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Influenza: Searching for Pandemic Origins

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Abstract

From a farming family of 13 children in New Zealand, I graduated with a Master of Science degree in microbiology from the University of Otago (Dunedin, Otago, New Zealand). I established the first veterinary virology laboratory at Wallaceville Animal Research Station. I subsequently completed my PhD degree at Australian National University (Canberra, Australia) and a postdoctoral fellowship at the University of Michigan (Ann Arbor, Michigan). While in New South Wales, Australia, a walk on a beach littered with dead mutton birds (shearwaters) with Dr. Graeme Laver led to the surveillance of influenza in seabirds on the Great Barrier Reef Islands and my lifelong search for the origin of pandemic influenza viruses. Subsequent studies established that (*a*) aquatic birds are a natural reservoir of influenza A viruses, (*b*) these viruses replicate primarily in cells lining the intestinal tract, (*c*) reassortment in nature can lead to novel pandemic influenza viruses, and (*d*) live bird markets are one place where transmission of influenza virus from animals to humans occurs.

THE WEBSTER HERITAGE

My grandfather, John Webster, was born in 1856 at Ballyknocken in County Wicklow, Ireland. In 1878, he immigrated to New Zealand on the ship *Dunedin* and married Annie Robertson, who was of Scottish descent. Together, they established a farm in the hill country at Waitahuna West in South Otago and raised 11 children; my father, Robert (Bert) Webster, who was born in 1888, was their second oldest child.

My grandfather went blind at a late age and must have taught his nine sons the skills of farming, such as ploughing and tilling the soil and raising sheep for wool and dairy cows for milk. My father excelled at shearing sheep, which provided a good income for a young person.

In November 1916, my father married Lilian Katherine Browning, and eight months later, he enlisted in the New Zealand Expeditionary Force of the army to fight in France during World War I. During the trench warfare, he experienced the 1918 influenza and the terror of poison gas clouds, and was wounded with a bullet in his shoulder and right elbow. He was discharged in February 1919 and was always reluctant to talk about his experiences during the war.

After World War I, soldiers who returned to New Zealand and had a farming background were given the option of leasing 120 acres of land to establish a farm. My father took up a lease and established our farm in the valley of the Clutha River, South Otago. Although 120 acres may seem like a large area, it was not sufficient for a productive farm in that area. When the veteran living next door returned his farm to the government, my father acquired that lease, thereby doubling his productive acreage.

My father's first wife died at age 34, during surgery for a goiter. Her death was a major tragedy in his life. He had seven young children to care for and a farm to run. My mother, Mary Ann (Mollie) Shirreffs, came to the rescue. The Shirreffs were silversmiths in Aberdeen, Scotland, before they immigrated to New Zealand and settled in Southland. My mother's family lived at Ryal Bush, near Invercargill, in Southland. She was a very gutsy lady for marrying a grieving widower with seven children, helping on the farm, and having six children of her own. I am my mother's oldest son, and I learned quite early that one had to be self-sufficient. The large family provided all the hands needed to run a farm and then some. From early childhood, I learned how to milk cows by hand, raise and shear sheep, grow and harvest grain, and hunt rabbits.

SCHOOLING

Schooling started at the age of five. My older siblings and I would walk about two miles across the farm, down a local road, and across two creeks to a single-room school in Pukepito. The school had only one teacher who taught all the grades. After about one year, a new school that served the entire region, the Clutha Valley District High School, was opened.

The bus ride to and from the Clutha Valley school played an important role in my education. Although the morning trip took about 30 minutes, the afternoon trip took 1.5 to 2 hours, over winding gravel roads. Those bus rides provided time for me to learn to read the many books I checked out from the school library. I was extremely fortunate to have superb teachers, especially in the sciences, at Clutha Valley District High School. In New Zealand, students had to pass the School Certificate examination to proceed to university entrance. However, the courses for university entrance were not taught at my school, so it was necessary for me to travel to Balclutha to attend South Otago High School.

South Otago High School also had terrific teachers and a large laboratory for conducting chemistry experiments. My goal at that time was to become a chemist like my older brother Harold. At the end of my final year in high school, I decided to join the Air Force, as New Zealand required that all young men spend six months on a military base receiving formal military training.

Since I was heading to university thereafter, the duration of my training on a military base was reduced to three months, but I was required to report each weekend for air training.

The first long vacation at Otago University was spent fulfilling my military training obligation at Taieri Airport, which served the city of Dunedin. One of the rules for student pilots was that you must never wear heavy drill boots while flying because they could get jammed in the rudder pedal slot. When I was told that we would begin aerobatics after the evening meal, I was so excited that I forgot to change out of my drill boots. Fortunately, we began with steep turns. My boot got stuck in the rudder slot, but the instructor kicked like crazy from the front seat and freed it. I was severely berated by the instructor and was lucky not to be grounded.

Entrance to the university was by accreditation—examination scores achieved in the sixth form determined acceptance by the university of one's choice. I opted to pursue a Bachelor of Science degree, majoring in chemistry with minors in botany and geology. Acceptance by Otago University meant that I would move to Dunedin to attend classes.

UNIVERSITY OF OTAGO YEARS

University was free of charge for courses and practicals, but students had to provide their own funds for room, board, and travel. Coming from a large family, I had to be self-sufficient. Therefore, to cover those costs, I had many different jobs during vacation periods, including cheese maker, pottery worker, kiln fireman, coal miner, and wharf worker. Those wide-ranging experiences were very educational.

Course and laboratory practical work were all-consuming during the first year at the University of Otago. One Saturday morning each month, students met for chemistry club, where an invited speaker would present topics, such as the application of chemistry to other fields. One of the most important events in my life occurred at one of those Saturday meetings. The speaker was Professor Mollie Marples, who spoke on a new program called microbiology that was being introduced at the medical school for science students. After the meeting, I talked to Dr. Marples, and the next semester I signed up to take classes. It was not long before I realized that microbiology would be my major. At the beginning of the second semester of microbiology, a recruiter from the New Zealand Department of Agriculture gave a presentation about the importance of microbiology to agriculture, the main industry in the country. The Department of Agriculture was offering scholarships to recruit young people to work at the Wallaceville Animal Research Station at Upper Hutt, near Wellington, on the North Island of New Zealand. The proposal was to work in the research laboratories during university vacations and join the laboratories after graduation. In return, they provided a basic stipend to support room and board at Otago University. I was interested in continuing my studies in microbiology to earn a Master of Science degree; therefore, I applied and was accepted into the program. Later, I became an employee of the Department of Agriculture at the Wallaceville Animal Research Laboratories. My time at Wallaceville was a terrific experience; samples from sick animals from all over New Zealand were tested for the causative agent of disease.

At Otago University, my mentor was Professor John Miles, the newly appointed Chair of Microbiology and a virologist. My project was to determine if the virus causing scabby mouth (contagious pustular dermatitis) in sheep was related to poxviruses like vaccinia virus. The studies on poxvirus in sheep led to my first publication describing the antigenic relatedness between contagious pustular dermatitis virus and poxviruses like vaccinia (1).

