5 MODELS AND THERAPIES: INFECTIOUS DISEASES, CARDIOVASCULAR DISEASE, AND CANCER

> From all the above tests it was clear that this substance possessed qualities which made it suitable for trial as a chemotherapeutic agent. Therapeutic tests were therefore done on mice infected with streptococci, staphylococci and *Cl.* [Clostridium] *septique*. . . . The results are clear cut, and show that penicillin is active in vivo against at least three of the organisms inhibited in vitro. It would seem a reasonable hope that all organisms inhibited in high dilution in vitro will be found to be dealt with in vivo.
>
> —CHAIN ET AL. (1940), P. 228

The two previous chapters introduced the principal in vivo and in vitro models used in biomedical research. By contrast, the present and following chapters will focus on several major human diseases, exploring how the various models were used to understand and develop treatments for those maladies. In many cases the research advanced from in vitro to in vivo, as it did in the work on penicillin (see the chapter's opening quote). Much of the research focused on mouse models, but other species were also used, especially in the historically older studies and in the development of cardiovascular surgery. Human tissues and cells have featured heavily in recent work, especially as transplants into mice. We return to these and other trends at the end of this chapter.

Given that it is neither possible nor desirable to cover all diseases equally, the present chapter focuses on three types of disease that, between them, account for the majority of human deaths: infectious diseases, cardiovascular disease, and cancer. Chapter 6 will address neurological disorders, which represent a huge drain on humanity because they tend to be long-lasting and difficult to treat. Both chapters focus on therapies that were historically very important or are particularly relevant to the topic of model use.

5.1 INFECTIOUS DISEASES

Infectious diseases are caused by parasites (e.g., malaria), bacteria, or viruses; I here focus only on the latter two. Both bacterial and viral infections can be prevented (and in some cases treated) with vaccines, but it has proven difficult to develop effective vaccines against some major viral diseases, notably human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and hepatitis C. Bacterial and viral diseases can also be treated with antibiotics and antiviral drugs, respectively.

5.1.1 Vaccines

Vaccine development was pursued most famously by Louis Pasteur, whose efforts were already mentioned in chapter 3. Briefly, Pasteur created his vaccines by weakening the original infectious particles by physical means, such as heat or exposure to dry air. He began with vaccines against several animal diseases, such as chicken cholera, swine flu, and cattle anthrax, but then developed a vaccine against the rabies virus, which can infect humans. In 1885, Pasteur gave his rabies vaccine to a boy who had been bitten by a rabid dog, and the boy survived (Rappuoli, 2014). Some say that Pasteur had previously tested this vaccine on many dogs, but others are not so sure and have uncovered doubts about several of Pasteur's vaccine-related claims (Anderson, 1993). In any case, Pasteur's research on vaccines brought him worldwide acclaim. Chapter 3 also included a brief review of polio vaccine development. As we discussed, this research initially required many nonhuman primates, both for virus production and vaccine testing. This changed with the development of in vitro systems, which significantly reduced the need for nonhuman primates in polio research.

The very first vaccines—predating Pasteur's—were directed against the smallpox virus, which had long been a global scourge and killed approximately 400,000 people a year in Europe during the 18th century (Riedel, 2005). The effort was spearheaded by Edward Jenner in the 1790s. He had heard stories of milkmaids becoming immune to smallpox after becoming infected with cowpox—a related but much milder disease. Acting on this idea, Jenner inoculated a boy with fluid from the sores of someone who had been infected with cowpox and then challenged the boy with an injection of smallpox. The boy was fine, but it took some time for Jenner to find additional volunteers for this somewhat daring procedure, which he named vaccination (*vacca* being Latin for "related to cows"). Still, Jenner's vaccine turned out to be quite effective and quickly gained worldwide recognition. After additional vaccine development and worldwide vaccination campaigns, the World Health Assembly declared the planet free of smallpox in 1980. For our purposes, it is interesting that Jenner did not have a detailed, mechanistic understanding of what causes smallpox and did not, apparently, test his vaccine on animals before experimenting with the boy.

Tuberculosis is another highly infectious disease, but it is caused by a bacterium rather than a virus. The bacterium causing tuberculosis was identified by Robert Koch in 1882 as *Myobacterium tuberculosis*. Koch managed to grow this bacterium in vitro and used these cultures to infect guinea pigs, which then developed the disease. Koch had also experimented with mice and dogs but found the guinea pigs to be more susceptible to tuberculosis. In addition, Koch developed a histological stain for the tuberculosis bacterium that he then used to demonstrate the bacterium's presence in infected guinea pigs as well as humans (Cambau & Drancourt, 2014). Koch attempted to develop a treatment for tuberculosis, but, despite some exaggerated claims, he ultimately failed. Emil von Behring, who in 1892 pioneered the development of serum therapy (which entails treating infected organisms with serum from others who previously conquered the infection), likewise failed in his attempts to develop a human tuberculosis vaccine (Grundman, 2001).

The first successful vaccine against tuberculosis was developed between 1908 and 1921. The researchers discovered, somewhat fortuitously, that cultivating the tuberculosis bacterium in a medium containing ox bile reduced its virulence. Exhibiting remarkable persistence, they subcultured their bacterial colony 230 times over the course of 11 years and, eventually, obtained a bacterium that did not cause progressive tuberculosis when injected into various animals, including horses, cattle, and guinea pigs (Luca & Mihaescu, 2013). This vaccine, commonly referred to as Bacillus Calmette–Guérin (or BCG), was first given to humans in 1921 and has been administered to more than 1 billion people since then (Behar & Sassetti, 2020). Because this vaccine's effectiveness wanes several years after the immunization, researchers are eagerly seeking improved vaccines or administration schemes. It is exciting, therefore, to learn that the BCG vaccine is more effective in rhesus monkeys when it is given intravenously, rather than injected into the skin, which is the traditional route (Darrah et al., 2019). Whether this will also be the case in humans remains to be seen.

