All macaques infected with the 1918 virus became ill within 24 hours with depression, nasal discharge and cough, and rapidly developed an increased respiratory rate and hypoxia, consistent with acute respiratory distress syndrome, while animals that received the seasonal virus developed only few, mild clinical signs. Because of severe illness, all were humanely killed by day 8. The seasonal virus was recovered only from the upper respiratory tract, but the 1918 virus was present at high titer throughout the lungs. Histologic examination of lung samples taken at necropsy showed diffuse infection and desquamation of alveolar lining cells and exudation of fluid into alveolar spaces. Much higher levels of proinflammatory cytokines, especially IL-6, were measured in the plasma of 1918 virus-infected macaques than in those that received the seasonal virus. No virus was detected in blood samples or in extrapulmonary tissues.

A second report from the same research group compared host responses in cynomolgus macaques infected with the reconstructed 1918 virus or a highly pathogenic H5N1 avian virus (Cilloniz et al., 2009). In contrast to the experiment described above, the animals were not permitted to develop full-blown illness, but were humanely killed at 12, 24 or 48 hours postinfection. Global transcriptional profiling revealed that the two viruses elicited

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significantly different patterns of host gene expression. Although both the 1918 and H5N1 viruses triggered an apoptotic response in pulmonary tissues, animals infected with the 1918 virus showed strong up-regulation of the key inflammasome components, NLRP3 and IL-1β, while the same genes were down-regulated early after infection in macaques given the avian virus.

In the third study, cynomolgus macaques were inoculated by multiple routes either with a seasonal H1N1 virus, a reassortant virus containing 1918 HA and NA genes, or an H5N1 avian virus and were humanely killed on day 1, 2, 4 or 7 post-challenge (Baskin et al., 2009). The 1918 reassortant virus replicated to a much higher titer than the seasonal virus, which produced no signs of illness. However, the H5N1 virus was even more pathogenic, causing greater tissue damage and eliciting a stronger proinflammatory response than the 1918 reassortant virus. Three follow-up studies employing tissue samples collected in this experiment examined host gene expression in extrapulmonary tissues (Tolnay et al., 2010), the proteome response (Brown et al., 2010) and microRNA expression in lung tissues (Li et al., 2011). Each report identified distinct patterns of host responses in macaques infected with the seasonal H1N1, reassortant 1918 or H5N1 avian virus.

9. Infection of NHPs with the 2009 swine-origin pandemic H1N1 virus—Since a novel pandemic H1N1 virus emerged in Mexico in April, 2009, eleven published articles have characterized the virulence of the virus for NHPs or examined their host responses to infection (Table S14). Seven studies were in cynomolgus, three in rhesus macaques and one in common marmosets. In most cases, the animals were inoculated by a combination of the IT, IN and conjunctival routes, sometimes accompanied by an oral dose.

In the first report, Itoh and colleagues compared the disease produced by the CA04 pandemic isolate or a seasonal H1N1 strain in cynomolgus macaques (Itoh et al., 2009). The authors provide no information on visible signs of illness, but report that animals infected with the CA04 virus developed a higher fever and had greater levels of viral replication in the respiratory tract, stronger proinflammatory cytokine responses and more severe and extensive lesions in lung tissues collected at necropsy. A subsequent report compared the severity of pulmonary pathology in macaques infected with a 2009 pandemic H1N1 or a seasonal H1N1 virus (Herfst et al., 2010). Tissues collected at necropsy on day 4 showed that the pandemic virus replicated to a greater extent in the lower respiratory tract, causing diffuse alveolar damage. A later study found that, although the pandemic virus infects mice, pigs and macaques, it elicits different sets of transcriptional responses in the three species (Go et al., 2012).

In a further evaluation of the pathogenicity of the 2009 pandemic H1N1 virus for cynomolgus macaques, Safronetz *et al.* found that two isolates were both more virulent than a seasonal H1N1 strain, but they differed from each other in clinical features, levels of viral replication and intensity of host responses (Safronetz et al., 2011). While the seasonal virus caused no apparent illness, the two pandemic strains produced moderate respiratory disease and infiltrates on thoracic radiographs.

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The same research group also published a unique report focusing on the use of thoracic radiography in research on influenza in NHPs, in which they compared the disease caused by a seasonal H1N1 virus and three different 2009 H1N1 pandemic virus isolates in cynomolgus macaques (Brining et al., 2010). All of the latter caused fever, loss of appetite and increased respiratory rate, with interstitial infiltrates and areas of consolidation in their chest radiographs, while the seasonal virus produced only loss of appetite, without other abnormalities.

More recently, Moncla *et al*. employed a new animal model for influenza studies, by inoculating four common marmosets with a strain of the 2009 pandemic H1N1 virus, influenza A/California/07/2009 (Moncla et al., 2013). Each animal was housed with a naïve cagemate. The inoculated marmosets all demonstrated signs of illness similar to clinical influenza in humans, with nasal discharge and sneezing. Transmission of infection was confirmed for one co-housed pair.

Five studies have examined immune mechanisms in macaques infected with the 2009 swine-origin pandemic H1N1 virus. In the first, Weinfurter and colleagues demonstrated that rhesus macaques previously infected with a seasonal H1N1 virus were able to clear the 2009 pandemic virus from the respiratory tract more rapidly than control animals (Weinfurter et al., 2011). This “priming” effect was linked to the development of cross-reactive T-cell responses. A subsequent report examined the ability of antibodies elicited by infection with a seasonal H1N1 virus to protect against the 2009 pandemic virus, and showed that antibody-dependent cellular cytotoxicity played a role in cross-protection (Jegaskanda et al., 2013c). In an attempt to identify a reproducible biomarker to employ in studies of vaccine efficacy, Skinner and colleagues found that rhesus macaques challenged with a small-particle aerosol of the 2009 pandemic virus developed no physical signs of illness other than fever, but whole-blood microarray analysis showed a type I IFN response (Skinner et al., 2014). Animals immunized with an experimental vaccine exhibited markedly reduced IFN activity, suggesting its value as a biomarker.

In a further evaluation of the basis of immune control, Pham *et al*. compared viral infections in normal and immunodeficient cynomolgus macaques, whose cellular immune responses were impaired by treatment with cyclophosphamide and cyclosporine A (Pham et al., 2013). They found that both the pandemic virus and a seasonal H1N1 strain replicated to higher titer and persisted longer in the immunodeficient animals, but the groups did not differ in duration of fever. Even though treated animals had fewer circulating immune cells, they showed higher plasma levels of proinflammatory cytokines.

