problems[5]. Thus, another strategy needs to be explored for neonatal seizure therapy.

Because NKCC1 is a key molecule in determining excitatory GABA actions in immature neurons[2,4,5], Dzhala *et al.* examined the molecule as a potential target for anticonvulsant therapy in the immature brain[1]. The authors tested the reasonable hypothesis that inhibition of NKCC1 activity would reduce the concentration of intracellular chloride in immature cortical neurons—which in turn could reduce $GABA_A$ receptor–mediated excitation, or convert the GABA response to inhibitory.

To address this hypothesis, the authors first confirmed that NKCC1 was highly expressed in both neonatal rat and human cortex, whereas KCC2 expression in neonatal rats was only 5–15 % of the adult expression levels. To examine possible therapeutic applications of blocking NKCC1, they used bumetanide, a diuretic drug with a unique selectivity for inhibition of NKCC1 over the related KCC2, if used at low doses[2]. They found that NKCC1 blockade by bumetanide inhibited cortical seizure activity in neonatal rats both *in vitro* and *in vivo*. Importantly, this inhibition was observed at doses that have already been extensively tested in human neonates in diuresis studies[6]. In contrast, bumetanide did not depress epileptiform activity in mature neurons, in which NKCC1 protein is present at less than 10% of that in neonatal tissue.

The effectiveness of bumetamide in inhibiting epileptic activity in these different tissues corresponded well with its effectiveness in reducing intracellular chloride concentration (estimated from the GABA reversal potential in experiments using patch-clamp recordings). To convincingly show that NKCC1 was indeed the molecular target of bumetanide's anticonvulsant effects, the authors tested bumetanide in hippocampal slices from neonatal NKCC1 knockout mice. The drug did not suppress epileptiform activity in these slices, although it was effective in control slices in which NKCC1 was intact.

During development, upregulation of KCC2 expression underlies the switch of GABAergic responses from excitatory to inhibitory[3]. Inhibition of NKCC1 is a feasible therapeutic strategy (**Fig. 1**) because of the availability and reversibility of bumetanide.

Another possible strategy is molecular knockdown of NKCC1 function, or upregulation of KCC2 expression and function. That strategy may achieve more exclusive neuronal effects, but is still far from clinical trials. Although there is some concern about just how specific bumetamide[2], like other diuretic compounds, is, Dzhala *et al.* have clearly shown that bumetanide targets NKCC1 by examining the drug's anticonvulsant actions in NKCC1 knockout mice.

NKCC1 is also expressed in astrocytes, unlike KCC2, which is strictly expressed in neurons[2]. Bumetanide's anticonvulsant effects were blocked by $GABA_A$ receptor antagonists and its cellular target was apparently the GABAergic synapse. Nonetheless, astrocytic NKCC1 might be involved in the control of the extracellular space, which can affect neuronal synchronization. Other diuretic drugs have suppressed epileptic activity in adult human brain by altering the extracellular space[7], and so whether bumetamide affects NKCC1 in glial cells and alters that milieu should be clarified.

Dzhala *et al.* also provide important data to help direct subsequent clinical trials. They investigated developmental changes in NKCC1 expression in human brains, showing that it peaks right before and after birth. One would therefore predict that bumetanide could treat seizures in the human neonate.

Manipulation of chloride transporters may also be effective in treating some specific adult epilepsies. Several pathological conditions in adults seem to involve accumulation of excess chloride in neurons as well as disruptions in the balance between NKCC1 and KCC2. For instance, neurons from individuals with adult temporal lobe epilepsy who have intractable seizures accumulate chloride such that $GABA_A$ receptor activation is excitatory[8]. Moreover, epileptic discharges in human cortex with focal malformation are initiated by a synchronizing mechanism that relies on depolarizing GABA actions[9].

Corresponding to these findings, altered expression of NKCC1 and KCC2 in the hippocampus of individuals with epilepsy[10], and downregulation of KCC2 expression in human epileptogenic focal cortical dysplasia tissue[11], have also been shown recently. Furthermore, a specific upregulation of NKCC1, without any accompanying changes in KCC2, has been reported in the dentate gyrus of the kindling model—seizures modeling anatomical and functional correlates of complex partial epilepsy—suggesting an increase in intracellular chloride concentration and a resultant reduction in GABAergic inhibition[12].