My main extracurricular activity while at Otago University was tramping (hiking). For my last two years at Otago, I was president of the Otago University Tramping Club. There were two events over the years that were quite memorable. The first event was when a sizable group of

student trampers missed the return ferry from Stewart Island to Invercargill, on the South Island. The Dean of the Home Services School was quite concerned when she got frantic telephone calls about missing students. The group arrived back about one week later, after being fed by the fishermen of Stewart Island. The second, more fortuitous event was when I helped a young lady named Marjorie Freegard cross a flooded river at Trotter's Gorge; she would subsequently agree to become my wife.

FROM THE WALLACEVILLE ANIMAL RESEARCH STATION TO THE AUSTRALIAN NATIONAL UNIVERSITY

After finishing my studies at Otago University, I joined the Department of Agriculture at Wallaceville and had the privilege of setting up the first agricultural virology laboratory in New Zealand (**Figure 1*c***). No one had tested for viruses in diseased animals in New Zealand, so it was not surprising that a plethora of viruses were isolated from the samples submitted: poxviruses and respiratory viruses of chickens, diverse viruses of calves and pigs, and respiratory viruses of cattle, along with many, many more. Interestingly, we also isolated viruses from pigs that caused no disease in the animals. It was not long before we were writing papers about the viruses in livestock in New Zealand (2).

Life as a diagnostic virologist was straightforward—isolate viruses from the samples submitted by the veterinarians, identify the viruses when possible, and report the results. The collection of uncharacterized viruses in the freezer grew, as did my desire to study them in more detail. My supervisors were happy with my work performance, but I had the feeling that I should do more than "collect butterflies." I saw an advertisement in the journal *Nature* about a new medical school that had opened in Canberra, Australia—the John Curtin School of Medical Research. They were offering scholarships to do a PhD with Dr. Frank Fenner, who worked on poxviruses and myxomatosis. I sent my CV and a letter of application requesting Frank Fenner as my mentor, and I was accepted. The Director of Wallaceville Animal Research Station was supportive of the idea but wanted me back at the end of my time in Canberra.

My first day at the Australian National University (ANU) was one that I will never forget, for it changed my life forever. Frank Fenner greeted me in his office and then informed me that I would not be working with him but would be a student in the laboratory of Professor Stephen Fazekas de St. Groth, who was working on influenza virus neutralization. Thus, it was Frank Fenner who decided that I would become an influenza virologist—and I was very upset. I had no knowledge of Professor Fazekas de St. Groth or influenza, although I later found that Stephen was a brilliant scientist, and it was a privilege and a challenge to work with him. He had a mathematical approach to biology, which was not my strong point. My first two months were a real struggle, but eventually I learned his modus operandi, and he learned my laboratory skills. He was an active bench scientist, and we soon worked well together.

About the same time that I joined the John Curtin School, so did Dr. Graeme Laver. Graeme had just finished his postdoctoral training in London. Frank Fenner had also assigned him to work with Fazekas de St. Groth. Graeme was a "dinky-di" Aussie (a genuine Australian), who was always up for an adventure. He had returned to Australia by driving a tiny Standard Family 10 saloon car across country, from London to India via Afghanistan and Persia, with his wife, Judy. Graeme and I hit it off from the first meeting and formed a lifelong working friendship, as did Marjorie and Judy.

My project at ANU was to determine the route of immunization that produced high-quality antibody responses in rabbits after immunization with influenza virus. To measure the quantity of antibody bound to a virus, we needed a quantitative protein stain. Since Australia was a major

Figure 1

New Zealand early years. (*a*) Sheep on the Webster farm at Pukeawa and Rob Webster (*inset*). (*b*) University of Otago. (*c*) Original virology laboratory at Wallaceville, with Dr. Sue Huang and Rob Webster. (*d*) Farm at Waeranga, where Marjorie (my wife) grew up. (*e*) Marjorie on home farm at Waeranga.

producer of fine wool from Merino sheep, we knew that lots of great dyes should be readily available. Fazekas de St. Groth contacted a chemist he knew in the wool industry who sent us many dyes. The best dye, by far, was Coomassie Brilliant Blue R.S., but we could not get the stain out of the support media, paper, or cellulose acetate, and had given up on it. That evening, I returned to

the lab and played with possible ways to get the stain out of the background. After hours of trial and error, I found that acidic methanol worked like magic. The problem was solved, the paper was published, and the quantitative stain used in studies today is still Coomassie Brilliant Blue R.S. (3).

After finishing my PhD I decided to continue in influenza research and wrote to the School of Public Health at the University of Michigan in Ann Arbor about doing postdoctoral training. Dr. Thomas Francis, Jr. and his group were working on "original antigenic sin" to influenza (see below). I was accepted and began to make plans to move my family to the United States.

To support my postdoctoral training at the University of Michigan, I applied for and was awarded a Fulbright Scholarship. Then disaster struck. I had been awarded a Fulbright Scholarship on the Australian quota, but I was a New Zealander, and the quota for New Zealand had been given out. The answer hit me instantly—I told the Fulbright Fellowship Committee to hold everything. I immediately applied for Australian citizenship, paid my 10 shillings, and in one day became an Aussie!

ANN ARBOR, MICHIGAN, POSTDOCTORAL PERIOD AND RETURN TO CANBERRA

The trip to the United States was on a Dutch ship, the *Johan van Oldenbarnevelt* or the JVO. The ship did not have stabilizers and was not air conditioned. To say that the trip was through rough seas would be an understatement. Added to this, most of the 1,200 passengers were students with young families. When the children interacted in the nursery, the childhood diseases of mumps, measles, and chickenpox spread like wildfire. The six-week voyage from Wellington, New Zealand, to New York, through the Panama Canal, was sheer hell. There is little wonder why Marjorie, my wife, will never go on another ocean cruise!

My mentor at the University of Michigan, in Ann Arbor, was Dr. Fred Davenport, a close colleague of Dr. Tommy Francis. It was Francis who first described "original antigenic sin," the concept that the first infection with influenza virus during childhood leaves an immunologic imprint that affects all subsequent vaccinations. My project was to study original antigenic sin in ferrets, and I immediately obtained publishable results. Fazekas de St. Groth joined me in Ann Arbor for several months, and we worked together on two papers, "Disquisitions on Original Antigenic Sin I and II," which were published in the *Journal of Experimental Medicine* (4, 5).

After a highly productive postdoc in Ann Arbor, I was recruited back to ANU as a junior staff member. Upon returning to Canberra, I focused my efforts on preparing and testing a subunit influenza vaccine with Graeme Laver. Intact influenza virus vaccine is quite toxic, especially in children, so people were reluctant to be vaccinated. A common statement at that time was that the vaccine was as bad as or worse than the flu. Graeme and I decided to test a virus-subunit vaccine. He prepared the vaccine by disrupting purified influenza virus with the mild detergent sodium deoxycholate, and I tested for toxicity and immunogenicity after intravenous injection into rabbits. The intact-virus vaccine caused a high temperature spike in rabbits, but the virus-subunit vaccine did not, yet both vaccines were immunogenic (6). We then moved the subunit vaccine into clinical tests for children; this work was done in conjunction with the Commonwealth Serum Laboratories (CSL) in Melbourne. The phase I trial was very revealing—the subunit vaccine was not reactogenic, in terms of causing areas of redness (erythema) around the site of injection or causing fever. However, the intact control vaccine was so toxic that that arm of the study had to be discontinued; two children injected with the intact virus vaccine had convulsions. The subunit vaccine was immunogenic and nonreactogenic. The results were so encouraging that the CSL started producing influenza virus-subunit vaccine and still does so today. ANU realized that this subunit vaccine had commercial value and patented the process. Proceeds from the patent would

later enable me to travel back to Canberra from Memphis, Tennessee, to participate in studies of migratory birds on the Great Barrier Reef (described below).