Because epidemiologic studies have shown that the 2009 pandemic H1N1 virus tended to cause less severe disease in older humans, Josset *et al.* compared its virulence for groups of rhesus macaques that were either 10–12 or 20–24 years of age (Josset et al., 2012). The older animals showed higher levels of viral replication and inflammatory cytokines, but neither group developed visible signs of illness.

In a recent report summarized above, Song et al. tested a monoclonal antibody in cynomolgus macaques challenged with the 2009 pandemic H1N1 virus; control animals

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developed fever, decreased blood oxygen saturation and extensive pulmonary damage (Song et al., 2014). In an interesting study, Clay and colleagues modeled the susceptibility of human infants to severe influenza by experimentally infecting infant and adult rhesus macaques with the 2009 virus (Clay et al., 2014). Primary airway epithelial cultures from infant macaques supported higher levels of viral replication than cells from adults and displayed weaker innate immune responses. Challenge of infant macaques by the IN and IT routes resulted in virus clearance by day 9, but lower airway inflammation persisted through day 14.

B. Specific aspects of the use of NHPs for research on seasonal and pandemic influenza

The preceding section has provided a chronological review of the past 120 years of experimental studies of seasonal and pandemic influenza in NHPs. Several aspects which cannot easily be examined in a chronological account are briefly discussed here: studies of influenza B virus infection; routes employed to expose NHPs to influenza viruses; research on viral/bacterial coinfection; and studies in which thoracic radiography was employed to assess the severity of pulmonary disease.

1. Influenza B viruses—Four papers have examined influenza B virus infection of NHPs (Table S15). In 1972, Paniker and Nair found that, although bonnet macaques could be infected with an influenza B virus, the animals did not become ill (Paniker and Nair, 1972). In 1989, Snyder et al. reported that a cold-adapted influenza B virus was replication-restricted in chimpanzees, similar to its behavior in ferrets and humans (Snyder et al., 1989).

In a recent study, Kitano and colleagues challenged cynomolgus macaques with two different influenza B viruses by a combination of the IT, IN and conjunctival routes (Kitano et al., 2010). They observed virus shedding from the respiratory tract, elevated plasma cytokine levels and fever, but no other signs of illness. Necropsies of infected animals on day 14 showed areas of alveolar wall thickening and interstitial pneumonitis. As described above, the researchers then used cynomolgus macaques infected with an influenza B virus to test the efficacy of the novel intravenous antiviral drug, peramivir (Kitano et al., 2011).

There are no reports of the experimental infection of NHPs with influenza C viruses.

2. Routes of exposure—In the early years of influenza research, before methods had been developed to propagate the virus in embryonated eggs, investigators attempting to reproduce human influenza in NHPs were obliged to make use of filtered or cultured respiratory secretions obtained from patients, which they administered to animals by SC, IV or intrathoracic injection or by the IN, IT or conjunctival route. Once egg-cultured virus became available, and researchers were no longer dependent on material from patients, they initially focused on IN inoculation of NHPs, reflecting contemporaneous success in producing disease in ferrets by the same route. However, Burnet reported in 1941 that cynomolgus macaques challenged IN with the mouse-passaged WS virus did not become ill, while those challenged IT developed lethargy and fever, and one died (Burnet, 1941). He concluded that “In order to produce symptoms, lung lesions or any considerable immunological response, it is necessary to administer the virus directly into the trachea…”,

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and that some of the inoculum must reach the smaller bronchi or bronchioles to cause signs of illness.

Despite Burnet's admonition, most studies performed after 1941 still made use of the IN route, and it was not largely abandoned until after Berendt *et al.* succeeded in producing an illness resembling human influenza in squirrel monkeys by IT inoculation of a seasonal virus (Berendt et al., 1975). Interestingly, capuchins and marmosets have been the only other NHP species to develop sneezing and rhinorrhea after IT challenge (Grizzard et al., 1978; Moncla et al., 2013).

Beginning in the 1960s, several investigators attempted to produce an influenza-like disease in various species of NHPs by exposing them to aerosolized virus. However, when cynomolgus and rhesus macaques were infected by the aerosol route, less than half of the animals became ill, developing fever, lassitude and anorexia (Saslaw and Carlisle, 1965). In two further studies in rhesus macaques, all animals remained well (Berendt, 1974; Berendt et al., 1974). As noted above, the only NHP species that has developed sneezing, cough and other visible signs of an influenza-like illness after aerosol exposure is the squirrel monkey, which is also susceptible to IT infection (Stephen et al., 1977b). A study that directly compared aerosol and IT challenge of squirrel monkeys with an H3N2 virus saw no difference in the resulting illness (Snyder et al., 1986c). The only report since that time of the infection of NHPs with aerosolized influenza virus is the study in rhesus macaques summarized above (Skinner et al., 2014).

As noted, investigators employing NHPs for influenza research from 1893–2000 usually inoculated the animals with infectious material by a single route, or occasionally by two routes simultaneously. Since 2000, however, investigators have frequently exposed animals to virus by two, three or even four routes, one of which has always been IT. This practice began with the first study of the newly emerged H5N1 avian influenza virus in macaques, in which the agent was inoculated IT and dripped onto the conjunctiva and tonsils (Rimmelzwaan et al., 2001). Similarly, when researchers first challenged macaques with the reconstructed 1918 H1N1 virus, they gave it by the IT, IN, conjunctival and oral routes (Kobasa et al., 2007). The rationale for employing multiple simultaneous infection pathways in the 2001 H5N1 study has never been explicitly stated. Perhaps the authors thought that this multi-route method more closely simulated natural exposures; more likely, it was to maximize viral exposure and increase the likelihood of producing illness.

3. Studies of viral/bacterial coinfection—It is now widely recognized that influenza virus infection predisposes human patients to the development of bacterial pneumonia, which in most fatal cases is the proximate cause of death (Morens et al., 2008). A number of studies, beginning in 1941, have attempted to replicate this scenario in NHPs (Table S16). However, of the many experiments performed over more than 60 years, employing inoculation of virus alone, bacteria alone or virus inoculated simultaneously with or followed by bacteria, the only one in which co-infection resulted in significantly enhanced disease was that of Berendt et al. summarized below (Berendt et al., 1975).

