

FROM THE GREAT BARRIER REEF TO A “CURE” FOR THE FLU

tall tales, but true

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ABSTRACT How we discovered that sea birds on the Great Barrier Reef are riddled with influenza viruses, and how one of these led to a new drug now being used in the battle against the flu.

I WAS IN SYDNEY, attending a cocktail party thrown by the pharmaceutical company Hoffmann-La Roche to launch its new anti-influenza drug Tamiflu[®].

As one does on these occasions, I got talking to a young lady from the marketing division of Roche, who asked, “Why are you here? What is your involvement with Tamiflu?”

I said, “If I told you, you wouldn’t believe me.”

“Try me,” she replied, so this is what I said:

One summer I went to a deserted coral island on Australia’s Great Barrier Reef with friends and colleagues. One of these was Adrian Gibbs, a veritable giant of a man, skilled in catching airborne objects. He caught a white-capped noddy tern, stuck a cotton wool swab up its backside, from which we isolated an influ-

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enza virus. The neuraminidase from this virus was sent into space to crystallize on the Soviet space station *Mir*, and from the crystals the structure of the virus's neuraminidase was discovered. This information was then used by Gilead Sciences in California to create the anti-influenza drug now called Tamiflu which was taken over by Roche and is what you are now toasting at this party.

"Oh," she exclaimed, and went off to talk to someone else. Clearly she did not believe a word I said.

The story started in the late 1960s, when Rob Webster and I were walking along a sandy beach on the south coast of New South Wales in Australia. We noticed that every 10 to 15 meters or so there was a dead mutton bird (shearwater) washed up on the beach. Knowing that terns in South Africa had been killed by an influenza virus in 1961, we wondered if these birds, too, had died from a flu infection.

Avian influenza, or fowl plague, was the first influenza virus to be isolated, in 1900, but fowl plague was not recognized as being caused by a type A influenza virus until 1955, years after the first human influenza virus was isolated in 1933. Then, in 1956, two other avian influenza A viruses were isolated from domestic ducks, following which an increasing number of influenza viruses were isolated from domestic chickens, turkeys, ducks, quail, pheasants, and pigeons. It was believed, however, that these bird viruses all originated from human strains of influenza and had got into the domesticated birds because of their close proximity to people.

Apart from the one incident in South Africa in 1961, where terns were found dying from influenza, there were no reports of influenza viruses being isolated from wild birds, and no accounts of attempts to do this. Two people, however, pushed this idea: Martin Kaplan of the World Health Organization, and Helio Pereira, who headed the Department of Virology at the National Institute for Medical Research, Mill Hill, London.

I was skiing with Pereira one winter in Argentiere, France, and we talked about trying to isolate flu from wild birds. We toyed with the idea of doing this on the coral islands of the Great Barrier Reef. Why there? Can you think of a more unlikely place to look for flu? Beautiful islands in an azure sea, hot sand, a baking sun, and a warm coral lagoon. What better place to do flu research! After skiing, we visited Martin Kaplan at the WHO in Geneva, and after a good deal of smooth talking managed to wheedle \$500 out of him to help pay for an expedition to the Reef.

This was just as well, as my Head of Department at the Australian National University, when asked for funds for an expedition to look for flu on the Great Barrier Reef, said, "Laver is hallucinating." He also said that in any case I wouldn't be able to catch the birds. But I knew that thousands upon thousands of mutton birds or shearwaters nested on the coral cays of the Reef in burrows in the

**FIGURE 1**

An aerial view of Tryon Island, an uninhabited coral cay in the Capricorn group at the southern end of the Great Barrier Reef, about 45 miles (72 km) off the Queensland coast (lat 23°15' S, long 151°47' E). Many thousands of birds nest on these islands during the breeding season, including shearwaters, which nest in burrows in the ground.

sand, and that all you had to do to catch these wild, free-flying sea birds was to bend over and pick them up.

As it turned out, Pereira wasn't able to come to Australia at the time the mutation birds were nesting so I went with an assistant, Alice Murdoch, for three weeks in December 1969. We set up camp on the uninhabited coral cay Tryon Island, 50 miles off the coast of Queensland (Figure 1). We collected sera from 201 shearwaters and tested these on the spot in double immuno-diffusion tests with a preparation of influenza type A ribonucleoprotein (RNP), which had been made in the lab before coming to the island. All type A influenza viruses have the same RNP antigen, and following infection, antibodies to RNP can be found in the sera of infected individuals.