A collapse of chloride homeostasis causes reduced GABAergic inhibition, and conversion to GABAergic excitation. This could have an important role in the genesis and maintenance of epileptiform discharges that warrants further investigation. Therefore, strategies to inhibit NKCC1—and perhaps to facilitate KCC2 activity—may also be useful in some adult epilepsies. In the meantime, Dzhala *et al.* have set the stage for clinical trials of bumetamide to treat seizures in newborns.

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Extinct 1918 virus comes alive

Robert A Lamb & David Jackson

The 1918 'Spanish' flu that killed 20–50 million people has been recreated from its gene sequence. The virus truly is a nasty beast.

The complete nucleotide sequence of the 1918 influenza virus genome has been determined[1] and the virus recreated from its gene sequences[2]. This virus caused the most deadly outbreak of infectious disease in recorded history, the 'Spanish' influenza pandemic of 1918 that killed 20–50 million people worldwide. Why was this virus so virulent? In molecular terms we do not know, but the pathogenicity of the 1918 influenza virus is clearly greater than the individual contributions of its separate genes.

Beginning in 1995, Taubenberger and colleagues began to obtain the gene sequences of 1918 influenza virus from preserved tissue samples and from the frozen cadaver of a victim buried in the Alaskan permafrost. Painstakingly, over the years the sequence of each gene—recovered from fragments under

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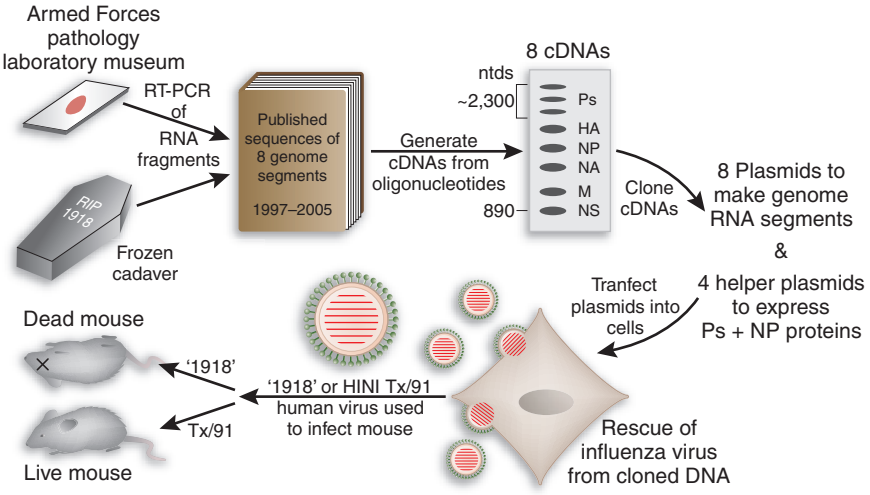

Katie Ris

Figure 1 Resurrecting the 1918 influenza virus. The nucleotide sequence of the eight RNA segments was obtained by the systematic use of random primers, reverse transcriptase and PCR. Using the published sequences, eight cDNAs were constructed from synthetic oligonucleotides and the cDNAs cloned in a plasmid such that influenza virus genome RNA can be synthesized. These eight plasmids, together with four helper plasmids that express the three polymerase proteins and the nucleocapsid protein, were transfected into cells. Virus rescued from cloned DNA was released and used to infected mice.

200 nucleotides in length—was determined[3–5]. Influenza viruses contain eight pieces of RNA, ranging in size from 890 to ~2,340 nucleotides, and each RNA segment encodes one or more proteins[6].

Now, 10 years later, Taubenberger *et al.* have completed the 1918 virus genome by publishing the sequence encoding the three polymerase subunits[1]. Nonetheless, the complete sequence of the 1918 influenza virus does not contain an obvious smoking gun for pathogenicity.

Two glycoproteins—hemagglutinin and neuraminidase—coat the surface of influenza viruses. Aquatic birds are the reservoir for influenza viruses and avian viruses with 16 antigenically distinguishable hemagglutinins and 9 antigenically distinguishable neuraminidases have been found.

Phylogenetic analysis of the 1918 virus genome sequence indicates that the 1918 H1N1 virus was derived entirely from an avian source[1], although it contains changes not found in any known avian influenza virus. However, the 1918 virus was not the result of gene mixing between existing human and avian influenza viruses, unlike what occurred for the viruses that caused the 1957 and 1968 pandemics. This exchange of avian and human virus genes is one scenario by which the highly pathogenic avian H5N1 'bird flu'—which is now killing millions of birds and some people and spreading from Asia to Europe's doorstep—could become the next human pandemic virus.