Another study I conducted after returning to Canberra was to determine whether the surface glycoprotein neuraminidase had any role in immune protection or releasing influenza virus from cells. Graeme isolated the neuraminidase from the rest of the virus components, and I prepared specific antisera in rabbits. The serum inhibited the enzymatic activity of the neuraminidase. Although the serum did not prevent infection of cells, it reduced the yield of virus in culture, provided that the antibody was left in the culture (7). This result provided an important early clue that neuraminidase functions in influenza virus release.

RECRUITMENT TO ST. JUDE CHILDREN'S RESEARCH HOSPITAL

Dr. Ed Kilbourne, the Chair of Microbiology at Cornell Medical School in New York City and a strong proponent of the importance of neuraminidase in influenza biology, invited me to work with him at Cornell for a couple of weeks. He wanted to use the neuraminidase-specific antiserum in several collaborative experiments. The collaboration was very successful, resulting in a publication that showed that the antineuraminidase serum reduced plaque size and, providing that the virus had a high number of neuraminidase molecules, inhibited hemagglutination (8). Neuraminidase also played a role in protective immunity and was important to include in vaccines.

During my visit to New York, Dr. Bernie Fields, who was at the Centers for Disease Control and Prevention (CDC), insisted that I come to Atlanta to give a talk. Dr. Allan Granoff, who was establishing a new Department of Virology at St. Jude Children's Research Hospital (St. Jude), heard about my visit to the CDC and persuaded me to come to Memphis during the same trip and present a seminar there. After my seminar at St. Jude, Allan offered me a junior faculty position in his new department.

When I returned to Australia, I asked Graeme his opinion. "You are thinking of going where? To Memphis, Tennessee? You would be committing scientific suicide, just when you are establishing yourself." Allan Granoff was a super salesman. He made several long-distance telephone calls, upping the offer and emphasizing the opportunity to establish an independent research program focused on influenza virus, as well as starting a new program on cancer-causing viruses. Allan convinced us to make the move.

Our introduction to Memphis in 1968 was less than auspicious. Soon after our arrival Dr. Martin Luther King, Jr. was shot, and troops and tanks were on the streets. Graeme, of course, pounced on the dangers of living in Memphis and organized a more senior faculty position back at ANU. Allan's salesmanship skills, once again, came to the fore. He persuaded me not to resign but to take a year's leave of absence. Allan was completely right; within six months, we were back at St. Jude for good. Having the opportunity to completely run my own research laboratory was only possible away from ANU. The connection with Graeme and ANU, however, continued for the rest of my career, with multiple visits to participate in studies of influenza viruses in migrating pelagic birds on the Great Barrier Reef and in multiple international scientific meetings.

During my first review by the National Institutes of Health after I joined St. Jude, I was asked how I justified working on influenza virus at a pediatric cancer institute. I asked the questioner, "What kills our kids, infectious diseases or cancer?" He thought that the answer would be cancer, but I explained that in immunosuppressed patients it is more often infectious diseases that cause death.

My laboratory's mission was to understand the origin and prevention of influenza epidemics and pandemics. At that time, no antiviral drugs were available, and participation in the World Health Organization (WHO) Influenza Program played an essential role in determining vaccine

Figure 2

World Health Organization (WHO), 1970. WHO Consultation on Influenza Ecology at which the animal reservoir for influenza viruses was discussed and potential threats identified. (*back row, right to left*) D'mitri L'vov, Rob Webster, Graeme Laver, Geoffrey Schild, Walter Dowdle, Claude Hannoun, Ken Shortridge, Dennis Alexander, Peter Bachman, M. Picard, and unknown. (*front row, right to left*) V.M. Milouchine, Ian Beveridge, Martin Kaplan, Bela Tumova, and Barney Easterday. Retrieved from the World Health Organization Library Archive section, volume 1970–1980. Reproduced with permission of the World Health Organization.

strains. In 1975, St. Jude was designated a WHO Collaborating Center on the Ecology of Influenza Viruses, and St. Jude continues to be part of the WHO's global network (**Figure 2**).

A WALK ON THE BEACH RESULTS IN THE INITIAL THEORIES ABOUT THE ORIGIN OF INFLUENZA PANDEMICS

During our years at ANU, we made many trips to the beaches on the New South Wales coast, north of Batemans Bay. Pretty Beach was one of my favorite places to snorkel, collect abalone, and go fishing. Perhaps the most important of these trips, from a scientific perspective, occurred in 1967 when Graeme and I found the beach littered with dead mutton birds [shearwaters (*Puffinus pacificus*)] that migrate around the Pacific Ocean. Knowing that influenza had killed terns in South Africa in 1961, we thought that flu had killed them. After returning to ANU, we approached the head of the Microbiology Department with a proposal to sample (swab) birds on the Great Barrier Reef. The immediate response was, "You have got to be joking. . .a scientific expedition, my foot! More like a junket to take your family on a holiday!" He was largely correct, but we did not give up. We sent the proposal to Dr. Martin Kaplan, who was part of the WHO Global Influenza Surveillance Network (GISN) (**Figure 2**). We were delighted to receive a positive response and support of $500 (US). ANU then decided there was scientific merit to the proposal and provided a vehicle for our travel from Canberra to Gladstone, the nearest port to the Great Barrier Reef.

Mutton birds are pelagic birds that spend their life at sea and come ashore only to raise their young. They nest in shallow burrows on the offshore islands south of New Zealand and on the Great Barrier Reef islands of Australia. The name mutton birds comes from early settlers, who found the young birds an easy source of food that could be salted and smoked. The islands of Tyron, Northwest, and Lady Elliot, at the southern end of the Great Barrier Reef, are sandy cays with scrubby vegetation, where mutton birds nest (**Figure 3**). There was no shortage of colleagues from around the world who joined the expeditions when invited over the years.

The short trip from Gladstone to the offshore islands could be a challenge and varied from glassy smooth to extremely rough water. Even scopolamine tablets did not prevent seasickness. Permission to sample the birds of the uninhabited islands had to be obtained from Australian authorities beforehand. Since there was no potable water or facilities on the islands, all equipment, tents, food, and water had to be ferried ashore by rowboat. During the day, participants would swim and snorkel in the surrounding coral reef and catch fish and lobster for dinner.

The workday began at dusk, as the parent birds returned to the colony with their bellies full of fish to feed to the ravenous developing chicks. Lanterns were lit, and adult birds were pulled from their burrows for sampling. Throats were swabbed, a small blood sample was collected from the wing vein, and then the birds were returned to their burrows. When new recruits were lying on the ground, with their arms deep in burrows, Graeme would say, "Beware of the snakes!" There are no snakes on the coral cays, but the recruits would hastily remove their arms. During the day, noddy terns (*Anous tenuirostris*) that nested in the scrubby trees (*Pisonia grandis*) also were sampled. These birds were easily caught from their nests, as they were unafraid of humans (**Figure 3**).