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In two reports from the 1940s in which rhesus macaques were infected first with influenza virus, then with *Streptococcus hemolyticus*, the animals developed only a transient fever, with no visible signs of illness (Merino et al., 1941;Wilson et al., 1947). Similarly, Verlinde and Makstenieks infected a large number of rhesus and cynomolgus macaques with influenza virus, followed by *Staphylococcus aureus* (Verlinde and Makstenieks, 1954). As in the previous study, the animals also developed only mild fever, but necropsies revealed extensive bronchopneumonia, indicating that clinical observation may not accurately measure the extent of respiratory tract disease in NHPs.

Twenty years later, Berendt and colleagues attempted to develop a system to model bacterial pneumonia in rhesus macaques infected with aerosols of influenza virus followed by *S. pneumoniae* (Berendt et al., 1974). No apparent illness was described in their report. Culture of pulmonary tissues collected at necropsy demonstrated the persistence of bacteria in co-infected animals, but no abnormalities were observed in radiographic studies or in histopathologic examination of tissue samples.

Efforts to reproduce the human syndrome of influenza leading to bacterial pneumonia were more successful when researchers began to use squirrel monkeys for their research. Berendt and colleagues showed that challenge with an H3N2 virus or with a small dose of *S. pneumoniae* produced a mild respiratory illness. When given alone, a large dose of bacteria was required to produce severe pneumonia, but when virus and bacteria were administered sequentially, a small dose of bacteria caused severe disease (Berendt et al., 1975).

In the only recent study of co-infection of NHPs with a seasonal influenza virus and bacteria, Kobayashi and colleagues were unable to demonstrate any increased severity of illness when they infected cynomolgus macaques with an H3N2 virus followed by methicillin-resistant *S. aureus* (Kobayashi et al., 2013). This finding for seasonal influenza is mirrored in a recent experiment using a highly pathogenic H7N7 avian virus, in which infection of cynomolgus macaques with the virus alone or with virus plus *S. pneumoniae* did not produce significant signs of illness (see section on avian viruses below) (Miyake et al., 2010).

It thus appears that squirrel monkeys are the only laboratory primates that develop a disease with the typical clinical features of human influenza complicated by bacterial pneumonia. However, the earlier study of Verlinde and Makstenieks showed that significant pathologic changes may be present in the lungs of animals that show few or no visible signs of illness (Verlinde and Makstenieks, 1954).

4. Use of thoracic radiography to detect pulmonary disease—Thoracic imaging can make an important contribution to research on influenza virus infection of NHPs, as a noninvasive means of detecting the development of pulmonary lesions and tracking the course of illness. By providing an objective measurement of the extent of lung disease in animals that do not display cough, respiratory distress or other visible signs of illness, sequential chest radiography may make it possible to reduce the number of animals sacrificed for pathology studies. As noted below, imaging of NHPs during the course of illness has recently been improved through the introduction of digital radiography;

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significant further improvement could be gained through the use of computer tomography, as has recently been shown in infected ferret (Veldhuis et al., 2011; Jonsson et al., 2012).

Eight reports cited above describe the use of thoracic radiography to detect evidence of pulmonary involvement in NHPs infected with seasonal or pandemic influenza viruses (Table S17). In 1939, Vieuchange reported that a rhesus macaque dosed IN with the WS virus developed a shadow in the right hilum two days after exposure, followed by the development of radiographic opacification and dyspnea on effort (Vieuchange, 1939). Two years later, Burnet used chest radiography to track the course of illness in cynomolgus macaques challenged IN or IT with the WS virus, and found that pulmonary lesions could be visualized before the animals became seriously ill (Burnet, 1941).

In 1974, Berendt *et al.* reported that rhesus macaques, which remained well after sequential exposure to aerosolized influenza virus and D. pneumoniae, had normal chest radiographs (Berendt et al., 1974). Similarly, thoracic radiographs of capuchin, owl and squirrel monkeys were unremarkable after infection (Murphy et al., 1980). However, a subsequent study in squirrel monkeys found radiographic evidence of pneumonia in some animals, correlating with the severity of the observed illness (Murphy et al., 1982a).

In the only report to focus entirely on imaging, Brining et al. used digital chest radiography to compare the illness produced in cynomolgus macaques by three different strains of the 2009 pandemic H1N1 virus or an earlier H1N1 seasonal virus (Brining et al., 2010). Imaging revealed infiltrates in the lungs of animals challenged with each of the pandemic virus isolates. Changes reached a maximum by day 6 postinfection and cleared by day 14. Chest radiography was also used in a recent study, in which animals co-infected with an H3N2 virus plus bacteria showed minimal radiographic changes (Kobayashi et al., 2013).

In the most recent report to employ chest radiography to evaluate the extent of pulmonary disease, cynomolgus macaques were exposed to the avian H7N9 virus (De Wit et al., 2014). Interstitial infiltrates were detected in the lungs beginning on day 2 postexposure, and became diffuse by day 6 in most animals.

C. Studies in NHPs of extrapulmonary complications of seasonal influenza

The vast majority of influenza research employing NHPs has focused on disease of the respiratory tract, but a few investigators have used the experimental infection of macaques to study two potential complications of human influenza. The first is the occasional observation of neurologic abnormalities in influenza patients, while the second examines the question of whether influenza virus infection of a pregnant woman can damage the developing fetus.

1. Neurologic complications—Three reports have described experimental influenza encephalitis in NHPs (Table S18). The first found that two rhesus macaques inoculated intracerebrally with filtered nasal secretions of influenza patients developed signs of both influenza and encephalitis (Gordon, 1933). Nearly 40 years later, a study found that squirrel monkeys rendered immunodeficient by administration of cyclophosphamide developed encephalitis following intracerebral inoculation of two neurotropic mouse-adapted H1N1

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influenza strains (Miyoshi et al., 1971). Animals that received only the drug or the virus remained normal, while those that received both developed a neurologic illness on days 3–9, characterized by lassitude, fine tremors, hunched posture and deadened responses to stimuli. Histologic studies showed neutrophils infiltrating the choroid plexus, leptomeninges and subependymal structures and the presence of viral antigen.

A later study examined the effect of intracerebral or intraspinal inoculation of rhesus macaques and patas and AGMs with H1N1 and H3N2 viruses and attenuated murine neuroadapted strains (Lussier et al., 1974). Similar to the small study cited above (Gordon, 1933), “80% of the rhesus macaques inoculated with either strain of virus developed clinical evidence of CNS involvement and 63% died.” All viruses caused a predominantly mononuclear ependymitis and choroiditis, often resulting in hydrocephalus. Interestingly, even though virus was inoculated intrathalamically, no inflammation or other evidence of infection was seen in the brain parenchyma; the authors do not explain this result. None of these studies demonstrated central nervous system infection with human influenza induced through a natural route of infection.