Several determinants of pathogenicity for influenza viruses have been well characterized at the molecular level[7]. Three of these concern cell entry. The first concerns hemagglutinin, which binds the virus to cells and mediates membrane fusion. Specific proteolytic cleavage of hemagglutinin is required for activation of membrane fusion. In highly pathogenic viruses, hemagglutinin contains a cleavage site that has many basic residues (cleaved intracellularly), whereas the hemagglutinin of less pathogenic avian viruses and all human viruses contains a single basic residue cleavage site (cleaved extracellularly).

The susceptibility of hemagglutinin to cleavage is thought to control the spread of virus in an infected organism, hence affecting pathogenicity. To grow most human viruses in tissue culture cells, exogenous trypsin-like protease has to be added to the medium.

The second determinant concerns the receptor for hemagglutinin, which is cell-surface sialic acid—a ubiquitous carbohydrate molecule added as the last sugar to carbohydrate chains on cellular proteins and lipids. The predominant chemical linkage of sialic acid to the sugar chains is molecularly different in avian cells and mammalian cells, and hemagglutinin can have specific amino acid residue changes in avian and mammalian viruses to aid in accommodating this difference.

Finally, the other influenza virus spike glycoprotein, neuraminidase, can also be involved in virulence. Some strains contain a neuraminidase that lacks a carbohydrate addition site that permits neuraminidase to bind plasminogen, the precursor to plasmin, a serum protease. Tethered plasminogen leads to ready cleavage of hemagglutinin by the protease at the single basic residue cleavage site, probably increasing the virulence of human influenza viruses. Other pathogenicity determinants for avian and human influenza viruses include mutations in a polymerase protein, PB2, and mutations in the interferon antagonist protein NS1.

To confound researchers, it turns out the 1918 sequencing project showed that 1918 hemagglutinin has a single basic residue in its cleavage site, generally associated with low-pathogenicity viruses[5]. Moreover, the 1918 neuraminidase is not predicted to bind plasminogen[5], the NS1 protein is not a special interferon antagonist[4] and hemagglutinin mostly binds mammalian-linkage sialic acid[8,9], even though the virus transferred directly from an avian species to humans. Thus, either the lethality of the 1918 influenza virus was not directly a property of the virus—or it holds a big surprise.

The answer is not complete, but genes encoding the polymerases may hold clues to part of the puzzle. Some of the ten amino acid changes between genes encoding avian and human polymerases identified in the 1918 analysis are also seen in the H5N1 'bird flu.' These changes are somewhat worrying because they may facilitate virus replication in human cells and increase pathogenicity. But sequence information itself can only yield so much.

The generation of influenza virus from cloned DNA is now a highly efficient process. The generation of viruses containing sequences of both contemporary human H1N1 viruses and 1918 H1N1 virus genes began to give a glimpse that the 1918 virus was special[10,11]. These studies have been conducted in inbred mice—a convenient, although imperfect, small-animal model for influenza virus.

Contemporary human influenza viruses replicate in the mouse lung without causing overt disease. Only strains of virus specially adapted to mice, by serial passage through mice brains allowing evolution of virus, cause death. A contemporary H1N1 virus carrying the 1918 hemagglutinin confers enhanced pathogenicity to the contemporary human virus[11]. This hybrid virus is nearly as efficient at killing the mouse as an adapted virus; it infects the entire mouse lung and induces high levels of macrophage-derived chemokines and cytokines. Production of these molecules results in infiltration of inflammatory cells and severe hemorrhage, similar to the post-mortem pathology of human victims of 1918 influenza[11].

The whole 1918 virus is even meaner (**Fig. 1**). The 1918 virus killed mice rapidly without needing adaptation. In two days these mice lost 13% of their body weight, whereas mice

infected with contemporary H1N1 virus did not lose weight. The mouse lung produced 39,000-fold more 1918 virus than contemporary H1N1 human virus and human lung cells released 50 times more 1918 particles than contemporary H1N1 virus. The 1918 influenza virus caused deep lung pathology: necrotizing bronchiolitis, severe alveolitis, severe alveolar edema and infiltration of inflammatory cells.