During the first year of our study, Graeme used agar gel double-diffusion plates to directly test the sera from the birds for the presence of influenza-specific antibodies. On several plates, a line of precipitation was detected between the sera and inactivated influenza virus, indicating previous influenza virus infection. Subsequent testing in the laboratory detected antibodies to N2 neuraminidase, but no virus was isolated from the hundreds of throat swabs. This result indicated that it was necessary to return to the islands the following years to attempt to isolate the virus. During the second year, we sampled more than 200 birds and isolated one influenza virus strain, A/Shearwater/Australia/1/72 (H6N5), that expressed a previously uncharacterized neuraminidase. The next year, we isolated eight influenza virus strains from the throat swabs of noddy terns and one influenza virus strain from the throat swab of a shearwater. All the isolates were from apparently healthy birds, and on subsequent testing for pathogenic potential in domestic ducks, chickens, and turkeys, the virus replicated well in the respiratory tract but did not produce disease signs. The isolates included an H5N3 virus that was antigenically like the virus that had killed terns on the coast of South Africa in 1961. The harmless versions of the H5NX influenza viruses (i.e., those influenza virus reassortants containing N1, N2, N5, N6, and N8 neuraminidase) were circulating in these birds.

Although sampling birds on the Great Barrier Reef was perhaps some of the most enjoyable fieldwork in my career, it was not risk free. Stone fish with deadly spines lurked in the sand. Sharks fed during the incoming tide, and giant sea turtles would disrupt your tent and awaken you from sleep if you were camping where they laid their eggs. Field rules were that hats, shirts, and sneakers had to be worn during the day, including while swimming, because the sun was always intense, and stonefish toxin is lethal. The detection of influenza A virus from apparently healthy shearwaters and noddy terns on the Great Barrier Reef led me to search for influenza viruses in aquatic birds globally. Additionally, Graeme crystallized the neuraminidase of an H11N9 influenza virus isolated from a noddy tern and helped Gilead Sciences (Foster City, California) design oseltamivir (trade name: Tamiflu®) (9).

Figure 3

Australian studies. (*a*) John Curtin Medical School. (*b*) Australian beach where dead mutton birds were found. (*c*) An island on the Great Barrier Reef. (*d*) Migration route of shearwaters (mutton birds) in the Pacific Ocean. (*e*) A noddy tern. (*f*) Mathew Barnard holding a noddy tern for Walter Dowdle on a Great Barrier Reef island.

ANTIGENIC RELATIONSHIPS OF ANIMAL INFLUENZA VIRUSES AND HUMAN PANDEMIC STRAINS

After we isolated influenza A viruses from migratory waterfowl, the question was whether influenza viruses in mammals and birds are antigenically related to those in humans. Since the largest

collection of animal influenza viruses was at the World Influenza Centre, National Medical Institute of Records (Mill Hill, London, UK), I contacted the director, Dr. Helio Pereira. Like me, Helio was a proponent of the animal reservoir hypothesis to explain the origin of human influenza pandemics, and he invited me to come to Mill Hill to study their repository of influenza viruses. Dr. Bela Tumova, the senior influenza virologist from Czechoslovakia, joined the study and provided rat antisera that recognized a wide range of animal influenza viruses, including the 1957 H2N2 pandemic virus from humans. These studies showed strong serological cross-reactivity between the human H2N2 pandemic influenza virus and an influenza virus isolated from turkeys in Massachusetts in 1965 (10).

Although this study provided some of the first evidence supporting the theory that human pandemic influenza viruses originate in animals, it did not establish which of the surface glycoproteins on the virus were shared. Specific antisera prepared to recognize isolated hemagglutinin or neuraminidase of human H2N2 influenza virus yielded the answer. The specific antisera to the neuraminidase of the 1957 human pandemic virus completely inhibited the enzymatic activity of the turkey influenza virus, thereby supporting the idea that the neuraminidase of the 1957 pandemic influenza virus could have come from an influenza virus in animals or vice versa.

SEARCHING FOR INFLUENZA VIRUSES IN CANADIAN WILD DUCKS

Since I had moved to St. Jude in Memphis, Tennessee, which is located on the Mississippi River, forming the Mississippi flyway for migratory waterfowl, it was logical to sample wild ducks. Duck hunting occurs in late November and early December in Memphis. The number of birds that hunters can shoot is determined by Canadian authorities, who conduct a yearly census at the breeding grounds across Canada. Obtaining throat swabs from wild ducks was easy; most hunters take their freshly killed birds to a dressing station, where the birds are plucked, eviscerated, and packaged. With permission from the owner, we collected throat swabs before the dressing began. From the very first sampling of wild ducks, we isolated an influenza virus strain designated A/Duck/Memphis/546/74 (H11N9). Like the antigenically similar virus from the Great Barrier Reef, this isolate did not cause disease in young domestic ducks. During the first sampling season, approximately 2% of samples contained influenza viruses of different subtypes. To determine the site of replication of these influenza viruses in ducks, a visiting Russian scientist, Dr. Maya Yakhno, dissected the organs of an influenza-infected bird. The results provided important ecological information and one of those "Eureka!" moments in the laboratory—influenza virus replicated predominantly in the intestinal tract and was shed in huge concentrations in the feces (11). For years we had been sampling the wrong end of the bird (**Figure 4**)!

We then wondered—if we sampled the wild ducks in Canada before their migration, would we detect even more influenza viruses? I wrote a letter to Bruce Turner of the Edmonton Office of the Canadian Wildlife Service (now part of Environment Canada). In response, he invited me to join the duck-banding team in July 1976.

All the ducks were fat, healthy, and ready for migration. Thus, the wildlife personnel thought they were hosting a nutty professor when I suggested that some ducks were infected with influenza virus. Therefore, we obtained throat and rectal swabs and a small blood sample from each bird. When tested for viral replication in embryonated chicken eggs, 18.5% of samples from juvenile ducks and 5% of samples from adults yielded influenza viruses. Both wildlife personnel and scientists were amazed at the results and decided to establish a collaboration that continues today. Although the number of influenza subtypes is limited, the dominant circulating influenza subtype changes yearly. It is noteworthy that in the past 46 years of sampling, no highly pathogenic influenza viruses have been isolated from Canadian ducks.

Figure 4

"The wrong end of the duck." For many years, Graeme Laver (*left*) and Rob Webster (*right*) swabbed the respiratory tracts of birds for influenza virus. However, the virus replicates in the intestinal tract. Reproduced with permission from Australian National University.

It will be most interesting to view the results from the 2023 sampling, as highly pathogenic H5N1 influenza virus strains have been isolated from multiple aquatic birds in Canada and the United States, and those birds are implicated in the global spread of this virus to domestic poultry. The surveillance studies in Canadian migratory ducks contributed to the concept that aquatic birds are a natural reservoir of influenza A viruses.

DELAWARE BAY: AN INFLUENZA VIRUS GOLD MINE

Each year on the first full moon in May, one of the most significant events in the ecology of influenza viruses occurs at Delaware Bay in the United States. Swarms of horseshoe crabs (*Limulus polyphemus*) come ashore to mate and lay their eggs in the sand. Just at that time, migrating shorebirds, including red knots (*Calidris canutus*) and ruddy turnstones (*Arenaria interpres*), arrive, having flown 6,600 miles nonstop from South America.