2. Teratogenic effects of seasonal influenza in pregnant women—Most experimental studies in NHPs of the potential teratogenic effects of influenza virus infection were published in the 1970s (Table S19). In the first report, researchers inoculated the anterior fontanelles of the fetuses of two groups of pregnant rhesus macaques with influenza A/Aichi/2/68 (H3N2) virus suspended in allantoic fluid (London et al., 1975). One group of females was at 105–111 days gestation and the other at 105–118 days. Hydrocephalus developed in 6 of 12 virus-inoculated fetuses, but was absent in the two controls inoculated only with allantoic fluid. Histologic studies showed evidence of a destructive ependymitis and/or choroiditis resembling lesions seen in the report cited above (Lussier et al., 1974).

In a similar study in 1978, the investigators inoculated the fetuses of female rhesus macaques intracerebrally with an attenuated H2N2 vaccine virus in allantoic fluid, or with allantoic fluid alone, and observed hydrocephalus only in virus-inoculated animals (Krous et al., 1978). In contrast to the earlier report, the aqueduct of Sylvius was also involved, possibly as a result of a difference in the route of inoculation. In a third study, Moreland *et al*. instilled influenza virus intra-amniotically at 90 days gestation in pregnant rhesus macaques. They recovered virus from 100% of the exposed animals, confirming fetal infection. They then went on to do fetal IC inoculations at the same gestation time and saw “no malformations or measurable fetal effects” during gross and histologic examination of the three fetuses delivered by caesarean section at 158 days gestation. Simultaneous experiments with mumps and western equine encephalitis viruses, yielded 100% fetal mortality and incidence of encephalitis, respectively (Moreland et al., 1979).

In the only recent study of the effect of maternal influenza virus infection on fetal development, Short and colleagues infected 12 pregnant rhesus macaques one month before term, and compared them with 7 control pregnancies at birth and 1-year post partum (Short et al., 2010). Differences were subtle in early infancy, manifesting as decreases in orientation and mother-infant interaction leading to more rapid autonomy in infected animals’ offspring. The degree of these differences also correlated with the virulence of the

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viral strain. At 1 year, neuroimaging of the affected offspring revealed a reduction in gray matter in the cortex and white matter in the parietal lobes and an increase in cingulate white matter. The authors concluded that these changes were probably not due to direct viral action, but to the host inflammatory response.

IV. Studies of avian influenza in NHPs

Because the human population lacks immunity to most influenza A subtypes, avian viruses other than H1 or H3 pose a potential threat of pandemic disease. The agent of greatest concern has been the highly pathogenic H5N1 virus, which emerged in poultry in Hong Kong in 1997, spreading to 18 people and killing 6 of them, then re-emerged in Southeast Asia in 2001 (Li et al., 2004; Gambotto et al., 2008). According to the World Health Organization website, the subsequent spread of the H5N1 virus to a number of countries and its establishment in local poultry had by January 6th, 2015, resulted in 694 confirmed human infections, of which 402 were fatal. Sustained person-to-person spread has not occurred.

The past two decades have also seen the emergence of highly pathogenic H7N7 viruses that have caused outbreaks in poultry in several countries. Infection has occasionally spread to humans in contact with the sick birds, but it has generally remained limited to conjunctivitis, and only one individual has developed severe respiratory tract disease (Fouchier et al., 2004). A more recent threat to human health is the H7N9 avian influenza virus that emerged in China in early 2013, which spreads among various poultry species without producing recognizable illness, but has caused fatal disease in humans (Gao et al., 2013). Most patients have a history of close contact with poultry.

Finally, several instances of human infection with an H9N2 avian influenza A virus following poultry contact have been reported (Butt et al., 2005;Uyeki et al., 2002; Peiris, 2009). As with H5N1, the H7 and H9 viruses have not acquired the capacity for sustained human-tohuman spread. Because they are too virulent to be administered to human volunteers, pathogenesis studies and vaccine evaluations have relied on experimental infections of a variety of laboratory animals, including mice, ferrets, guinea pigs, cats, pigs and NHPs.

A. H5N1 avian influenza virus infections of NHPs

Recognized cases of H5N1 influenza in humans typically begin 2–4 days after exposure to infected poultry, with fever, cough and shortness of breath that quickly progress to fulminant, bilateral pneumonia with respiratory failure (Gambotto et al., 2008). Postmortem studies have shown extensive viral pneumonia with diffuse alveolar damage. In some fatal cases, necropsies have detected high levels of virus in the blood and non-pulmonary tissues; gastrointestinal disease, including vomiting and diarrhea, and involvement of the central nervous system are less common. Mild or asymptomatic infections also occur (Powell et al., 2012; Le et al., 2013; Gomaa et al., 2014; Morens and Taubenberger, 2014) .

As described below, a number of investigators have observed severe pulmonary disease in cynomolgus macaques infected with H5N1 viruses (Table S20). Animals typically become febrile, and visible signs of illness have included anorexia, cough, diarrhea and abnormal

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behavior. Only in two studies in African green monkeys have no signs of illness been noted (see below).

1. Pathogenesis studies—The first study of H5N1 virus infections in macaques inaugurated a new practice in influenza research. In all work performed before 2001, animals were exposed to infectious material by the IN, IT or aerosol route, or occasionally by two of these routes simultaneously. Beginning with the first H5N1 experiments, however, investigators have challenged NHPs simultaneously by three or even four routes, one of which has always been IT (Table S20). In the first report, the virus suspension was inoculated IT and dripped onto the conjunctiva and tonsils of cynomolgus macaques (Rimmelzwaan et al., 2001). Since that time, one study of H5N1 infection used only IN challenge, one used only IT challenge, two used both IT and IN challenge, and the remainder have administered virus simultaneously by the IT, IN, conjunctival and the oral or tonsillar routes.