Human papilloma virus (HPV) can cause anogenital warts and cervical cancer. Scientists found it difficult to prove this causal relationship, however, because HPV does not infect nonhuman species, and intentionally infecting humans with HPV would be unethical. To get around this problem, researchers in the 1980s exposed human cervical tissue to HPV in vitro and then implanted it into immunodeficient mice; several weeks later the grafted tissue exhibited several features reminiscent of the anogenital warts typically observed in HPV patients (Kreider et al., 1985). This experiment helped to prove that HPV causes the warts; together with analogous experiments using grafts of human foreskin from circumcised infants (Kreider et al., 1987), it also gave researchers a way to cultivate the virus in animals. This rather cumbersome approach was necessary at the time because HPV was extremely difficult to cultivate

in traditional two-dimensional cell cultures, even with human cells. However, studies in the 1990s revealed that HPV can go through its complete life cycle when it is cultivated in 3D human cell cultures that closely resemble epidermis (Doorbar, 2016; De Gregorio et al., 2020).

The early 1990s also saw the development of the first HPV vaccine. In contrast to traditional vaccines, which use weakened or killed viruses, this HPV vaccine employed viruslike particles. Specifically, researchers expressed one of the two main HPV proteins in *Escherichia coli* or an insect cell line and found that the viral proteins self-assembled into large viruslike particles. When injected into animals, these particles trigger a strong immune response that then results in good protection against future HPV infections (Roldão et al., 2010). Because the vaccine contains no viral genes, there is no risk of the viruslike particles replicating and becoming infectious. This novel approach was pioneered using rabbits, dogs, and viruses closely related to HPV (Kirnbauer et al., 1992; Suzich et al., 1995). The human vaccine, called Gardasil, was approved for the prevention of human HPV in 2006. Other, similar vaccines were developed later.

Novel approaches to vaccine development have also been employed to fight the COVID-19 pandemic (van Riel & de Wit, 2020). In addition to traditional vaccines that use weakened versions of the responsible virus—severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)—some researchers developed vaccines that consist of viral messenger RNA (mRNA) encoding just a single SARS-CoV-2 protein. Encapsulated within special nanoparticles, this mRNA can enter host cells and then be translated into the viral protein, which triggers the desired immune response. Other promising COVID-19 vaccines use weakened versions of relatively harmless viruses that have been engineered to express one of the SARS-CoV-2 proteins. When those viruses infect host cells, they cause them to make the SARS-CoV-2 protein, transport it to the cell surface, and trigger an immune response. Both of these vaccine types are presumed to be relatively safe because the SARS-CoV-2 protein cannot form complete SARS-CoV-2 particles. That said, the new vaccines might well cause some unexpected adverse reactions, which is why they are tested in animals, including monkeys, for both safety and efficacy (Corbett et al., 2020; Doremalen et al., 2020). An intriguing aspect of COVID-19 vaccine development is that the animal tests are often run concurrently with early phase human trials, rather than sequentially. This extraordinary step seems justified, given the great urgency created by this lethal pandemic.

Overall, we can conclude from this brief sampling of vaccine development history that the field has become much less reliant on animal models for basic virus research and production. Advances in cell culture technique and genomics now make it possible to study most bacteria and viruses without infecting animals. However, animal models continue to play a role in the development of all vaccines, especially when it

comes to testing for safety and efficacy (Pardi et al., 2018). Given the regrettably widespread skepticism toward vaccines that already exists, humanity can ill afford vaccines that cause substantial harm or do not work.

5.1.2 Antibiotics

Antibiotics are used to fight bacterial infections. They are what Paul Ehrlich called "magic bullets" because, unlike real bullets, they kill the pathogen without harming the host (Bosch & Rosich, 2008; Strebhardt & Ullrich, 2008). Some antibiotics are synthesized by scientists, but many others are naturally produced by other microbes, which is consistent with the idea that different microbial species often compete aggressively with one another.

The first widely used antibiotic was discovered in the quest to conquer syphilis, a sexually transmitted disease whose symptoms begin with genital ulcers, followed by painful rashes and abscesses; in the long run, syphilis often leads to cardiovascular and neurological problems as well. This disease, sometimes referred to as "the great pox" (Bowater, 2016), erupted into devastating epidemics throughout much of Europe in the late 1400s. Diverse treatments for syphilis had been proposed over the years, including ointments containing potentially toxic levels of mercury (Abraham, 1948).

Significant progress came only after 1905, when scientists discovered that syphilis is caused by a spirochete bacterium called *Treponema pallidum*. Inspired by this discovery, Paul Ehrlich and his Japanese student Sahachiro Hata in 1909 infected rabbits with syphilis and then tested hundreds of arsenic-related compounds that Ehrlich and his collaborators had already synthesized in the quest to treat a different disease (African sleeping sickness). One of these compounds, arsphenamine, turned out to cure the rabbits of syphilis very effectively. Curiously, two of Ehrlich's assistants had previously tested this compound and failed to see an effect (Williams, 2009), thereby illustrating how false negatives and issues with replicability (see chapter 1) have frustrated scientists for a long time.

By the end of 1910, 65,000 doses of arsphenamine (trademarked as Salvarsan) had been administered to more than 20,000 patients with syphilis; these were unprecedented numbers at the time (Williams, 2009). Indeed, arsphenamine was more effective than any previous syphilis treatment. Unfortunately, treatment with arsphenamine involved a complex, protracted regimen, and unpleasant side effects were commonplace. Researchers tried to avoid these problems by slightly modifying the drug's chemical structure, but problems persisted (Bosch & Rosich, 2008). In the long run, arsphenamine was eclipsed by penicillin, which proved to be a simpler, even more effective treatment for syphilis.