The migration of these birds is timed so that they can refuel on horseshoe crab eggs, gaining up to 30% of their body weight before flying to Churchill Bay, Canada, en route to the far-north of Canada, where they will mate and breed. In Delaware Bay, the migrating birds deliver influenza viruses to beaches that are shared with the resident gulls and wading birds that also feed on horseshoe crab eggs.

On my first visit to Delaware Bay in 1985, I located Reeds Beach and was amazed, as the beach was littered with overturned horseshoe crabs and packed solid with shore birds in a feeding frenzy. We collected individual fecal samples with Dacron swabs from the sand at the waterline, put the samples in a portable nitrogen freezer, and tested them back in the laboratory for influenza viruses using embryonated chicken eggs. The results were astonishing: Approximately 20% of the samples tested positive for influenza viruses (12). In the following two years, we isolated influenza viruses belonging to 10 of the 12 subtypes of influenza viruses known at that time. Polymerase chain reaction testing of the fecal samples and subsequent testing of birds revealed that most influenza virus isolates were from ruddy turnstones; fewer were from red knots, gulls, and wading birds. We realized that we had stumbled on a goldmine of influenza viruses, and we have continued to mine them every year since.

Surveillance of the same species of birds in different parts of the world for influenza viruses by other investigators has detected much lower frequencies of virus isolation, raising the question of why the birds at Delaware Bay shed influenza viruses more frequently. To this day, the answer remains unclear. As in migratory ducks, the dominant subtype of influenza viruses in the birds at Delaware Bay changes yearly. The H7N3 virus was isolated quite frequently from surveillance at Delaware Bay and was avirulent when tested in chickens. However, these H7N3 viruses evolved into the highly pathogenic strains that killed domestic poultry in Chile in 2002 and Mexico in 2012.

The studies of influenza viruses in wild ducks and shorebirds provided the background information that enabled us to propose in the late 1990s that wild aquatic birds are the natural reservoirs of most influenza A viruses found in other species, including humans. Two subtypes of influenza virus have since been found in bats, thereby extending the natural reservoirs of influenza viruses.

REASSORTMENT OF INFLUENZA A VIRUSES UNDER NATURAL CONDITIONS

Influenza viruses possess eight segments of single-stranded RNA. Therefore, we expected that they would reassort their genomes if two or more influenza A viruses coinfected the same cell. Sir Macfarlane Burnet and Dr. Patricia Lind, both in Melbourne, Australia, demonstrated this property in 1952. They showed that when two different influenza A subtypes were inoculated together into the same chicken embryo, hybrid viruses were obtained (13). The question we asked was whether this would occur in nature. Since attempting to isolate novel influenza viruses by reassortment might yield strains that are dangerous to humans, mammals, and birds, we decided that these studies should be conducted in a high-biosecurity facility. No such facilities were available at St. Jude at that time, so I approached Dr. Jerry Callis, Director of the Agricultural Biocontainment Facility at Plum Island, New York, about the possibility of conducting the research there. The Plum Island facility, located on an offshore island, is designed to protect US livestock against exotic animal viruses, such as foot and mouth disease virus, that might be inadvertently introduced into the United States. After we presented a seminar to the staff at Plum Island and explained the details of the proposed study, they approved it.

Getting onto Plum Island required that you be accompanied by a staff member at all times. Dr. Charles Campbell and his staff provided laboratory space and training. Over the next four years, we conducted a series of experiments demonstrating that different influenza A viruses could transmit to a contact animal, reassort their genetic information in that animal, cause disease, and depending on the viruses used, kill the recipient animal (14). The ease with which influenza A viruses reassorted led us to question why there are not more frequent pandemics in humans or outbreaks of diseases in lower animals. The experiments at Plum Island established that reassortant viruses in contact animals are always in the minority. For a reassortant virus to become dominant, it must have additional properties of dominance, fitness, and transmissibility (15). Thus, it is not surprising that influenza pandemics are relatively infrequent in humans.

A VISIT TO CHINA LEADS TO 50 YEARS OF COLLABORATIVE RESEARCH

Both the 1957 H2N2 and 1968 H3N2 influenza pandemics first emerged in southern China, so that was the logical place to start my search for their origins. The large population of ducks, pigs, and people frequently intermingling seemed optimal for the genesis of reassortant influenza viruses. In 1972, Graeme and I had the opportunity to join a group of Australian medical scientists on an exchange visit with the Chinese Medical Association. Additionally, we had permission to bring swabs and vials to sample and bleed animals to detect influenza viruses. The WHO fully

supported our visit, as they were most interested in establishing a collaboration with influenza virologists in China.

From September 9 until October 4, 1972, we visited medical institutions, from Guangzhou in the south to Shenyang in the north and then back to Shanghai (16). The highlight of our visit was the friendly welcomes we received from people on the street. Because our visit was a medically based exchange, we spent most of our time in hospitals, but in Beijing and Shanghai, Graeme and I met with local virologists. We were most impressed when we arrived in Beijing at 4 AM by train and were met by Dr. Chu Chi Ming, who had trained as an influenza virologist in Cambridge and London. Both Graeme and I were invited to present lectures at the National Vaccine and Serum Institute and were concerned that our hypothesis—that human influenza pandemics originate from ducks and pigs in China—would be considered a slight to our Chinese hosts. However, we were well-received.

We were greatly impressed by the willingness of the Chinese scientists to give us samples of their influenza viruses and openly discuss their findings. Their consensus was that pandemic influenza viruses were not from an animal reservoir but arose by excessive mutation. The virologists provided us with bridging strains, which also were shared with the WHO network. We found that these strains were very sensitive to serum inhibitors, giving the erroneous impression of strong antigenic relationships between viruses of different subtypes in serological tests.

Our attempts to isolate influenza virus from various animals were not as successful. At Shijiazhuang, a large military base en route to Beijing that had a large pig farm, we were permitted to sample one pig, with the assurance that "All pigs are the same." We then realized that animal testing was a sensitive issue.

Whether our visit played any part in the release of information about the re-emergence of the H1N1 virus A/USSR/90/77 (H1N1) in northern China in 1977 or the eventual acceptance of the hypothesis of an animal origin of pandemic human influenza, we cannot ascertain. However, there were many more exchange visits of scholars from China to our laboratories, and in November 1982, a meeting about the origin of pandemic influenza viruses was held in Beijing and sponsored by the Institute of Virology of the Chinese Academy of Medical Sciences and ANU. These connections have continued to the present, with the full participation of China as a WHO Collaborating Center for Influenza.

THE IMPORTANCE OF LIVE BIRD MARKETS

After visiting China in 1972 and having seen live bird markets (LBMs) in the streets, I thought these sites would be optimal for surveillance of influenza viruses. When the National Institutes of Health in the United States issued a request for the scientific community "to elucidate the origin and possible control strategies for pandemic influenza," I contacted Professor Kennedy Shortridge at the University of Hong Kong about a collaborative study on influenza virus surveillance in wild birds, domestic poultry, and pigs in the United States and Hong Kong. The proposal provided the funding to ascertain the importance of LBMs in the genesis of influenza viruses and many of the ecological principles of influenza in wild birds.