The mixing of different genes between 1918 virus and contemporary H1N1 virus showed that although there are individual roles for the three genes encoding polymerases and major roles for hemagglutinin and neuraminidase in pathogenesis, the severe 1918 virus pathogenicity is the result of its constellation of genes and is clearly greater than the contribution of its separate genes.

Perhaps the most unexpected finding was that to get the virus to grow efficiently in tissue culture, a trypsin-like protease did not have to be added to the medium. That is surprising because the 1918 hemagglutinin lacks a cleavage site containing many basic residues and the 1918 neuraminidase contains the carbohydrate site thought to block binding of plasminogen. Nonetheless, multiple rounds of 1918 virus growth is dependent on the presence of 1918 neuraminidase, and when a hybrid virus containing a 1918 neuraminidase and a contemporary hemagglutinin was made, it also grew without addition of exogenous trypsin. A novel pathogenicity mechanism now remains to be unraveled.

Some researchers had predicted that the 1918 influenza virus would not be more pathogenic than other human influenza viruses—and that its massive human toll in 1918 was the result of secondary bacterial infections and an absence of antibiotics, along with stress from World War I. The death of mice by 1918 virus makes that scenario less likely.

Many questions remain to be answered. Did the 1918 virus transfer from an unknown avian species or have all avian viruses evolved since 1918 to explain the sequence differences between current avian viruses and 1918 virus—or was there an intermediate host such as a pig? The molecular archaeology of influenza virus genomes is just beginning. Stepwise mutagenesis studies of the differences between the sequences of 1918 virus and contemporary H1N1 viruses and inoculation of these viruses into mice should yield information on the gene and specific sequences responsible for the increased pathogenicity. Furthermore, analysis of RNA abundance in infected cells may help address whether 1918 virus polymerase replicates faster than other influenza virus polymerases.

How will studying the 1918 virus help us fight contemporary flu viruses and provide insight into prevention, treatment and surveillance?

For combating influenza virus pandemics we will still depend on vaccination as the primary means of protection. The existing vaccine protects mice from 1918 influenza virus infection[12] and in the United States, a trial vaccine to H5N1 'bird flu' has been shown to be immunogenic in humans. Development of the H5N1 vaccine for general populations will depend on political and economic considerations.

The 1918 influenza virus is sensitive to several antiviral drugs, but the current H5N1 strain has shown signs of resistance to some drugs, including amantadine and in one recent isolate, the neuraminidase inhibitor oseltamivir (Tamiflu)[14]. Clearly, it would be useful to have other antiviral drugs such as inhibitors of the influenza virus polymerase to combat an influenza virus pandemic. Studies of the gene sequences of influenza virus, including 1918 and H5N1 viruses, are not particularly helpful for drug discovery.

The 1918 sequence, on the other hand, is likely to be most useful in the arena of virus strain surveillance. Central to such efforts will be tracking the changes needed to generate a pandemic virus in humans. But we still don't know what those changes are. That understanding will be aided by a comparison of emerging virus sequences with 1918 virus sequences, and by the large-scale sequencing of influenza virus genes[13].

Such information should be useful in mapping the evolutionary progress of the genes of H5N1 'bird flu' in case they accumulate mutations that enable human-to-human transmission, possibly causing a pandemic of unknown but possibly devastating consequences.

Future experiments should enable an understanding of why the unique sequence differences—only about 40 in the genes encoding the polymerase subunits of the 1918 virus—enable it to replicate faster and to a higher titer, and to cause such severe inflammation in the lungs. These studies will require extensive examination of the interplay between hemagglutinin and neuraminidase, and the cytokines released from cells of the respiratory tract. Recreating the 1918 influenza virus now makes it possible to investigate in detail how a relatively few nucleotide changes can convert a virus from a mild threat into a major killer.

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Cancer: the matrix is now in control

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The extracellular matrix is traditionally regarded as a facilitator of tumor progression, providing an environment in which cells can grow and metastasize. Three new studies take the matrix out of this context and suggest that the microenvironment can not only subsidize, but also initiate, the oncogenic conversion of epithelial cells.

The extracellular matrix (ECM) is a complex molecular milieu that provides cells with the tensile scaffold necessary for appropriate assembly into three-dimensional macroscopic structures. In addition, the ECM behaves as a reservoir of soluble and insoluble signaling molecules that integrate positional cues and translate them into fundamental cellular fates, including growth, differentiation, survival and movement[1]. This orchestration of topographical and biochemical parameters is disrupted in cancer development. The classic view is this: in the chaotic context of incipient

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