Penicillin was discovered by Alexander Fleming in 1928, when he noticed a bacteria-free area around a bit of mold that had contaminated one of his bacterial culture dishes

(Fleming, 1929). The mold turned out to be of the genus *Penicillium*, and Fleming showed that it secreted a compound capable of killing *Staphylococcus* and several other kinds of bacteria. Fleming also showed that the mold's secretions were not toxic to rabbits or mice, but he did not examine whether they could halt ongoing infections. Part of the problem was that Fleming could not produce large amounts of the mold's secretions, which he called penicillin; nor was he able to isolate its active component. These problems were solved 11 years later by Howard Florey and Ernst Chain, who then demonstrated penicillin's antibacterial effectiveness in mice and, one year later, in humans (Chain et al., 1940; Science History Institute, 2016). After penicillin production had been scaled up (Bentley, 2009), the drug was used widely to treat innumerable patients, including many soldiers wounded in World War II.

Although the development of penicillin clearly involved extensive animal research, animal rights advocates sometimes point out that the drug is toxic to guinea pigs. This observation supposedly undermines the notion that biologists can extrapolate findings from animals to humans. It is important to note, however, that penicillin is not toxic to germ-free guinea pigs (Formal et al., 1963) (figure 5.1). Indeed, penicillin and some related antibiotics alter the gut microbiome of ordinary (i.e., not germ-free) guinea pigs in such a way that highly toxic coliform bacteria come to dominate, thereby killing the guinea pigs indirectly (Farrar et al., 1966). Apparently, this shift toward a toxic gut microbiome does not happen in other mammalian species. Therefore, we can conclude

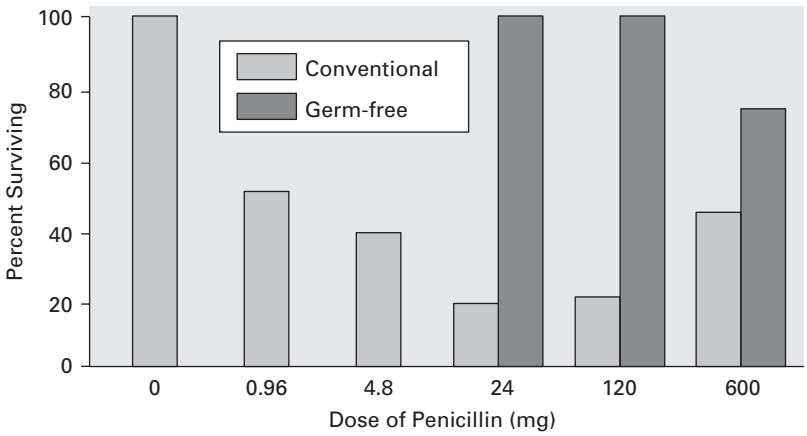

Figure 5.1

The microbiome's effect on penicillin toxicity in guinea pigs. Formal et al. (1963) gave varying doses of penicillin to guinea pigs that were either raised in a germ-free environment (and thus without microbes in their gut) or raised conventionally. Considering the three highest doses penicillin together, only 1 out of 20 animals died within seven days in the germ-free group, whereas 41 out of 54 animals succumbed in the conventional group. These data demonstrated that it is something about the microbiome that makes penicillin toxic to guinea pigs. Adapted from Botting (2015).

that species differences are real and important—indeed, antibiotics can only work as magic bullets because of species differences between hosts and their pathogens—but that those differences can be explained (at least sometimes). Nor do they negate the utility of all animal research. Indeed, the species differences in penicillin sensitivity argue in favor of working with multiple animal models, rather than just one.

Roughly coincident with the discovery of penicillin, Gerhard Domagk tested a variety of sulfur-containing azo dyes for their ability to kill highly virulent streptococcal bacteria. One of these compounds, eventually marketed as Prontosil, did not kill the bacteria in vitro but was able to cure streptococcal and other bacterial infections in mice and rabbits (Domagk, 1947). Domagk and his collaborators then tested Prontosil on humans, including Domagk's own daughter. The results were positive, with minimal side effects, and were published in 1935 (Bentley, 2009; Bailey, 2010). Although Prontosil's antibacterial effectiveness was discovered before that of penicillin, penicillin kills a broader range of bacteria and has, therefore, been more widely used. Another interesting twist in this story is the relatively late discovery that Prontosil is metabolized to sulfanilamide, which turns out to be the active antibacterial agent. That discovery explains why Prontosil did not kill the bacteria in vitro, where the requisite metabolic enzymes are lacking. It also led to the development of second-generation sulfa drugs based on slight modifications of sulfanilamide. Also of interest is that the sulfanilamide tragedy of 1937, which killed more than 100 people (see chapter 4, section 4.3), involved sulfanilamide dissolved in diethylene glycol; it was the latter molecule that killed, not the sulfanilamide!

5.1.3 Antiviral Drugs

AIDS was first identified in 1981 and was characterized by a progressive decrease in the number of helper T cells, which are type of white blood cell. AIDS is transmitted primarily through sexual intercourse; left untreated, it leads to a slow and painful death by other diseases such as tuberculosis or cancer. Researchers in the early 1980s determined that AIDS is caused by a complex retrovirus called human immunodeficiency virus (HIV) (Vahlne, 2009). This virus probably evolved from simian immunodeficiency virus (SIV), which is quite similar to HIV but infects a variety of nonhuman primates, rather than humans. Indeed, HIV easily infects only humans and chimpanzees, and infected chimpanzees rarely develop AIDS. In the words of Varki et al. (2011), "more than 100 chimpanzees in the United States and Europe were experimentally infected with HIV. Surprisingly, after more than 10 years, only one chimpanzee progressed to a full-blown acquired immune deficiency syndrome (AIDS)-like syndrome" (p. 376).