Experimental exposure of cynomolgus macaques to the 1997 H5N1 avian virus was first described by Rimmelzwaan et al. and further examined by the same authors in two subsequent articles (Rimmelzwaan et al., 2001; Kuiken et al., 2003; Rimmelzwaan et al., 2003). Four animals were infected by the IT, tonsillar and conjunctival routes, and two were euthanized on day 4 and two on day 7. Three became febrile within 2–3 days, and one that died on day 7 developed signs of acute pulmonary disease, with respiratory distress and rapid breathing; necrotizing interstitial pneumonia was seen at necropsy. The other three animals shed virus from the trachea and had virus in the lungs on day 4 or 7, but showed no other signs of illness. This model was recently employed to evaluate the efficacy of prophylactic administration of low-dose oral interferon-alpha in preventing pulmonary injury after H5N1 virus challenge (see section on antiviral therapy above) (Strayer et al., 2014).

In a 2009 report, Baskin and colleagues compared the disease produced in cynomolgus macaques by an H5N1 avian virus to that caused by two reassortant seasonal viruses encoding the 1918 HA (Baskin et al., 2009). Eight animals were infected with each virus, and 2 were euthanized on each of days 1, 2, 4 and 7. Compared to the 1918 HA viruses, H5N1 caused more severe illness, including anorexia, depression, fever, cough and diarrhea; replicated to higher titers in the upper and lower respiratory tract; caused more extensive pathologic changes in pulmonary tissues; and induced stronger proinflammatory responses than the 1918 reassortant viruses. One H5N1-infected animal died on day 6 postinoculation, and showed extensive pulmonary damage at necropsy. Tissue samples from these animals were used in three subsequent studies. The first characterized the proteome response to infection (Brown et al., 2010); the second identified unique patterns of host microRNA expression (Li et al., 2011); and the third found that only the H5N1 virus spread extensively to extrapulmonary tissues (Tolnay et al., 2010).

In a similarly designed experiment, Cilloniz *et al.* compared gene expression in pulmonary tissues of cynomolgus macaques infected with either an H5N1 or the 1918 virus, and found that genes encoding inflammasome components were down-regulated in the former and up-regulated in the latter animals (Cilloniz et al., 2009). In another investigation in the same

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year, Chen and colleagues infected four Chinese rhesus macaques IN with an H5N1 avian virus and observed fever, anorexia and behavioral changes, beginning on day 4 (Chen et al., 2009). Histologic studies at necropsy showed that infection was localized to pneumocytes and macrophages of the lower respiratory tract.

In an interesting recent study, researchers found that deletion of the multibasic cleavage site (MBS) from an H5N1 virus reduced its virulence for mice and ferrets, but neither the parent nor the modified virus caused visible signs of illness in AGMs (Suguitan et al., 2012). The only effect of MBS deletion was a prolongation in virus shedding. Another investigation employing cynomolgus macaques describes a striking mobilization of myeloid and plasmacytoid dendritic cells into the bloodstream of animals infected with viruses from two H5N1 clades; no illness is described (Soloff et al., 2014). According to the authors, such a cellular response had not been seen in other respiratory viral infections. In a 2013 report from Japan, researchers examined the virulence for rhesus and cynomolgus macaques of a H5N1 virus recovered from a local whooper swan, delivering the agent either by combined IN, IT and oral delivery or by droplet spray into the trachea (Fujiyuki et al., 2013). A low-dose challenge resulted in decreased activity, with tachypnea in one animal, but all showed interstitial pneumonia at necropsy on day 7. In contrast, delivery of higher doses resulted in fever, tachypnea, cough and depression.

As described below, a study of cold-adapted vaccines against a number of avian influenza viruses found that challenge of AGMs by the IN + IT routes with an H5N1, H6N1, H7N3 or H9N2 virus resulted in significant levels of replication in the upper and lower respiratory tract, but did not cause fever or visible signs of illness (Matsuoka et al., 2014). In contrast, researchers who assessed the efficacy of IV peramivir for the treatment of H5N1 virus infection in cynomolgus macaques observed fever, diminished appetite and weight loss in control animals; the peak titer and duration of viral shedding from the upper and lower respiratory tract were greater than previously observed with seasonal influenza virus challenge (Kitano et al., 2014). In another 2014 report, cynomolgus macaques challenged with an H5N1 virus were partially protected from illness by a neutralizing mab administered the day before or after infection, but the same treatment was less effective in immunodeficient animals (see paper by Itoh et al. below).

In the most recent article describing H5N1 infection in NHPs, Muramoto et al. exposed groups of 3 cynomolgus macaques to 6 different viruses isolated in Viet Nam in 2004–5, challenging animals simultaneously by the IN, IT, oral and conjunctival routes (Muramoto et al., 2014). All animals developed some degree of illness, ranging from nasal discharge alone through cough, depression, huddling and decreased activity; one died on day 9. There was no systemic spread of virus and no evidence of bacterial superinfection. Gene expression studies suggested that early, strong IFN-induced activation of innate immune responses prevents the development of severe illness.

2. Evaluation of candidate H5N1 vaccines—The occurrence of fever and other clinical signs, measurement of virus shedding and histopathologic changes in the lungs at necropsy following challenge with H5N1 avian viruses have provided a sufficient number of markers to assess the protective effect of candidate vaccines in NHPs (Table S21). Because

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of the difficulty of growing highly pathogenic avian viruses in chicken eggs and their virulence for humans, most vaccines tested in NHPs have not been derived from live H5N1 virus, but have been based on the recombinant expression of the viral HA, either in a DNA vaccine or in a variety of live viral vectors, including baculovirus, vaccinia, vesicular stomatitis and Newcastle disease viruses. In a different approach, some researchers have evaluated the ability of an inactivated low-path H5N1 virus to induce protective immunity against virulent H5N1 strains (Itoh et al., 2008). A similar method of achieving protection against a highly pathogenic H7N7 influenza virus is described below.

Fifteen reports of the experimental evaluation of H5N1 vaccines in NHPs have been published to date. In contrast to pathogenesis research, which has almost exclusively employed cynomolgus macaques, eight vaccine studies have used cynomolgus, five have used rhesus macaques and two have used AGMs. Four reports have examined safety and immune responses to vaccination, including the ability of elicited antibodies to neutralize a range of H5N1 subclade viruses, without performing virus challenge. Antibody responses predicted to be sufficient for protection were measured in animals immunized with a recombinant Newcastle disease virus (DiNapoli et al., 2007), with a live, NS1-deleted virus (Romanova et al., 2009), with a whole, inactivated, adjuvanted vaccine (Heldens et al., 2010) and with a recombinant vesicular stomatitis virus vaccine (Schwartz et al., 2011).