To our great surprise, we saw faint precipitin lines in some of the gel diffusion plates. These were too weak and fuzzy to form the basis of a publication, but they were strong enough to encourage further testing of the sera for flu antibodies. Back in the lab the question was which viral antigen to test the sera against. We ruled out hemagglutination inhibition tests, the ones most people would have used, because sera often contain high levels of non-specific inhibitors of flu hemagglutinin, and these might have muddied the waters. Instead, we chose to look for the ability of the shearwater sera to inhibit influenza virus neuraminidase. But which neuraminidase? Several antigenically distinct influenza type A neuraminidase subtypes were already known, and we had to guess which was the right one to use.

A previous experiment by Webster pointed the way. In 1967, with Pereira and Bela Tumova, Webster had found that some avian influenza viruses possessed neuraminidase antigens that were immunologically similar to that of the "Asian" (1957) H2N2 strain of human influenza. We mixed samples of influenza virus neuraminidase of the human 1957 N2 subtype with sera from the shearwaters on Tryon Island and looked for inhibition of neuraminidase activity. We tested about 30 sera, and in each case the test gave the familiar bright red color produced by active neuraminidase. And then, suddenly, we got one test that was completely colorless. Something in that bird's serum had completely eliminated the activity of the neuraminidase. It was one of those rare "Eureka" moments that make scientific research so exciting.

It didn't take long to prove that this inhibition of the neuraminidase was due to specific antibody to human influenza virus neuraminidase of the N2 subtype. This led to the inescapable conclusion that this shearwater bird had been infected sometime in the past with a virus possessing N2/1957 neuraminidase. Out of the 201 shearwater sera collected on Tryon Island in December 1969, and 119 sera from neighboring Heron Island, collected by my research assistant Catherine Dasen, a total of 18 had antibody that inhibited N2/1957 neuraminidase.

It then became imperative that we should try to isolate live virus from the birds, and a number of expeditions were organized to do just that. At the end of 1970, Webster and I collected 172 sera and tracheal swabs from shearwaters on Phillip Island near Melbourne, Australia, and a total of 321 sera and 148 tracheal swabs from a variety of pelagic birds on the Great Barrier Reef. We found a number of sera with antibody to "Asian" (N2/1957) neuraminidase, confirming the previous findings, but no virus was isolated from any of the tracheal swabs.

Then, in 1971, another expedition was undertaken to Tryon Island, to collect 201 tracheal swabs from shearwaters nesting on the island. During the day, the shearwaters spent their time out at sea fishing, returning to their nesting burrows when the sun went down. The daily routine for us, therefore, was to swim and sunbake during the day and then, following the traditional sherry party on the beach at dusk, to spend two hours or so catching and swabbing the birds before returning to camp for dinner prepared by our excellent cooking team. The swabs were then stored in liquid nitrogen before being transported back to the lab.

Finding volunteers to undertake the arduous work involved was not difficult. Small children who came along were particularly helpful, as their light weight enabled them to walk among the burrows and capture the birds without breaking through and damaging the nests (Figure 2).

Material from the swabs was inoculated into 10-day-old embryonated chicken eggs, and after two days' incubation at 37 degrees, the allantoic fluid around the embryo was harvested and tested for influenza virus. Most of the eggs were negative, but you can imagine our excitement when we eventually found one egg full of an influenza type A virus, which had come from the trachea of a completely healthy shearwater bird nesting on Tryon Island, remote from human



FIGURE 2

Dr. Walter Dowdle, CDC Atlanta, collecting serum samples and swabs from White Capped Noddy Terns on Lady Musgrave Island on the Great Barrier Reef. The children who caught the terns are (left to right) Judith Skeat, Rowan Laver, Tim Perry, and Penny Laver.

habitation. This finding suggested that the natural hosts of influenza A might be wild aquatic birds, and that many more type A viruses might exist in these pelagic bird populations.

Human influenza is, of course, a respiratory virus, and it is therefore understandable that we looked for viruses in the bird's respiratory secretions. However, Webster then found that in domestic ducks, avian influenza viruses replicated in cells lining the bird's gut, rather than in the lungs or trachea, and he suggested that it might be a better idea to swab the other end of the bird, and to collect material from the cloaca rather than from the trachea. So in later expeditions to the Reef we did just that, and this eventually led to the isolation of a number of influenza A viruses from the Reef birds, some of which had not been characterized previously.

One virus was of particular interest and importance. The 70th cloacal swab collected by Gibbs from a white capped noddy tern on North West Island in December 1975 yielded a type A influenza virus of subtype H11N9. Type A influenza viruses exist in a number of subtypes with serologically quite different surface antigens, hemagglutinin (H) and neuraminidase (N). So far, 15 H and nine N subtypes have been discovered, and viruses can be classified into H1N2, H3N2, H3N6, and so on. Viruses of the H1N1, H2N2, and H3N2 subtypes are known to have caused human flu pandemics.