Given the ethical problems inherent in chimpanzee research and the fact that these animals rarely develop AIDS, researchers have tried to understand the human disease by

studying SIV-infected macaques, many of which do develop AIDS-like symptoms (Letvin et al., 1983). Efforts are also underway to modify HIV's genetic sequence so that the virus can infect monkeys, but this modification is nontrivial (Thippeshappa et al., 2020). In addition, AIDS researchers frequently study immunodeficient mice that have been implanted with HIV-infected human tissue (Namikawa et al., 1988; Hatziioannou & Evans, 2012). These humanized mouse models are ethically less troubling than the nonhuman primate models, but they are expensive, difficult to work with, and limited insofar as the virus will infect only the transplanted human cells, not the rest of the mouse. Finally, scientists have long been able to cultivate the HIV virus in cell lines derived from human T cells (Mitsuya et al., 1985), but those in vitro models cannot reveal the more complex aspects of AIDS.

In short, most models of HIV/AIDS are rather limited. This constraint largely explains why, despite long-standing efforts to develop an AIDS vaccine, none has been forthcoming. At least as important is that, as a retrovirus, HIV can "hide" from antibodies in the host's genome and then re-emerge later. Yet another factor is that HIV can spread not only through the intercellular fluid but also via direct cell-to-cell contact, which can make it inaccessible to most antibodies (Agosto et al., 2015).

Although it is frustrating to have no AIDS vaccine, scientists have had considerable success with antiviral drugs that target HIV. The first and most influential of these antiviral drugs is azidothymidine (AZT). It was discovered in the mid-1980s by screening several related drugs in an in vitro assay where AZT prevented HIV from infecting and killing T cells (Mitsuya et al., 1985). We now know that this protective effect results from AZT's ability to inhibit an enzyme (a reverse transcriptase) that HIV requires for replication. We also know that AZT must be phosphorylated before it can exert its antiviral activity, but fortunately the cells in the original in vitro assay possessed the requisite enzymes; otherwise, the discovery of AZT might well have been delayed. Once the in vitro data were in hand, AZT moved very quickly to clinical trials. Because of intense societal pressure to find a treatment for AIDS, which by 1985 had killed 20,000 people worldwide, human trials of AZT were begun in 1985 without prior testing in animals (Yarchoan & Broder, 1987; Mitsuya et al., 1990; Wyand, 1992). However, some animal testing was conducted concurrently (Ruprecht et al., 1990). High doses of AZT turned out to have substantial adverse effects, but lower doses were reasonably safe and effective. Nowadays, AZT is usually taken in combination with other antiviral drugs.

Hepatitis C is another major disease that has resisted vaccine development but can now be treated with antiviral drugs. Plaguing roughly 3% of the world's population, the hepatitis C virus (HCV) mutates at a high rate in human liver cells and thus tends to evade the host's immune response. The resulting chronic infection often leads to liver cancer, cirrhosis, or other forms of liver failure. Like HIV, HCV naturally infects

only chimpanzees and humans. Tree shrews (which are closely related to primates) can be infected with HCV, but only if they are severely immunosuppressed, and mice are normally resistant to HCV. To overcome the latter limitation, biologists sometimes study HCV infection in immunodeficient mice implanted with human hepatocytes. However, humanized mouse models of hepatitis C are (like the mouse models of HIV/AIDS) expensive and limited insofar as only the transplanted cells are infected. Perhaps the biggest obstacle to hepatitis C research was, for many years, that HCV is extremely difficult to propagate in cell culture.

This in vitro cultivation problem was overcome in 1999 when researchers developed a "replicon system" that allows most of the viral genome to replicate efficiently in a human liver cell line. Early versions of this model did not produce complete HCV particles, but this was later rectified (Woerz et al., 2009). Moreover, even the early versions allowed researchers to screen numerous drugs for their ability to interfere with many (though not all) aspects of HCV's life cycle. Most notably, these screens yielded drugs like sofosbuvir and simeprevir, which inhibit enzymes needed to replicate the viral genome and cleave a viral protein precursor, respectively (Horscroft et al., 2005; Eltahla et al., 2015). These and several other drugs that directly interfere with the life cycle of HCV have been approved for the treatment of hepatitis C since 2011 (Horsley-Silva & Vargas, 2017). They are relatively simple to administer and have fewer negative side effects than earlier treatments. Although they were initially discovered through in vitro drug screens, they were subsequently tested in rats and monkeys for safety and to estimate their effective tissue concentrations and excretion/degradation rates (i.e., pharmacokinetic parameters) (Rosenquist et al., 2014; Spera et al., 2016). Similarly, newer generations of anti-HCV drugs usually undergo safety and pharmacokinetic tests in diverse animal models but are tested for efficacy in humanized mouse models and in vitro assays (e.g., Rajagopalan et al., 2016).

A third important infectious disease that can be treated with antiviral drugs is COVID-19. Specifically, a randomized placebo-controlled clinical trial with 1,063 COVID-19 patients indicated that the drug remdesivir can reduce the duration of hospital stays (Beigel et al., 2020). Earlier research had already shown that remdesivir reduces lung damage in monkeys infected with Middle East respiratory syndrome coronavirus (MERS-CoV), which is similar to SARS-CoV-2, and that it inhibits SARS-CoV-2 replication in a monkey kidney cell line (M. Wang et al., 2020). Rodents, it turns out, do not make good animal models for testing remdesivir (and some other antiviral drugs, such as Tamiflu), because they express high levels of an enzyme that interferes with remdesivir's bioavailability (Bahar et al., 2012; Warren et al., 2016). In primates, however, remdesivir causes replication of the viral genome to be aborted prematurely; that is, it interferes with the ability of SARS-CoV-2 to replicate.

5.1.4 Sepsis

Sepsis is an extreme inflammatory response to bacterial or viral infections that harms internal organs; it often develops in trauma patients because their immune system is weakened. In the US, sepsis is responsible for more than 250,000 deaths per year, and approximately 6 million people die from sepsis every year worldwide (Korneev, 2019). Attempts to treat sepsis have a poor track record, including an apparently successful clinical trial that could not be replicated and at least one drug that was withdrawn after having been approved (Fink, 2014).

