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16. The mother of this fetus had the highest antenatal risk score (55). The risk factors included a positive VDRL (serum and cerebrospinal fluid), moderate consumption of alcohol, smoking, and emotional problems. The mother was treated for neurosyphilis 2 months before delivery.
17. A high-pass filter was selected [(*12*), p. 300] so that the frequency response function was < 0.5 at frequencies below the upper half-maximum point on the dominant peak. At the frequency of maximum spectral density on the dominant peak, the frequency response of the filter was between 0.03 and 0.31 (0.16 ± 0.09).
18. The occurrence of movement in bursts is common in the behavior of neonates and young infants, including coordinated motor patterns such as sucking (*3*) and kicking [E. Thelen, G. Bradshaw, J. A. Ward, *Behav. Neural. Biol.* **32**, 45 (1981)].
19. We thank the staff and patients of the Perinatal Clinical Research Center for their cooperation; J. Nobbe for help in data analysis; and A. Fanaroff, J. Kennell, and M. Robertson for their comments. Supported in part by Public Health Service grant M01RR00210 and Major Research Program grant P50HD11089.

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Reassortant Virus Derived from Avian and Human Influenza A Viruses Is Attenuated and Immunogenic in Monkeys

Abstract. *An influenza A reassortant virus that contained the hemagglutinin and neuraminidase genes of a virulent human virus, A/Udorn/72 (H3N2), and the six other influenza A virus genome segments from an avirulent avian virus, A/Mallard/New York/6750/78 (H2N2), was evaluated for its level of replication in squirrel monkeys and hamsters. In monkeys, the reassortant virus was as attenuated and as restricted in its level of replication in the upper and lower respiratory tract as its avian influenza virus parent. Nonetheless, infection with the reassortant induced significant resistance to challenge with virulent human influenza virus. In hamsters, the reassortant virus replicated to a level intermediate between that of its parents. These findings suggest that the nonsurface antigen genes of the avian parental virus are the primary determinants of restriction of replication of the reassortant virus in monkeys. Attenuation of the reassortant virus for primates is achieved by the inefficient functioning of the avian influenza genes in primate cells, while antigenic specificity of the human influenza virus is provided by the neuraminidase and hemagglutinin genes derived from the human virus. This approach could lead to the development of a live influenza A virus vaccine that is attenuated for man if the avian influenza genes are similarly restricted in human cells.*

There is renewed interest in the development of a live attenuated influenza A virus vaccine, since current inactivated influenza virus vaccines do not provide complete protection (*1*) and they do not appear to retain their effectiveness when administered annually (*2*). Live attenuated influenza A viruses have been produced by transfer of genes from an attenuated donor virus to new epidemic influenza A viruses (*3*). Since resistance to influenza A virus is mediated by the development of an immune response to the hemagglutinin and neuraminidase glycoproteins (*4, 5*), live, attenuated, reassortant vaccine strains were selected that derived genes for these surface antigens from the epidemic virus, while the attenuating genes were derived from the attenuated parent. This process of gene exchange is readily achieved with influenza A viruses since they possess a segmented genome consisting of eight negative-stranded RNA segments that code for at least ten proteins (*6–8*). Although genes bearing temperature-sensitive mutations have been transferred to a series of new epidemic wild-type viruses and have rendered such viruses satisfactorily attenuated for man, the genetic instability of the attenuated phenotype represents an insoluble problem (*1, 9, 10*).

There is a need for stable attenuated viruses that are unable to escape their attenuated phenotype. Many of the influenza A virus genes that have evolved over a long period in birds differ significantly in nucleotide sequence from corresponding genes of human influenza A virus (*11–15*). Because of these marked differences, we would expect some avian influenza viruses to replicate inefficiently in human cells and thereby be attenuated. We would also expect such attenuated avian viruses to retain their attenuated characteristics after limited replication in man.

This concept was evaluated initially in squirrel monkeys because these primates develop illness after experimental infection with human influenza A viruses (*16*). Ten avian influenza A viruses were evaluated in squirrel monkeys, and a spectrum of virulence was observed (*17*). Although some of the avian viruses induced illness comparable to that caused by human influenza A viruses (*17*), an avian virus was identified that was infectious but avirulent for the monkeys (*17*). We now report that a virulent human influenza A virus can be attenuated for monkeys by substituting the six nonsurface antigen genes of the attenuated avian influenza virus for the corresponding genes of the human virus. This method of generating attenuated viruses by the production of a reassortant virus containing the hemagglutinin and neuraminidase genes from a human influenza virus and the remaining genes from a nonhuman virus represents a new approach to the production of viral vaccines for man.