The tradition of LBMs originated during the Ming Dynasty in the sixteenth century (17). The LBMs operating in Hong Kong and southern China until 1997 are the most relevant to influenza elucidation (**Figure 5**). After the 1997 emergence of H5N1 bird flu, LBMs in Hong Kong changed dramatically; e.g., the number and size of LBMs were reduced, and different species of birds were housed separately. In mainland China, however, LBMs changed more slowly. In Hong Kong, the LBMs established in each district were the size of huge supermarkets. On the ground floor of these multistory city markets were dozens of individual stalls, with vendors selling live poultry or fresh fish, freshly slaughtered livestock (mainly pork), and a vast variety of fresh vegetables.

Figure 5

A live bird market in Hong Kong in the mid-1970s. Chickens and ducks were often caged together.

It was common to find ducks and chickens in the same cage. Cages were always stacked on top of each other, five or six high. Although each cage had a water trough and litter pan, spillage always occurred when the cages were opened, providing an opportunity for viruses to spread from cage to cage. Furthermore, although the market areas were regularly hosed down with water and kept quite clean, individual stall owners rarely, if ever, emptied or cleaned their cages. In Hong Kong LBMs, stall owners obtained their birds from two central wholesale markets that received land-based birds by truck from farms in southern China or the new territories of Hong Kong and aquatic birds by boat from coastal duck farmers in China.

LBMs contain a wide variety of both land-based birds and aquatic birds. The land-based birds were mainly varieties of chicken (red, yellow, white, and silky chicken), with some quail and pigeons and the occasional pheasant, chukar, and guinea fowl. The aquatic birds included domestic ducks of various varieties (white, Peking, khaki Campbell, and Muscovy), a few wild ducks (mainly mallards), and a wide range of white, gray, and black geese. Customers shopping for fresh chicken or other poultry would survey the birds and sometimes handle them to feel their size and plumpness. The stall owner would then process the chosen birds on the spot, killing, defeathering, and eviscerating the birds in the back of the stall. Although the splattering of blood, feathers, and entrails was kept to a minimum, it was inevitable that aerosols (i.e., airborne particles) were produced.

Kennedy Shortridge established a laboratory for influenza virus surveillance and research at the University of Hong Kong in 1976, and by 1977, we had found a plethora of influenza A virus subtypes in the LBMs. All the virus isolates came from apparently healthy birds—mainly ducks, but also some chickens, quail, and geese (18). We detected viruses in up to 10% of the birds: Half of the isolates were influenza virus, and the rest were Newcastle disease virus (19). Different subtypes of influenza viruses were isolated; some were related to the Hong Kong H3N2 of 1968, but most were influenza viruses from avian sources. Some viruses were closely related to influenza viruses from wild ducks in Canada, suggesting global spread. Most of the influenza viruses we found were from cloacal samples rather than respiratory tract samples, in keeping with the preferred replication niche of influenza virus in birds.

Shortridge continued the study for a second year. He not only confirmed the findings but also detected nearly all known subtypes of influenza viruses, including those found in humans, horses,

and swine. Of the 136 influenza viruses isolated, 126 were from domestic ducks originally from China (19). Thus, counterparts of influenza viruses found in wild ducks in the Americas were found in domestic ducks in China, suggesting that ducks constituted a global reservoir of influenza virus and further supporting the idea that LBMs are a hotbed for the mixing of influenza virus genomes, the development of new influenza viruses, and possible transmission of influenza virus to humans.

Studies of pigs at a slaughterhouse in Hong Kong in 1976 were equally rewarding. From 356 nasal swabs obtained from apparently healthy pigs, we isolated 11 influenza viruses. All were H3N2: Six were antigenically identical to the H3N2 1968 pandemic virus, and five were similar to the H3N2 Hong Kong variant A/Victoria/3/75 that was circulating in humans that year. By 1976, pandemic H3N2 influenza virus had disappeared from humans, yet it was still circulating in pigs (20). Our study in Hong Kong also showed that the currently circulating influenza virus in humans had spread to pigs.

TRANSMISSION OF BIRD FLU TO HUMANS

When a three-year-old boy died of H5N1 influenza virus on May 21, 1997, in Queen Elizabeth Hospital in Hong Kong, it received our undivided attention. The child had been perfectly healthy before the sudden onset of disease. Five days after admission, he had high fever and pneumonia and died.

At the time of the boy's death, H5N1 infections had broken out on three poultry farms in Hong Kong; the death rate among the birds was 70–100%. However, the child who died did not have known contact with those farms. H5N1 viruses had never been reported to infect humans, which made this first recorded case a cause for enormous concern. Like my colleagues, I thought that this human infection might be a pandemic warning (21). Fortunately, the H5N1 virus did not spread to the boy's family or to the staff taking care of him, and there were no immediate additional cases. However, six months later in November and December 1997, 17 more people became infected with H5N1 influenza and five died. When Dr. Nancy Cox from the CDC informed me about the surge in human cases of H5N1 in Hong Kong, I immediately telephoned Ken Shortridge and asked if I could join him there.

On arrival in Hong Kong, I knew exactly where to search for the origin of the human H5N1 cases—in the LBMs. However, the University of Hong Kong laboratories were understaffed, as Hong Kong was preparing for the transfer of the territory from Britain back to China. Because many of the young influenza virologists in China and Japan at that time had trained in my laboratory at St. Jude, I called them and asked that they come to Hong Kong and bring all the equipment and supplies needed for influenza surveillance, including fertile chicken eggs. The initial team included Shortridge, Dr. Peng Gao from China, Drs. Toshi Ito and Ayato Takada from Japan, and Dr. Yoshi Kawaoka and me from St. Jude. Since we were going into the LBMs, which were the likely source of the H5N1 influenza viruses, we vaccinated ourselves by puffing inactivated H5N1 virus from the first human case up our noses.

In 1997, there were more than 1,000 LBMs in Hong Kong. With approval of the Department of Agriculture and Fisheries, Ken and the international team concentrated our studies on the six large municipal markets in Kowloon and in the central districts of Hong Kong, including the markets that the patients' families had visited prior to developing influenza. These included Central Market and Smithfield on Hong Kong Island. At Central Market, the typical species were present: chickens, ducks, geese, quail, and pigeons, with a few guinea fowl, chukar, and pheasants. All the birds were healthy.

Two days after the first samples were injected into fertile eggs, influenza viruses were isolated from the apparently healthy chickens and ducks from the Central Market LBM. The very first

virus was a surprise—it was H9N2 not H5N1. As the tests went on, H5N1 and H9N2 were the major influenza viruses present. The significance of finding H5N1 was obvious, but we did not initially realize the relevance of H9N2.

We reported immediately to the health, agricultural, and fisheries departments that H5N1 was present in the LBMs. Their question was whether this was indeed the virus that was spreading to humans with severe and even fatal consequences. To answer that question, we sequenced the genomes of the H5N1 viruses from the LBMs and those from the patients: The viruses were essentially identical. Remarkably, H5N1 influenza virus was present in all six of the LBMs, with up to 20% of the chickens infected (22). We knew we had identified the most likely source of the human influenza outbreak.