In the other 11 studies, the animals were vaccinated, then challenged after an appropriate interval with an H5N1 virus. The approaches have included inactivated split virus plus adjuvant (Ruat et al., 2008); an inactivated low-path H5N1 avian virus (Itoh et al., 2008); a recombinant baculovirus expressing the H5 HA (Jin et al., 2008); modified vaccinia Ankara virus expressing the HA (Kreijtz et al., 2009a;Kreijtz et al., 2009b); a DNA vaccine administered by electroporation (Laddy et al., 2009); a live, attenuated virus (Fan et al., 2009); a recombinant Newcastle disease virus (DiNapoli et al., 2010); inactivated virus with polyIC12U as adjuvant (Ichinohe et al., 2010); and an optimized HA administered as virus-like particles, together with alum (Giles et al., 2012). Vaccine efficacy was assessed by comparing immunized and control animals following challenge for signs of visible illness, fever, virus shedding and histologic changes of bronchopneumonia at necropsy. Partial or complete protection was reported in all cases.

In the most recent report of countermeasures against avian influenza, Matsuoka et al. describe the testing of live, attenuated cold-adapted H5N1, H6N1, H7N3 and H9N2 viruses as vaccines against the corresponding wild-type agents (Matsuoka et al., 2014). The authors compared the outcome of IN + IT infection of AGMs with the wild-type and cold-adapted viruses, and found that the former replicated throughout the respiratory tract, while the latter were restricted to the upper respiratory tract. When vaccinated animals were challenged with the corresponding virus, there was a significant decrease in viral shedding; the absence of fever and other signs of illness in the control animals prevented further assessment of protective efficacy.

3. Evaluation of therapeutic agents against H5N1 avian influenza viruses in NHPs—Four studies have evaluated antiviral drugs, antibodies or type I interferon in macaques challenged with H5N1 avian influenza viruses. In the first, Stittelaar et al.

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inoculated cynomolgus macaques IT with an H5N1 virus and treated the animals with i.v. zanamivir, beginning either before or 4 hours after infection (Stittelaar et al., 2008). There was no reduction in fever or virus shedding, but treated macaques showed fewer pulmonary histologic abnormalities at necropsy. More recently, Kitano et al. found that cynomolgus macaques challenged with an H5N1 virus by multiple routes developed diminished appetite, weight loss and fever, while those given five daily doses of IV peramivir showed fewer signs of illness and had a reduced peak and duration of viral shedding (Kitano et al., 2014).

The third report described the protective efficacy of the chimeric human-mouse monoclonal antibody (mab) m61, which neutralized a range of H5N1 isolates, when administered postexposure to cynomolgus macaques (Itoh et al., 2014). Treatment with IV m61 on days 1 and 3 postinfection prevented fever and resulted in reduced peak viral titers and shedding, compared to controls given an unrelated mab. One control animal died on day 4, but all treated macaques survived through day 7, when they were sacrificed. When the same approach was used with macaques rendered immunodeficient with cyclophosphamide and cyclosporin, all three animals that received a control mab died, while three of five given m61 survived. M61 plus peramivir also protected immunodeficient animals, while peramivir treatment alone did not prevent death.

In the fourth study, H5N1-infected cynomolgus macaques were used to evaluate the prophylactic oral administration of a range of doses of IFN-alpha (Alferon-N®) (Strayer et al., 2014). The researchers found that, although treatment had no effect on virus shedding, animals that received the highest dose showed a marked reduction in gross and microscopic pulmonary pathology when necropsied at day 5 postinfection.

B. H7N7 avian influenza virus

In the only fatal human case of H7N7 avian virus infection reported to date, a Dutch veterinarian exposed to sick chickens in a poultry outbreak in 2003 developed fever and headache, followed by the development of respiratory distress (Fouchier et al., 2003). Radiographic evaluation initially showed lobar pneumonia, but he rapidly developed bilateral pulmonary infiltrates and died from respiratory failure. The autopsy showed severe diffuse alveolar damage.

Two reports have described experimental infection of cynomolgus macaques with the H7N7 virus recovered from this case. In both studies, the authors were evaluating the suitability of formalin-inactivated low-path avian H7N7 reassortant viruses as candidate vaccines (Itoh et al., 2010; Miyake et al., 2010). Control animals shed virus and developed a mild illness, characterized by fever, anorexia, and weight loss. Although no signs of respiratory tract disease were observed, postmortem pathologic analysis revealed alveolar inflammatory infiltrates. Vaccinated animals had less fever and virus shedding. When some macaques were infected simultaneously with the H7N7 virus and *Streptococcus pneumoniae*, no significant worsening of illness was observed, but vaccinated animals showed less bacterial replication in the lungs. Combined IN and conjunctival inoculation were employed in all these experiments; based on earlier findings, the use of IN instead of IT challenge may have lessened the severity of disease (Saslaw et al., 1946).

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C. H7N9 avian influenza virus

Cases of severe illness in humans infected with an avian H7N9 influenza virus that has no apparent pathogenicity for poultry were first reported in China in early 2013, and have continued into 2014. Patients have presented with fever, cough and dyspnea and showed rapid progression to diffuse alveolar damage and fatal ARDS, in a pattern reminiscent of infection with high-path H5N1 viruses (Gao et al., 2013).

In the first paper to describe experimental infection of NHPs with an H7N9 virus, Watanabe and colleagues found that challenge of cynomolgus macaques by multiple routes resulted in fever, but no visible signs of illness (Watanabe et al., 2013). Two different virus strains replicated efficiently in the upper and lower respiratory tract. Viral antigen was detected in tracheal and bronchial epithelial cells, and inflammatory changes extended to alveolar spaces. Another research group found that six of eight cynomolgus macaques challenged with an H7N9 virus developed an increased respiratory rate, labored breathing and cough (de Wit et al., 2014). Chest radiography revealed pulmonary infiltrates in all animals by day 2, becoming diffuse in most of them by day 6. Based on these observations, the researchers concluded that the H7N9 virus is more virulent for macaques than seasonal influenza viruses or most strains of the 2009 H1N1 pandemic virus. However, it appears to be less pathogenic than the 1918 or H5N1 avian virus. In the most recent report, Pan and colleagues found that serum from macaques vaccinated with an inactivated recombinant H7N9/PR8 virus protected mice against lethal H7N9 virus challenge (Pan et al., 2014).