Because N9 neuraminidase had not previously been described, we were curi-

ous to examine this in more detail. But first pure N9 neuraminidase had to be isolated from the virus. To do this, the N9 neuraminidase of the H11N9 noddy tern virus was first segregated into a re-assortant virus, H1N9, using Webster's famous recipe: "Antigenic hybrids of influenza A viruses with surface antigens to order." It was much easier to purify the neuraminidase from the re-assortant virus than from the parent. N9 neuraminidase "heads" were then isolated from the H1N9 virus, purified, and crystallized using a high-salt phosphate buffer recipe suggested by Peter Colman. How these neuraminidase crystals were used in the creation of the anti-viral drug, Tamiflu, will now be described.

In 1978, I had crystallized N2 neuraminidase from the human H2N2 influenza virus, and Peter Colman and his colleagues had determined its three-dimensional structure by X-ray crystallography. This showed the enzyme to have a conserved catalytic site, which meant that if a neuraminidase inhibitor, a "plug drug," could be developed as an anti-viral drug for viruses with N2 neuraminidase, this drug should be effective against all influenza viruses—even those that had not yet appeared in man.

From a knowledge of the three-dimensional structure of the catalytic site of N2 neuraminidase, Mark von Itzstein and his colleagues designed and synthesized a potent and specific inhibitor of the enzyme. This inhibitor, known as Relenza, is now being used worldwide to treat influenza infections. However, Relenza is not orally bioavailable; it is a powder that has to be puffed into the lungs, a procedure that does not appeal to many people. Gilead Sciences in California and BioCryst pharmaceuticals in Alabama therefore set out to create an influenza virus neuraminidase inhibitor that could be administered as a pill, or a suspension or solution that could be swallowed.

The story now shifts back to the Great Barrier Reef birds. The virus from Gibbs' noddy tern on North West Island, possessed neuraminidase of the N9 subtype, which formed the most beautiful large crystals that diffracted X-rays to 1.9 Å resolution. N9 crystals were, in fact, the best influenza neuraminidase crystals ever obtained. In an attempt to get N9 crystals of even bigger size and of higher quality, we grew them in space in conditions of microgravity. The first crystals were grown on the American space shuttle, but these experiments came to an end following the *Challenger* accident. I then travelled to Moscow and arranged to have N9 neuraminidase crystals grown on the Soviet space station, *Mir*. Growing crystals in space for X-ray diffraction analysis was a new experience for the Russians, and the appropriate apparatus for this had to be designed and built. This was accomplished in a remarkably short time, and on June 8, 1988, N9 neuraminidase was put on a rocket and sent into space to crystallize on *Mir* in microgravity.

Three months later the N9 crystals were returned to earth and a sample sent to BioCryst and the University of Alabama for X-ray analysis. The results showed that the crystals grown in space were no bigger, and of only slightly higher quality, than N9 crystals grown on earth. We therefore abandoned further attempts

to grow crystals in space, and although the data set from the *Mir* N9 crystal was used by Biocryst in their initial drug design experiments, further work used N9 crystals grown in Australia, which is a long way away from the United States, but not yet in space. The influenza virus neuraminidase inhibitor developed by Bio-Cryst has not so far been approved for clinical use.

Gilead Sciences in California also used N9 neuraminidase crystals grown in Canberra in the design and synthesis of a carbocyclic, orally bioavailable neuraminidase inhibitor, now marketed under the name Tamiflu. Tamiflu is administered as a pill for adults or a suspension for children, and is being used worldwide for the treatment of influenza infections. It is, in fact, the best defense we will have if the H5N1 bird flu virus, currently causing havoc in Southeast Asia, ever acquires the ability to spread in the human population and kill people as efficiently as it kills chickens. Tamiflu might, of course, have been developed using N2 neuraminidase crystals, but when Adrian Gibbs caught the noddy tern that yielded the N9 crystals, it made the job a whole lot easier.

So the story I told the young lady at the Tamiflu launch celebration was indeed more or less true. But the isolation of influenza viruses from birds on the Great Barrier Reef was of as much importance in understanding the ecology of influenza as it was in the design of anti-viral drugs. It led to Rob Webster's demonstration that healthy wild ducks on lakes in Northern Canada are infected with every known subtype of influenza type A virus, and that these viruses can be isolated, not only from the birds themselves, but also from the lake water on which they swim. Further work by Webster and others has now established that wild aquatic birds are indeed the natural hosts of type A influenza, and probably have been for many millions of years. They act as a reservoir of antigens for the formation of "new" human pandemic influenza viruses. Knowing this now gives us a better understanding of how to control pandemic influenza.

ADDITIONAL READING

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