One potential explanation for this woeful approval history is that the most commonly used animal model of sepsis may not reflect the human condition very well. Specifically, much of the research on sepsis is based on mice that have been injected with lipopolysaccharides (LPS), which are found in the outer membranes of toxic bacteria. Injections of LPS do trigger strong, systemic immune responses in both humans and mice, but the median lethal dose of LPS in mice is "about 1000-fold to 10,000-fold greater than the dose of LPS that is required to induce severe illness and hypotension in humans" (Fink, 2014, p. 148). Moreover, the pattern of up- and down-regulated genes observed in white blood cells after LPS injection in humans differs substantially from that observed in LPS-injected mice (Seok et al., 2013) (figure 5.2). Major burn injury or blunt trauma, which also cause intense inflammatory responses, likewise elicit surprisingly different responses in mice and humans. In the words of the study authors, "Although acute inflammatory stresses from different etiologies result in highly similar genomic responses in humans, the responses in corresponding mouse models correlate poorly with the human conditions and also, one another" (Seok et al., 2013, p. 3507). In short, mice appear to make poor models for human sepsis.

This conclusion caused quite a stir in the scientific community, and several objections were raised. Some critics argued, for example, that one cannot compare a single inbred strain of mice to a highly heterogeneous human population (Osuchowski et al., 2014). However, this criticism could be leveled at most of the existing mouse models and is, therefore, a poor defense of mouse models. More interesting is the suggestion that Seok and colleagues should have excluded from their analysis any genes that are up- or down-regulated only in humans, not in mice (Takao & Miyakawa, 2015). The rationale for this recommendation was that mouse models are always merely partial models of the human condition (see chapter 6, section 6.5.3) and thus would never mimic all the human genomic responses. The critics argued that excluding the human-only genomic responses from the comparative analysis is standard practice in the field, but others disagree (Shay et al., 2015).

I, too, believe that it is more appropriate to compare all the available data between a model and its target, at least as a first step. If the model system is then found to

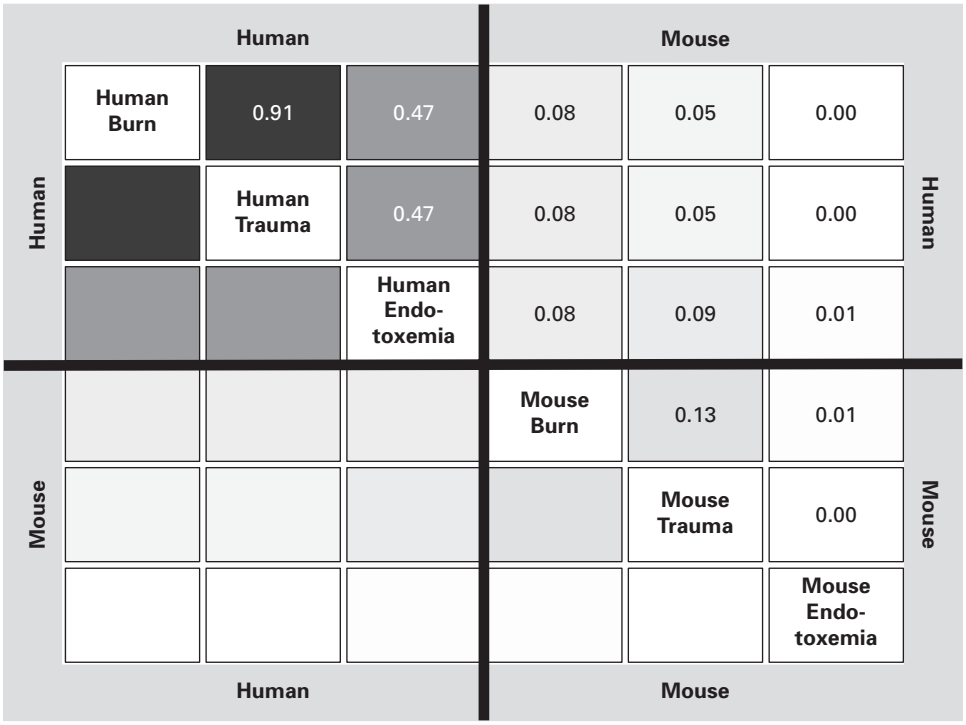

Figure 5.2

Sepsis-related gene expression changes reveal species and model differences. Seok et al. (2013) compared how gene expression in white blood cells was altered in human patients suffering from serious burns, major blunt trauma, and bacterial toxins (endotoxemia), all of which cause serious systemic inflammation (aka sepsis). They found that gene-expression changes in these three conditions are positively correlated with one another (Pearson's correlation coefficients shown as numbers and reflected in gray levels). However, the changes observed in these human conditions correlated poorly with corresponding mouse models. Nor did the mouse models correlate well with one another. The analyses covered almost 5,000 genes. Adapted from Seok et al. (2013).

mimic only restricted aspects of the target system, this finding can be pursued and specified in more detail. In other words, it is better to identify and, if possible, explain specific differences between a model and its target than to bias one's analyses a priori in favor of similarities. That said, an unbiased reanalysis of the data published by Seok et al. (2013) supposedly suggests that the similarities between mice and humans with regard to sepsis are greater than originally reported (Shay et al., 2015).

Whether one sees the mouse models of sepsis as being similar to or different from their human target may well be a matter of seeing the glass half-full or half-empty. However, the record of translational failures for potential therapies emerging from these models suggests that the species differences are meaningful. Indeed, the immune systems of mice and humans are already known to exhibit a great number of

morphological, cellular, and molecular differences (Haley, 2003; Mestas & Hughes, 2004; Gibbons & Spencer, 2011). More generally, the immune system of vertebrates has undergone extensive divergent evolution, which is not surprising given that immune systems are continually engaged in an evolutionary arms race with rapidly evolving pathogens (Bailey et al., 2013; Bitar et al., 2019).