D. H9N2 avian influenza virus

Since 1999, sporadic influenza-like illness in humans in contact with poultry have been attributed to H9N2 avian influenza viruses. Cases in children have consisted of mild, self-limited upper respiratory tract infections; human-to-human infection remains unconfirmed (Uyeki et al., 2002; Butt et al., 2005).

In a recent report, Zhang *et al*. found that combined IN and IT inoculation of rhesus macaques with an H9N2 virus resulted in mild clinical disease, characterized by small temperature spikes at days 1–2, decreased appetite and reduced mental state at days 5–6, and mild cough and dehydration (Zhang et al., 2013). Pathologic examination of the lungs showed gross and microscopic lesions consistent with influenza, and immunohistochemistry revealed viral antigen in the upper and lower respiratory tracts. Virus was isolated from the respiratory tracts of all four infected animals. In contrast, a study of live, attenuated vaccines in AGMs observed no fever or other signs of illness in control animals infected with an H9N2 virus (Matsuoka et al., 2014).

V. Differences between the respiratory tracts of humans and NHPs

Comparative studies of influenza virus infections of humans and NHPs would clearly benefit from knowledge of similarities and differences in the gross and microscopic anatomy of the respiratory tract in the various species. While hundreds of autopsy records from the San Diego Zoo have shown that the microanatomy of the respiratory tract of apes, monkeys and prosimians is similar at all ages to that of humans, there are broad inter-species differences between primates and man at the gross anatomical level, which tend to increase

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when the comparison includes the great apes (Scott, 1992). Chimpanzees and gorillas have the same lung lobe configurations as humans, but curiously, orangutans lack lung lobes.

Humans also have a mutation in the cytidine monophosphate-N-acetylneuraminic acid hydroxylase gene that results in the inability to produce cell surface N-glycol-neuraminic-acid (Neu5Gc) in the respiratory tract, where it would likely serve as a receptor for influenza A and B viruses, as it does in other mammals. Genomic analyses indicate that this mutation occurred sometime after the divergence of humans from great apes (Gagneux and Varki, 2001). Interestingly, through parallel evolution, New World owl monkeys (*Aotus nancymaae*) also primarily express Neu5Ac and have no detectable Neu5Gc as seen in humans (Martin et al., 2005). Additional studies reported that the sialic acid distribution in the upper airways of chimpanzees and other NHPs correlates better with sialic acid distribution in birds, i.e., lacking the abundance of α2,6-linked sialic acids seen in human upper airways (Varki et al., 2011).

Recent research on the distribution of influenza receptors in uninfected respiratory tissues, using formalin-fixed, paraffin-embedded sections, corroborates these differences between humans and macaques, with the exception of receptors for avian viruses (van Riel et al., 2013a; van Riel et al., 2007). In their 2007 study, van Riel et al. observed moderate binding of H5 and H6 avian viruses, but not human seasonal H1N1 and H3N2 viruses, to pulmonary alveolar lining cells (van Riel et al., 2007). In two further studies of the recently emerged H7N9 avian virus, the same group observed that the pattern and degree of attachment of the Shanghai and Anhui strains in the upper and lower respiratory tract of cynomolgus macaques resembled that in humans (van Riel et al., 2013b; Siegers et al., 2014). However, the recent study noted above, which focused on the development of cold-adapted vaccines against a number of avian influenza viruses, found that the distribution of sialic acid receptors in the respiratory tract of AGMs resembled that in humans (Matsuoka et al., 2014).

VI. Conclusions and directions for future research

We have reviewed more than 120 years of published reports of influenza virus infections in nonhuman primates, resulting either from the natural exposure of wild or captive animals to humans with influenza or from the experimental exposure of laboratory primates to human or avian viruses. Our goals have been to make investigators aware of this lengthy history of research, little of which is cited in current publications; to help them avoid “re-inventing the wheel”, by describing work that has already been performed; and to provide information helpful to assess the advantages and limitations of NHP studies.

The first section of this article summarizes more than 30 reports examining the natural transmission of influenza viruses from humans to wild and captive primates. Taken together, those studies provide evidence that a wide variety of primate species can become infected with human viruses, generally without developing visible manifestations of respiratory tract disease. Interestingly, despite commonly expressed concerns that zoo primates are at risk of severe illness if exposed to humans with influenza, the absence of published reports of such events suggests that they are in fact rare. Further investigation of the natural transmission of infection from humans to NHPs would benefit from focused surveillance in zoos during

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influenza seasons, repeated serologic testing of long-term resident animals and the publication of findings in the scientific literature.

The majority of this article has been devoted to a chronological review of experimental infections of captive primates with human seasonal influenza viruses. In general, this work supports the conclusion of observational studies, that seasonal viruses are able to replicate in the upper airway and pulmonary tissues of many species of nonhuman primates, but seldom produce visible signs of illness referable to the respiratory tract. Only in the experiments in squirrel monkeys performed by Berendt and colleagues in the 1970s and Murphy et al. in the 1980s did investigators observe a syndrome of cough, sneezing, tachypnea and other signs resembling human influenza. However, although other NHP species may respond to the inoculation of virus with no more than fever and diminished activity, numerous reports have shown that visual observation may underestimate the extent of pulmonary involvement. Beginning with the work of Gibson et al. in 1919, continuing with Burnet's report from 1941 and the extensive study by Verlinde and Makstenieks in 1954, investigators have found that animals that show few visible signs of illness may in fact display extensive mucosal infection and inflammatory changes in the upper and lower respiratory tract at necropsy. The presence of underlying illness in apparently normal animals may be detected by imaging; for example, Vieuchange found pulmonary infiltrates by radiography in a rhesus macaque several days before the animal became visibly ill (Vieuchange, 1939).

The mild or inapparent disease produced by seasonal influenza viruses in most species of NHPs appears to reflect the benign, transient illness that they cause in otherwise healthy humans. However, it is well known that the same seasonal viruses pose a much greater threat to infants, the elderly and persons with chronic underlying disease, in whom bacterial superinfection of damaged respiratory mucosa may lead to fatal pneumonia. Perhaps because laboratory research typically makes use of healthy young adult animals, experimental studies in captive primates have generally failed to reproduce the illness seen in humans vulnerable to severe seasonal influenza. Of the seven reports of experimental co-infection with influenza virus followed by bacteria published from 1941–2013, only one that utilized squirrel monkeys resulted in significantly enhanced disease (Berendt et al., 1975).