Reassortant virus was produced by mating a human influenza A virus, A/Udorn/307/72 (H3N2), with an avian virus, A/Mallard/New York/6750/78 (H2N2), at 37°C in primary chick kidney (CK) cultures (*18*) at a multiplicity of infection of 1. Virus produced by this coinfection was next passaged at 41°C in the presence of antiserums to the avian hemagglutinin (H2) and neuraminidase (N2). Goat antiserum to avian hemagglutinin with a hemagglutination inhibition titer of 1:800 against the avian whole virus, was used at a dilution of 1:400 (fluid overlay) or 1:1000 (agarose overlay). Goat antiserum to neuraminidase, with a neuraminidase-inhibition titer of 1:4000 against the avian virus, was used at this dilution in both fluid and solid overlays. The virus harvested from the passage at 41°C was next passaged at 42°C without antiserum in the fluid overlay. The incubation temperature of 42°C was chosen because avian influenza viruses replicate efficiently at this temperature, whereas human viruses do not (*17*). Progeny virus from the CK cultures at 42°C was next plated on CK cell monolayers with an agarose (0.8 percent) and L15 medium overlay containing antiserums to the avian hemagglutinin and neuraminidase. Plaque progeny were similarly plated at 42°C and then passaged once in CK cells (37°C) maintained with a fluid overlay. Subsequently, virus was grown in 10-day-old embryonated eggs (37°C). Under these selective pressures, we recovered a virus containing the hemagglutinin and neuraminidase genes of the human virus and the six other RNA segments from the avian virus.

Antigenic analysis showed that the reassortant virus contained the surface antigens (H3N2) of the human virus. The origin of the genes at the other six loci was determined by comparing electrophoretic mobility of virion RNA segments of the parental and progeny viruses (*19*). Differences in migration of each of the corresponding genes of the two parental virion RNA's were detected with the following conditions of polyacrylamide gel electrophoresis. For RNA segment 7, a 4 percent acrylamide gel (20 cm) containing 6*M* urea was run

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Table 1. Virulence and efficacy in squirrel monkeys of reassortant influenza virus clone 1A produced by mating A/Mallard/New York/78 (H2N2) avian virus with A/Udorn/72 (H3N2) human virus; the reassortant virus has the hemagglutinin and neuraminidase genes of the human virus and each of the six other genes of avian virus. Virulence was determined by infecting each monkey with $10^{7.0}$ $TCID_{50}$ of virus (0.5 ml transtracheally), and infection was indicated by recovery of virus. Immunized and unimmunized monkeys were challenged with $10^{7.0}$ $TCID_{50}$ of A/Udorn/72 wild-type virus (0.5 ml transtracheally), with infection indicated by recovery of virus or development of a fourfold or greater rise in antibody titer. Nasopharyngeal swab specimens were taken daily for 10 days, and tracheal lavage was performed on days 2, 4, and 6; the amount of virus in the specimens from each monkey was determined by titration in MDCK (Madin-Darby canine kidney) tissue culture; the maximum amounts of virus shed by each monkey were averaged, and values are given as mean titers (log_{10}) per milliliter. The serum hemagglutination inhibition antibody titer (reciprocal mean titer) for the monkeys infected with the avian virus was measured with purified virus disrupted with 5 percent Sarkosyl. The clinical response of the monkeys shown in experiment 1 was determined in a separate group of 16 monkeys that were not swabbed or manipulated. There were four monkeys for each virus group, and four other monkeys received a placebo inoculum transtracheally. Upper respiratory tract illness (rhinorrhea) was observed only in animals infected with the wild-type virus. In experiment 2, the clinical response shown was obtained from the monkeys that were sampled. Clinical responses were also determined for ten control, placebo-inoculated animals that were swabbed and manipulated in the same manner as the monkeys challenged with the A/Udorn/72 wild-type virus. The ten control monkeys had an average duration of "illness" of 1.3 ± 0.7 days. Values are expressed as means ± standard error.