Dr. Margaret Chan, the head of the Health Department in Hong Kong during this period, convened a panel of senior staff of all the relevant government departments, including health, environmental services, and agriculture and fisheries. She also included senior academic scientists and representatives from WHO laboratories in Geneva, Atlanta, and Memphis. This impressive lineup showed Chan's recognition of the importance of having all interested parties on board to draw together all available information and develop a plan.

After reviewing the data, Dr. Chan and the panel recommended to the health secretary that all LBMs be closed and all poultry in Hong Kong be culled and buried. This was a huge task and an enormous societal disruption, but the impact was dramatic. There were no more human cases of H5N1 avian influenza, and that particular strain of H5N1 virus was stamped out. The LBMs in Hong Kong were closed for seven weeks while strategies were devised to reduce the risk of reintroducing H5N1 virus. The strategies included cleaning and disinfecting all markets, implementing one cleaning day per month, when all stalls were empty of poultry and cleaned. Wild waterfowl were banned from the markets, and domestic waterfowl were sold at a separate market as killed and processed birds. The farms providing land-based poultry were inspected, and each shipment was inspected following arrival in Hong Kong.

These strategies kept H5N1 out of the LBMs in Hong Kong from late 1997 until 1999, when avian influenza virus was detected once again, and the whole process of LBM closure, depopulation, cleaning, and compensation was repeated. One surprising finding in the Hong Kong LBMs in 1997 was the absence of dead birds. Yet, when the H5N1 isolate was inoculated experimentally into chickens (in biosecurity facilities), it killed 100% of the birds. We thought that the cocirculation of H9N2 and H5N1 influenza viruses in the LBMs and the acquisition of H9N2 gene segments by H5N1 virus were the key events in transmission to humans (23). H9N2 was widespread in poultry in Asia and occasionally transmits to humans, causing mild respiratory disease (24).

The number of H5N1 isolates from the waterfowl market increased from 4 in 1999 to 18 in 2000 and much higher in 2001, indicating that waterfowl were a major source of H5N1 virus. Farms in coastal Chinese towns in Guangdong, Guangxi, Fujian, Zhejiang, and Shanghai provinces were sampled from 1999 to 2002, and these apparently healthy ducks were infected with H5N1 viruses, showing that those viruses were widespread (25).

H5N1 virus had become entrenched in domestic ducks and then reinfected wild ducks, which contributed to its spread. During the winter of 2003–2004, the H5N1 virus spread across Asia and eventually to Europe. The virus had acquired several new internal components and was designated genotype Z. It still contained the original hemagglutinin gene from goose isolates in Guangzhou but had acquired its seven other genes from aquatic birds in Asia (26).

The H5 influenza viruses have a high propensity to reassort with other influenza A viruses in nature. H5NX influenza viruses have been detected in wild and domestic birds, with occasional spillover into humans. It was not until September 2014 that H5N8 spread to the Americas. Soon after its arrival, the H5N8 virus reassorted with influenza viruses present in wild waterfowl to

produce H5N1, H5N2, and H5N8 strains. Despite warnings about the need for high levels of biosecurity, H5N1, H5N2, and H5N8 successfully infected more than 220 poultry farms in the United States. However, no human infections were detected. The great concern was whether these H5 viruses would become established in the wild ducks in Canada and spread to domestic poultry during their southern migration. However, there were no outbreaks of H5 influenza in poultry farms in North America in the years immediately following the 2014–2015 outbreaks.

The apparent disappearance of the lethal H5 influenza viruses from wild aquatic birds at that time is a mystery. My research group has been surveying influenza in wild ducks in the United States and Canada for more than 40 years, and highly pathogenic H5 or H7 influenza viruses have not been detected (27). We think that wild ducks have an as-yet unknown mechanism to keep the killer H5 and H7 influenza viruses out of their breeding areas. However, less virulent versions of H5 and H7, as well as almost all influenza viruses found anywhere in the world, are regularly found in apparently healthy young ducks.

In 2022, another H5N1 reassortant, HP H5N1, emerged in Eurasia and was spread to poultry in Newfoundland, Canada, presumably by migratory waterfowl. Following this introduction, it became widespread in poultry in North America. This H5N1 strain has become a global problem, as it is established in wild aquatic waterfowl, where it can cause neurologic disease and death. Predatory birds, like eagles, feeding on HP H5N1–infected waterfowl also die of infection. It is a virus of major concern because it has spread to domestic poultry worldwide and to multiple species, including foxes, seals, and porpoises. Most concerning is the capacity of some H5N1 reassortants to cause neurologic diseases in ferrets, but to date, it has not acquired the ability to spread from ferret to ferret. If reassortants of this H5N1 acquire the capacity to spread among humans, it will be catastrophic.

A second type of avian influenza (H7N9) emerged in Shanghai in 2013. The initial virus was not highly virulent in chickens, but in the first wave of H7N9 infections in humans in the Shanghai region, 135 people were infected and 45 died. When the LBMs were closed, the number of human cases fell dramatically, again emphasizing the role of LBMs in the genesis of influenza viruses with potential to spread to humans. Laboratory examinations of the virus showed a remarkable similarity to H5N1. Six of the eight gene segments of the H7N9 virus had come from the H9N2 influenza virus. The hemagglutinin gene came from a wild duck influenza virus, and the neuraminidase gene from a different wild duck influenza virus (24). Thus, the importance of H9N2 influenza viruses in the emergence of potential human pandemics was again demonstrated. During each wave of this H7N9 virus, from 2013 to 2018, there was a peak of human infection. During this period, there was a total of 1,623 human cases, resulting in 620 deaths. To date, the H7N9 virus has not spread to countries adjacent to China, but humans infected in China have taken the virus to Taiwan. In China, a nationwide poultry vaccination program against the H7N9 influenza virus was successful in controlling its spread. This virus has not been detected in poultry or humans since 2021.

THE BENEFITS OF THE EIGHT-PLASMID REVERSE-GENETICS SYSTEM FOR RAPID VACCINE PRODUCTION

While working with the influenza group at St. Jude as a postdoctoral fellow, Dr. Eric Hoffman developed the eight-plasmid reverse-genetics system, which allows rapid recovery of influenza vaccine seed viruses (28, 29). One problem with the availability of traditional influenza vaccines is that it takes up to six months to produce the vaccine after the WHO makes its recommendation. This process involves preparing reassortants containing gene segments encoding the hemagglutinin and neuraminidase of the WHO-recommended influenza virus and as many as six segments from

the A/PR/8/34 (H1N1) virus, which provides growth potential in chicken embryos. This process is followed by growth in embryonated chicken eggs or mammalian cells, purification, production of subunits, standardization, and safety testing.

After the emergence of H5N1 avian influenza in Hong Kong in 2003 and infection of two family members, one of whom died of infection, the WHO issued a pandemic alert. In response to the alert, Dr. Richard Webby and the team at St. Jude used the eight-plasmid reverse-genetics system to produce a vaccine seed stock suitable for human use in less than four weeks (30). The H5N1 influenza virus is highly pathogenic; it kills 100% of chickens and chicken embryos and has the potential to spread to humans. Therefore, all the work was done in biosafety level-3 (BSL-3) facilities. To attenuate the virulence of the H5N1 virus, the series of basic amino acids at the cleavage site of the hemagglutinin was replaced with the sequence from an avirulent H5 virus. The modified hemagglutinin and the N1 neuraminidase genes were then cloned individually into vector pHW 2000. The two resulting plasmids and the six plasmids encoding the remaining proteins of PR8 were transfected into WHO-approved Vero cells (i.e., kidney cells derived from an African green monkey), under good manufacturing practice (GMP) conditions, to rescue the vaccine seed virus. The resulting vaccine virus replicated to high titers in chicken embryos (hemagglutinin titers of 1,024 to 2,048), was nonpathogenic in chickens and ferrets, and was stable after 16 passages in embryonated chicken eggs (30).