Because the 1918 pandemic H1N1 virus, the H5N1 avian virus and other recently emerged avian influenza virus cannot be administered to human volunteers, experimental studies in NHPs may make important contributions to our understanding of their mechanisms of virulence and the development of effective vaccines and therapies. The few studies that have been performed using the 1918 virus or reassortant viruses encoding the 1918 HA have produced a severe illness in most infected macaques, with rapidly progressive respiratory tract disease. In contrast, the outcomes of challenge in experiments employing H5N1 viruses and the recently emerged H7N9 virus have been more variable, supporting that avian viruses may in fact cause a wide range of severity of illness in humans, including mild or asymptomatic infections that may not be included in calculations of case fatality rates. Variations in outcome may also reflect differences in virulence of various challenge virus strains, and the fact that laboratory primates are outbred animals, with differing individual responses to infection.

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Despite more than a century of research, a number of questions remain to be investigated that will help to more clearly define the utility of laboratory primates as models of human influenza. In this regard, it would be especially useful for researchers to perform side-by-side comparisons of the course of infection of humans and different animal species, to determine the accuracy of animal models. Investigators who are able to work with both ferrets and NHPs might compare their response to infection with the same viruses by the same route of exposure. Even more informative would be three-way comparisons of the illness produced in ferrets, NHPs and human volunteers by viruses approved for experimental human challenge. The parallel infections of humans and NHPs performed by Murphy's group in the 1980s provide an example of how such studies could be conducted. The vast increase in laboratory resources that has occurred since that time, including rapid detection and quantitation of shed virus, measurement of cytokine responses and other immunologic, genomic and proteomic analyses, would greatly enhance the value of this comparative approach.

Although our survey has described the use of some 10 different species of captive primates in laboratory research on influenza, the great majority of studies during the past decade have made use of cynomolgus and rhesus macaques. It is not clear that these two Old World species are necessarily optimal for the experimental replication of the human disease, or that they are of equal value for research employing seasonal, pandemic and avian viruses. It is possible that different primate species will be better suited to modeling the syndromes produced by different influenza viruses.

Further research is also needed to determine the optimal route of infection to simulate human influenza in NHPs. At present, investigators typically inoculate animals by multiple routes simultaneously, but this "shotgun" approach does not necessarily mimic natural exposures, but may be useful for experimental pathogenesis and therapeutics studies. Similarly, although the direct introduction of virus into the lower respiratory tract is likely to maximize the severity of disease, it may be less than optimal for studies modeling typical seasonal infections. Another question that has not been a focus of research in captive primates is the animal-to-animal transmission of influenza, a major concern in the investigation of newly emerging avian viruses. As described above, comparative studies in humans, NHPs and ferrets employing the same viruses would be especially valuable to elucidate mechanisms and determinants of virus spread. Such experiments could be made even more informative by the inclusion of sequential thoracic imaging, including the use of computed tomography and positron-emission tomography, as has been performed in infected ferrets.

Although laboratory primates have been used to a limited extent to test antiviral drugs for influenza, and more frequently to assess the efficacy of vaccines, such studies have not been a recognized part of the regulatory process leading to licensure. The utility of NHP experiments in product development could be more clearly defined by performing parallel studies in humans, captive primates, ferrets and rodents, to determine if studies in small animals are sufficient to predict safety and efficacy, or if experiments in large animals that more closely resemble humans would actually produce more accurate information. For this and other questions, we hope that our survey of the history of the use of laboratory primates

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for research on influenza will help investigators make appropriate choices of experimental animals for their work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We provide a chronological summary of 240 articles on natural or experimental influenza in nonhuman primates.
- NHPs naturally exposed to human influenza may become infected, but rarely show signs of illness.
- In 1893, Pfeiffer first described the inoculation of NHPs with material from human influenza patients.
- Seasonal influenza viruses generally cause few or no visible signs of illness in most species of NHPs.
- NHPs appear to be most useful for research on the 1918 pandemic and recently emerged avian influenza viruses.

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Table 1

Scientific and common names of nonhuman primates discussed in this article, based on (Nowak and Walker, 1999).

Family	Genus	Species	Common Name
Lemuridae	*Eulemur*	*fulvus rufus*	Red-fronted brown lemur
	Eulemur	*fulvus*	Common brown lemur
	Lemur	*catta*	Ring-tailed lemur
Indriidae	*Propithecus*	*diadema*	Diademed sifaka
	Propithecus	*verreauxi deckeni*	Von der Decken's Sifaka
Cebidae	*Cebus*	*apella*	Tufted capuchin
	Cebus	*albifrons*	White-fronted capuchin
	Saimiri	*sciureus*	Squirrel monkey
	Cebus	*capucinus*	White-headed capuchin
Aotidae	*Aotus*	*trivirgatus*	Northern owl monkey
Callitrichidae	*Sanguinus*	*nigricollis*	White lipped tamarin
	Sanguinus	*mystax*	Moustached tamarin
	Saguinus	*oedipus*	Cotton-top tamarin
	Callithrix	*jacchus*	Common marmoset
Cercopithecidae	*Papio*	*cynocephalus*	Yellow baboon
	Macaca	*mulatta*	Rhesus macaque
	Macaca	*sinica*	Toque macaque
	Macaca	*radiata*	Bonnet macaque
	Macaca	*nemestrina*	Pig-tailed macaque
	Macaca	*tonkeana*	Tonkean macaque
	Macaca	*sylvanus*	Barbary macaque
	Macaca	*fuscata*	Japanese macaque
	Macaca	*fascicularis philippensis*	Philippine macaque
	Cercopithecus	*sp.*	Guenon
	Macaca	*fascicularis*	Cynomolgus macaque
	Presbytis(Semno pithecus)	*entellus*	Hanuman Langur
	Cercopithecus	*aethiops*	African green monkey
	Cercopithecus	*ascanius*	Red-tailed monkey
	Papio	*cynocephalus*	Yellow baboon
	Papio	*sp.*	Baboon
	Erythrocebus	*patas*	Patas monkey
Hylobatidae	*Hylobates*	*agilis*	Black-handed gibbon
	Hylobates	*lar lar*	White-handed gibbon
Hominidae	*Pongo*	*pygmaeus*	Bornean orangutan
	Pan	*troglodytes*	Chimpanzee
	Gorilla	*gorilla*	Western gorilla

Family	Genus	Species	Common Name
	Gorilla	*gorilla gorilla*	Western lowland gorilla
	Gorilla	*beringei beringei*	Mountain gorilla
	Homo	*sapiens*	Human

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