In order to minimize the species differences, one can try to humanize the immune system of mice (or other species), but the transformations are likely always to remain partial (Yong et al., 2018). A potentially complementary approach is to work with animals that, unlike most laboratory mice, have faced multiple immune challenges as they grew up (Tao & Reese, 2017). The immune system of such animals will likely be more similar to that of their wild counterparts (Abolins et al., 2017) and, by extension, more similar to that of humans. However, significant differences between humans and mouse models will probably remain for the foreseeable future, suggesting that the modeling of human immune responses in mice should be done cautiously. In some cases, nonhuman primate models may be appropriate (Messaoudi et al., 2011).

5.2 CARDIOVASCULAR DISEASES

Heart attacks and strokes (defined as disruptions of the brain's blood supply) are the world's top two killers of humans, accounting for a combined 15.2 million deaths in 2016 (World Health Organization, 2018a, 2018b). Cardiovascular death rates have been dropping in high-income countries but, globally, cardiovascular disease still kills more people than cancer (Dagenais et al., 2020) (figure 5.3).

5.2.1 Heart Medications

The principal cardiac health problems are heart failure, defined as insufficient cardiac pumping ability and an irregular heartbeat (arrhythmia). These problems are sometimes addressed with coronary bypass surgery and the implantation of cardiac pacemakers, both of which were developed in the 1950s with heavy reliance on dogs as test subjects (Callaghan & Bigelow, 1951; Zoll, 1973; Konstantinov, 2000). However, heart failures and arrhythmias are most commonly treated with medication. For our purposes, therefore, it makes sense to focus on four very influential heart medications, namely digoxin/digitalis, nitroglycerin, propranolol, and angiotensin-converting enzyme (ACE) inhibitors.

Digoxin and digitalis belong to a class of plant-derived drugs that has been used to treat heart conditions for more than 200 years. William Withering (1785) first extracted the drug digitalis (aka digitoxin) from the common foxglove plant (*Digitalis purpurea*) and recommended it for the treatment of many conditions, including what we now call

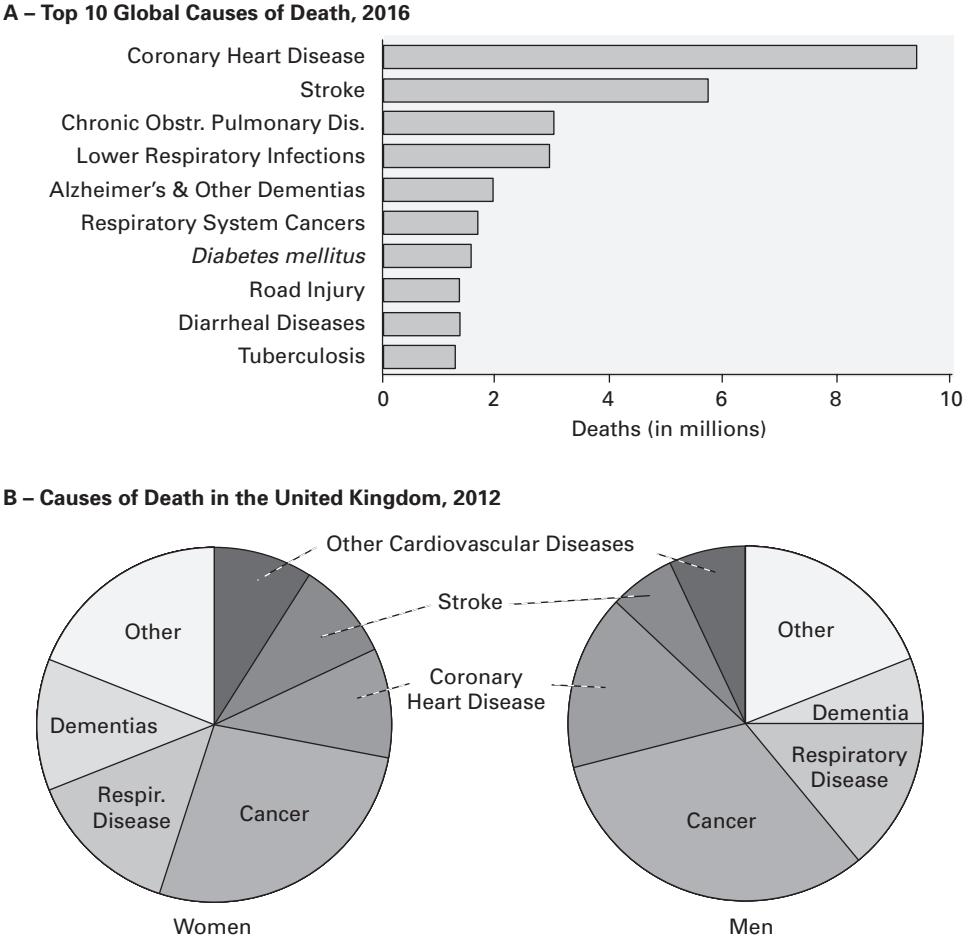

Figure 5.3

Statistics on causes of death. (*A*) Top 10 global causes of death, 2016. According to the World Health Organization, coronary heart disease and stroke were the two top causes of death globally. The respiratory cancers in this chart include lung, bronchial, and tracheal cancers. (*B*) Causes of death in the United Kingdom, 2012. Cancer was the most common cause of death, and women were more likely than men to die from Alzheimer's disease or other dementias (12% versus 6%). Cardiovascular diseases were equally common causes of death in men and women, but within that category men were more likely to die of coronary heart disease (16% versus 10%). Based on (A) World Health Organization (2018a); (B) adapted from Bhatnagar et al. (2015).

congestive heart failure (Silverman, 1989). A very similar drug, called digoxin, was isolated in 1930 from a different species in the foxglove genus (Hollman, 1996); it is sometimes prescribed instead of digitalis because it has a longer half-life in the body. Both drugs are cardiac glycosides that block the cellular sodium-potassium pump, which indirectly leads to an increase of intracellular calcium, which then leads to more forceful and regular contractions of the heart muscle. Human use of these drugs predates animal testing, but both clinical trials and animal testing later revealed potentially serious side effects. The principal problem is the relatively small difference between the therapeutically effective dose and one that is toxic. Indeed, the world's most prolific serial killer, Charles E. Cullen, used his position as a nurse to kill an estimated 400 patients with digoxin in the 1980s and 1990s (Harrington, 2018). Nowadays, digoxin and digitalis are used infrequently, mainly in combination with other heart medications.