To prepare seed stock vaccines for humans in less than four weeks, the following requirements were in place: (*a*) permits to ship highly pathogenic influenza viruses, (*b*) access to a BSL-3 facility, (*c*) WHO-approved Vero cells, (*d*) a GMP facility, and (*e*) approval of a genetically modified vaccine for use in humans (US Food and Drug Administration).

Although the H5N1 influenza virus did not transfer among humans in 2003, it is noteworthy that the H5N1 virus continues to evolve and spread in wild aquatic birds in 2022, causing catastrophic lethal outbreaks in domestic poultry in North America. It may yet shuffle its genes to cause a pandemic in humans!

ORIGIN OF THE 2009 H1N1 INFLUENZA PANDEMIC

In 2009, influenza experts around the world were blindsided when an influenza virus emerged that, in all of our tests, looked alarmingly like the H1N1 1918 Spanish influenza. Mother Nature had indeed been preparing this surprise for many years, and it illustrated the importance of the wild bird reservoir of influenza viruses, pigs as an intermediate host, and the role of reassortment in the genesis of influenza pandemics in humans.

One of the first events that led to the genesis of the 2009 H1N1 pandemic virus was the transmission of an avian H1N1 influenza virus to pigs in Europe in 1979 (31). These avian-like H1N1 viruses subsequently reassorted with human H3N2 viruses that were present in Italian pigs (32). The reassorted H3N2 viruses, which contained internal gene segments from the European avian virus, had the capacity to transmit irregularly to humans, thereby causing mild influenza, but the viruses did not spread (33, 34).

The next contributor to the 2009 H1N1 pandemic virus was detected in 1998, when a new influenza virus emerged in pigs in the United States. This virus caused severe infection in pigs in Texas, Minnesota, and Iowa, and it replaced the descendants of the 1918 swine influenza virus that had caused influenza in pigs for almost a century. When we investigated its composition, we found a triple-reassortment virus—it had three gene segments from human H3N2 influenza viruses (PB1, HA, NA), three from the classic 1918 influenza virus (NP, M, NS), and two from an American wild duck influenza virus (PB2, PA) (35). The H1N1 virus that emerged in Mexico

in 2009 contained five genes from the triple American swine influenza virus (PB2, PB1, PA, NP, NS), two from the European swine influenza virus (NA, M), and the hemagglutinin from pigs in Mexico. We do not know where all these parent viruses met. The simplest explanation is that European and American pigs were probably imported into Mexico.

Despite its similarity with the 1918 H1N1 Spanish influenza, the 2009 H1N1 pandemic virus was milder than other pandemics, with an estimated 284,000 deaths globally. The WHO was taken to task about overestimating the severity of the 2009 H1N1 pandemic. However, such criticism was unjustified, as we do not know how to accurately predict the severity of each pandemic. In these cases, it is better to be safe than sorry.

FUTURE DIRECTIONS

The global impact of a new infectious disease has been fully illustrated by COVID-19. The world is still poorly prepared for coping with such events. At the time of this writing, there have been more than 625 million cases of COVID-19 and 6.57 million deaths globally. The one positive outcome of the COVID-19 pandemic has been the rapid development of mRNA vaccines that are safe and efficacious. However, more than one-third of people declined to be vaccinated, a problem that must be solved.

Turning back to influenza, the following key issues must be resolved to ensure an improved outcome when the next influenza pandemic occurs: (*a*) development and safety testing of a universal influenza vaccine, (*b*) global availability of vaccines, (*c*) strategies to convince so-called anti-vaxxers that vaccines save lives, (*d*) closure of all LBMs and live animal markets, (*e*) accurately predicting which influenza viruses have pandemic potential and determining their severity, and (*f*) development of poultry and pigs that are genetically resistant to influenza infection. All these goals are achievable, but ethical issues must be considered. A universal vaccine for influenza is already being tested in clinical trials, and the wisdom of closing LBMs was demonstrated in Hong Kong and Shanghai after the emergence of H5N1 and H7N9 influenza viruses. However, alternative food supplies must be available. Additional information is needed to predict pandemics and their severity. The genetic basis of resistance to influenza will be elucidated, but that work will require genetically modified intermediate hosts, which will pose an ethical issue. Perhaps the most difficult task will be to convince people of the value of vaccination, even if vaccines are widely available.

CONCLUDING STATEMENT

Despite my initial chagrin when Frank Fenner decided I would work on influenza, it turned into a superbly fulfilling lifelong journey. A stained-glass window depicting the natural history of influenza viruses (**Figure 6**) resulted from a walking holiday along Hadrian's Wall in England. Marjorie and I had stopped for lunch at a pub and saw the superb work of a local stained-glass artist, Jenny Hammond. We visited the artist, and after sending her multiple research papers on influenza, we commissioned the stained-glass window. The window, which formerly adorned our home, now resides in the gallery at St. Jude and appeared on the cover of the journal *Emerging Infectious Diseases* in 2016.

In recognition of my contributions to influenza research, I was awarded a Fellowship of the Royal Society and the US National Academy of Sciences. One of the greatest moments in my life occurred in 1975, when I received an invitation from the WHO for St. Jude to join the GISN, the predecessor of today's Global Influenza Surveillance and Response System, and become a WHO Collaborative Center on the Ecology of Influenza in Lower Animals and Birds. This invitation opened doors to collaborations all over the world and continues today, with the center now directed by Dr. Richard Webby.

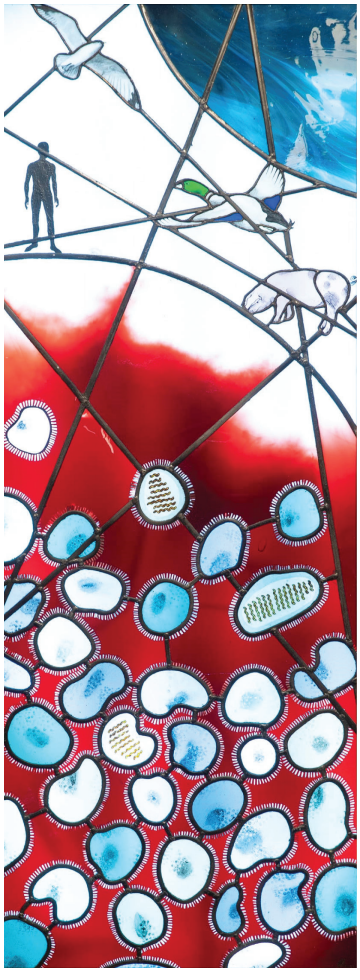

Figure 6

Stained-glass window depicting the evolution of influenza viruses from the aquatic bird reservoir (36). Artist: Jenny Hammond.

DISCLOSURE STATEMENT

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