Virus administered	Number of monkeys	Viral replication				Hemagglutination-inhibiting antibody titer		Clinical response (days of illness)
		Nasopharynx		Tracheal lavage				
		Duration of virus shedding (days)	Peak titer	Duration of virus shedding (days)	Peak titer	Day 0	Day 28	
Experiment 1: evaluation of virulence								
A/Mallard/New York/78	5	3.0 ± 0.5	2.7 ± 0.3	2.8 ± 0.5	2.4 ± 0.3	≤ 1.0 ± 0.0	3.8 ± 0.6	0.0
Reassortant 1A	6	3.0 ± 0.4	3.4 ± 0.5	2.0 ± 0.5	2.2 ± 0.5	≤ 1.0 ± 0.0	5.5 ± 0.4	0.0
A/Udorn/72	15	6.0 ± 0.5	5.2 ± 0.3	5.2 ± 0.4	5.9 ± 0.3	≤ 1.0 ± 0.0	6.4 ± 0.4	5.5 ± 1.9
Experiment 2: challenge with A/Udorn/72 wild-type virus								
Reassortant 1A	6	2.7 ± 1.1	1.2 ± 0.4	0.7 ± 0.4	0.8 ± 0.3	5.5 ± 0.4	8.3 ± 0.4	2.3 ± 1.3
None	15	6.0 ± 0.5	5.2 ± 0.3	5.2 ± 0.4	5.9 ± 0.3	≤ 1.0 ± 0.0	6.4 ± 0.4	7.3 ± 0.7

at 100 V for 17½ hours at 30°C. For the other RNA segments, a 3 percent acrylamide gel (20 cm) containing 6*M* urea was run also at 100 V, but for 16 hours at 27°C. Each of six clones appeared homogeneous in that they received the hemagglutinin and neuraminidase genes from the human virus and the other six genes from the avian virus.

One reassortant virus, clone 1A, was characterized in greater depth. This reassortant replicated to high titer in eggs [median tissue culture infectious dose ($TCID_{50}$), $10^{8.5}$ per milliliter] and, like its avian influenza virus parent, produced plaques at 42°C with high efficiency. This indicates that one or more of the avian influenza genes that code for nonsurface proteins is responsible for growth at high temperature (42°C).

The level of replication of reassortant 1A in the upper and lower respiratory tract of squirrel monkeys was compared to that of its parents (experiment 1 in Table 1). The magnitude and duration of virus replication in the nasopharynx and trachea were similar for the avian influenza virus parent and reassortant 1A, and both were significantly lower than that of the human influenza virus parent. Illness was observed only in animals infected with wild-type influenza virus. Monkeys that were infected with reassortant 1A were challenged 35 days later with wild-type human influenza virus (experiment 2 in Table 1). Significant reduction in replication of wild-type virus and in illness was observed in the monkeys previously infected with reassortant 1A. Thus, despite the restricted replication of the reassortant, significant resistance was induced to virulent virus.

The level of replication of the parental and reassortant viruses was also examined in hamsters (Table 2). The reassortant virus replicated in the lungs and nasal turbinates of hamsters to a level intermediate between that of its parents. These observations indicate that the avian influenza hemagglutinin or neuraminidase, or both, as well as the products of one or more of the other six avian influenza genes contribute to the restriction of viral replication in the hamster. It appears that the genetic determinants of virulence of influenza for hamsters and monkeys are not identical. The A/Mallard/New York/6750/78 virus was recently shown to be virulent in BALB/c mice (*20*), but we have found that the reassortant 1A was avirulent in these mice. Differences in virulence of influenza A viruses in primates (man) and rodents (mice) have been described (*21*). The hamsters infected with the reassortant virus resisted challenge with the human wild-type virus, whereas those infected with the avian parent virus did not (data not shown). This indicates that the resistance induced by infection with reassortant virus is immunologically specific.

The pathogenicity of the reassortant 1A for ducks was examined as described (*22*). The reassortant did not infect the respiratory and gastrointestinal tracts of ducks, indicating the importance of the surface antigens of avian influenza virus in infectivity for birds and in enterotropism (*22*). Neither reassortant 1A nor the

Table 2. Replication of A/Mallard/New York/78 and A/Udorn/72 parental viruses and their reassortant virus clone 1A in the lungs and nasal turbinates of hamsters. Each hamster received approximately $10^{5.0}$ $TCID_{50}$ of virus intranasally. Six animals receiving A/Udorn/72 virus were killed on days 1 and 2; six hamsters from each of the other two groups were killed on days 1, 2, 3, and 4. Values are given as mean titers (log_{10}) ± standard error and represent the highest mean titer reached on one of the 4 days of study.