In contrast to digoxin and digitalis, other heart medications tend not to increase the force of cardiac contractions. Instead, they decrease heart rate and increase the flow of blood through the coronary blood vessels. This (initially counterintuitive) approach reduces the metabolic stress placed on the heart and thus decreases heart-derived chest pain (i.e., angina pectoris) and the risk of a subsequent heart attack. The first drugs of this kind were organic nitrates, including nitroglycerin, which had originally been synthesized as a novel explosive (Fye, 1986). After becoming aware that licking a spot of freshly synthesized nitroglycerin induces violent headaches, a physician called Constantin Hering in the 1840s supposed that nitroglycerin might cure headaches (on the homeopathic principle that "like cures like") and proceeded to test it on himself and numerous patients (Marsh & Marsh, 2000). Another physician, William Murrell, noticed in the 1870s that self-administering nitroglycerin not only gave him headaches but also affected his heart, which prompted him to test the drug as a possible treatment for angina pectoris (Fye, 1995). The drug worked well for this purpose, and we now know that it does so, at least in part, by dilating the coronary veins. Later studies showed that nitroglycerin can have some serious side effects and that prolonged treatment leads to drug tolerance (Parker & Parker, 1998; Thadani & Rodgers, 2006). Experiments on rats further suggest that prolonged treatment with nitroglycerin may make the heart more vulnerable to subsequent attacks (Sun et al., 2011). Still, the drug remains in use for the temporary treatment of angina pectoris.

Propranolol is another influential drug that reduces heart stress. Because it selectively inhibits beta-adrenergic receptors, it is referred to as a beta-blocker. Propranolol was discovered in 1964 after an impressively systematic search for a novel drug that would selectively decrease heart rate and blood pressure (Black et al., 1964). This effort was carried out in a variety of material models, ranging from isolated, spontaneously beating guinea pig hearts to anesthetized dogs and cats (Black, 1989). The first clinical

trial of propranolol was conducted in 1968 and provided positive results (Hebb et al., 1968). Over the years, several additional beta-blockers have been developed, but propranolol is still often prescribed for angina pectoris and several other conditions (e.g., migraines and stage fright).

An important breakthrough in the treatment of heart conditions was the discovery of drugs that inhibit angiotensin-converting enzyme (i.e., ACE inhibitors). These drugs lower blood pressure by dilating blood vessels, both directly and indirectly via the hormone aldosterone. They were originally discovered in 1967 by researchers who tested extracts of pit viper venom—already known to kill prey by causing its blood pressure to plummet (Péterfi et al., 2019)—for their ability to inhibit angiotensin II activity. The assays used in these early experiments relied on cell-free extracts of dog lung to obtain the enzyme, and on in vitro pieces of rat colon to test for its inhibition (Ng & Vane, 1968; Bakhle, 1968). The first ACE inhibitors were tested in animals and even a few humans, but they could not be taken orally and, therefore, attracted little commercial interest (Cushman & Ondetti, 1991). However, the scientists then tested a strategically synthesized series of peptides in an in vitro assay (based on guinea pig intestine) to optimize the drug; in 1975, they ended up with a compound that could be taken orally. This drug, called captopril, was subsequently tested in intact rats and approved for human use in 1980. Additional refinements to reduce unwanted side effects led to enalapril (aka Vasotec), which became the first billion-dollar drug in 1988 (Bryan, 2009). Enalapril has now been eclipsed by even newer ACE inhibitors, but as a class the ACE inhibitors remain a mainstay of cardiovascular therapy.

5.2.2 Atherosclerosis

Atherosclerosis (derived from the ancient Greek words *athḗra* and *sklērōsis*, meaning gruel and hardening, respectively) is a progressive inflammatory disease characterized by the buildup of lipids and cells inside of arteries. The resulting accumulations, called plaques, narrow the arterial cavity and thus obstruct blood flow. Plaques can also rupture, which then creates a blood clot that tends to obstruct the vessel completely. If prolonged, the blockage will cause extensive cell death in the supplied tissues. Most seriously, the obstructions can lead to heart attacks (myocardial infarcts) or brain damage (i.e., ischemic stroke). The treatment of atherosclerosis typically involves changes in diet, cholesterol-lowering drugs, and, in advanced cases, endovascular surgery. In the following paragraphs, I discuss those therapies in turn.

Cholesterol is a lipid that helps to keep cell membranes pliable. It is synthesized mainly in the liver but can also be obtained by eating animal products (plants do not make cholesterol). Cholesterol is not water soluble and must, therefore, be transported in the blood by special carrier proteins (i.e., lipoproteins). Most of the cholesterol in

the blood is carried from the liver to other tissues by low-density lipoprotein (LDL), which is often referred to as the "bad cholesterol." Some cholesterol is carried from the tissues back to the liver by high-density lipoprotein (HDL).

The link between atherosclerosis and cholesterol was first suggested in 1908–1910, when scientists discovered that rabbits fed a diet rich in meat, fats, and milk developed atherosclerosis and that human arterial plaques contain high levels of cholesterol. An influential study in 1912 then showed that high levels of dietary cholesterol correlate with plaque formation in rabbits (Kritchevsky, 1995). As it happens, the choice of rabbits in these early experiments was fortuitous because rabbits are far more sensitive to dietary cholesterol than rodents or dogs (Getz & Reardon, 2017). The link between cholesterol and atherosclerosis in humans was firmly established by the Framingham Heart Study, which followed 5,209 men and women over many years (Castelli et al., 1992). It showed that, indeed, high levels of LDL-bound cholesterol are associated with coronary heart disease (i.e., constriction of the coronary arteries that supply the heart). It also revealed that having low levels of HDL augments the risk.