Virus administered	Number of animals	Maximum level of replication	
		Nasal turbinates	Lung
A/Mallard/New York/78	24	2.5 ± 0.3*	4.9 ± 0.4†‡
Reassortant 1A	24	5.0 ± 0.7*	7.5 ± 0.3†
A/Udorn/72	12	7.6 ± 0.2*	8.9 ± 0.2†‡

*Value differs from each of the other two values at a significance level of $P < .001$ (Student's *t*-test). †Value for reassortant 1A differs from the values for the parental viruses at a significance level of $P < .01$. ‡The parental viruses differ from each other at $P < .001$ (Student's *t*-test).

avian parent virus was recovered from rectal swabs of infected squirrel monkeys.

These findings have implications for the production of an influenza A vaccine virus that is attenuated for man. Since the genetic determinants of attenuation of the avian virus for primates reside on one or more of the genes that do not code for the surface antigens, it should be possible to produce an avian-human reassortant virus that has the surface antigens of a new epidemic human virus and the attenuating genes derived from an avian influenza virus. If such a reassortant virus behaves in man as it does in monkeys, it should be sufficiently restricted in replication to be attenuated. To be useful for immunoprophylaxis, reassortant virus would have to replicate well enough in man to induce resistance to illness caused by the epidemic wild-type virus.

These findings also have implications for the production of live virus vaccines for other animal or human viral pathogens.

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Benzodiazepine Receptor–Mediated Experimental "Anxiety" in Primates

Abstract. *The ethyl ester of β-carboline-3-carboxylic acid has a high affinity for benzodiazepine receptors in the brain. In the rhesus monkey this substance produces an acute behavioral syndrome characterized by dramatic elevations in heart rate, blood pressure, plasma cortisol, and catecholamines. The effects are blocked by benzodiazepines and the specific benzodiazepine receptor antagonist Ro 15-1788. The benzodiazepine receptor may consist of several subsites or functional domains that independently recognize agonists, antagonists, or "active" antagonists such as β-carboline-3-carboxylic acid ethyl ester. These results suggest that the benzodiazepine receptor is involved in both the affective and physiological manifestations of anxiety, and that the administration of β-carboline-3-carboxylic acid ethyl ester to monkeys may provide a reliable and reproducible animal model of human anxiety.*

The brain contains specific receptor sites for benzodiazepines that are functionally (and perhaps structurally) coupled to a recognition site for γ-aminobutyric acid and a chloride ionophore (*1*). Both direct and indirect evidence suggests that this "supramolecular receptor complex" mediates the pharmacological actions of benzodiazepines and of many structurally unrelated compounds that share common pharmacological properties with the benzodiazepines (*2*). Whether the benzodiazepine receptor complex has a physiological role in the absence of an exogenous ligand (that is, a drug) has been a source of considerable speculation, particularly regarding its function in the pathophysiology of anxiety and related disorders (*3*).

A study by Braestrup *et al.* (*4*) demonstrating that the ethyl ester of β-carboline-3-carboxylic acid (β-CCE) possesses a high affinity for the benzodiazepine receptor stimulated investigations of the pharmacological actions of this and related compounds. Although previous studies suggest that β-CCE antagonizes the pharmacological actions of diazepam and related benzodiazepines in rodents, with no apparent behavioral actions by itself (*5*), our data show that in primates β-CCE elicits a profound behavioral and physiological syndrome reminiscent of "fear" or "anxiety." Furthermore, both the behavioral and physiological effects of β-CCE are blocked by prior treatment with the specific benzodiazepine receptor antagonist Ro 15-1788 (*6*). The benzodiazepine receptor may not only be involved in the "anxiolytic" actions of benzodiazepines, but may also play a pivotal role in both the pathogenesis of anxiety and its pathophysiological sequelae in humans.

Adult male rhesus monkeys (*Macaca mulatta*) weighing 7 to 9 kg were restrained in chairs under ketamine anesthesia and allowed to adapt to this condition for at least 24 hours before drug administration. Previous behavioral and neuroendocrine studies with "chair-adapted" rhesus monkeys have validated this procedure for measuring stress-related hormones (*7*). All animals were fitted with femoral venous catheters, which were kept patent during the experiment by a slow infusion of sterile 0.9 percent NaCl. The animals were administered β-CCE (2.5 mg/kg) intravenously in vehicle (*8*). A control infusion of the vehicle alone was carried out 2 hours before the β-CCE infusion. The animals either received β-CCE alone or were treated with the benzodiazepine receptor antagonist Ro 15-1788 (5 mg/kg, intravenously) 20 minutes before β-CCE administration. In other experiments selected animals were treated with diazepam (1 to

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