Aside from changing one's diet, the principal treatment for atherosclerosis consists of medications that lower cholesterol (i.e., statins). The initial discovery of these drugs dates back to the 1960s, when Akira Endo hypothesized that some *Penicillium* fungi might secrete compounds that inhibit cholesterol synthesis in other organisms (but do not harm the fungi because they rely on ergosterol rather than cholesterol to accomplish similar functions). After screening several thousand "fungal broths" in in vitro rat liver assays, Endo discovered compactin, the first statin (Endo, 2010). Surprisingly, this compound does not reduce cholesterol synthesis in intact rats—a finding later confirmed by other researchers—but Endo persisted. Eventually, he found that compactin does reduce plasma cholesterol levels in chickens, dogs, and monkeys. The drug also showed promising results in humans, but at very high doses it caused cancer in dogs and was, therefore, abandoned.

Nonetheless, work on statins continued. In the late 1970s two groups of researchers independently discovered lovastatin, which is structurally quite similar to compactin but derived from a different fungus. This second major statin was shown to inhibit cholesterol synthesis in human cells and intact rats; it also reduced plasma levels of cholesterol, including LDL cholesterol, in dogs (Alberts et al., 1980, 1989). Importantly, long-term toxicity in dogs and several other animal species was relatively low. Clinical trials of lovastatin began in 1982 using healthy volunteers as well as subjects with congenitally high levels of cholesterol. Overall, lovastatin was found to reduce LDL cholesterol reliably with few negative side effects. It was approved by the US Food and Drug Administration (FDA) in 1999 and remains available, even though several additional statins have been developed in the intervening years. An important,

relatively recent addition to the arsenal of cholesterol-lowering drugs is the use of synthetic antibodies that lower LDL cholesterol by inhibiting PCSK9, an enzyme that helps degrade the LDL receptors. These therapeutic antibodies were shown to reduce LDL cholesterol in mice and monkeys (Chan et al., 2009). They have been approved for human use since 2015 and are usually taken in conjunction with statins.

When atherosclerotic plaques threaten the blood supply to vital organs, surgeons may try to dilate the obstructed vessels. The surgical technique for this procedure, called angioplasty, was originally developed in the 1960s and 1970s and involves the insertion of long catheters through the relevant blood vessels into the constriction (Payne, 2001). In early versions of angioplasty the surgeon inflated a small balloon at the tip of the inserted catheter. This inflation temporarily expands the vessel's interior, stretching its walls. Because the constriction often returns after such balloon angioplasties, surgeons invented stents, which are wire mesh cages that could be left in place to keep the blood vessel open. Unfortunately, material tends to accumulate inside regular stents, causing renewed constriction (i.e., restenosis). In response, scientists created drug-eluting stents, which release molecules (e.g., sirolimus) that reduce inflammatory responses and reduce restenosis rates substantially. Some of these drug-eluting stents appear to be effective for at least five years (Morice et al., 2007; Kastrati et al., 2007). However, there is some risk of blood clots forming inside of stents. One should also note that, in cases of stable coronary heart disease, stents appear to work no better than less invasive treatments (Maron et al., 2020).

The surgical techniques and devices involved in angioplasty were extensively tested and refined in large animal models, such as dogs, pigs, and cattle (Feng & Jing, 2018). This makes practical sense because the hearts and blood vessels of these animals are similar to ours in size, even if their anatomical details differ in some respects. It is worth noting, however, that the very first angioplasty was performed on patients without prior animal tests (Payne, 2001). Indeed, surgeons are generally given more latitude than other medical professionals in developing new therapies without prior testing in animals (Darrow, 2017). The reasons for this difference are unclear, but variations in surgical skill level are probably involved. It is also generally accepted that surgeons need to "learn in practice," which is to say that they must, from time to time, try new ways of doing things (Morlacchi & Nelson, 2011; Gardner, 2013). In any case, surgical devices, including stents, are typically subjected to more extensive animal tests than the surgical procedures themselves.

Although angioplasty was developed mainly in humans and large animal models, mouse models of atherosclerosis have contributed significantly to our understanding of the underlying disease mechanisms (Shen et al., 2017). Nonetheless, it is important to note that mice tend to develop plaques in different locations than humans, and that

these tend to rupture only in response to additional mechanical disturbance (Emini Veseli et al., 2017). Moreover, many treatments that are effective in the mouse models of atherosclerosis have not done well in clinical trials. One possible explanation for this discrepancy is that the manipulations performed on blood vessels in the mouse models do not mimic human angioplasty very precisely (Libby, 2015). Even the large animal models differ from the human condition in the time course of disease progression and response to therapies. In addition, the animal models are generally young and otherwise healthy, which tends not to be the case for humans with cardiovascular disease. In part because it is so difficult to develop good animal models of human cardiovascular disease, there is considerable interest in using human data to construct in vitro and in silico (i.e., computational) models of the human heart and blood vessels (Z. Li et al., 2019; Savoji et al., 2019). Although such models hold significant promise, their ability to deliver novel types of therapies remains to be seen.

5.2.3 Stroke and Neuroprotection

Because strokes cause damage to the brain, it is justifiable to classify stroke as a neurological disorder (Shakir, 2018). However, I here discuss stroke as a cardiovascular disease because it results from problems with cerebral blood vessels. Specifically, hemorrhagic stroke involves the rupture of cerebral blood vessels, and ischemic stroke occurs when the brain's blood supply is blocked, depriving the downstream neurons of the oxygen and nutrients they need for survival.

Some strains of rats, a few transgenic mouse lines, and even some dogs develop ischemic strokes spontaneously (O'Collins et al., 2017; Hermann et al., 2019), but these natural stroke models are difficult to work with because their strokes are unpredictable in time and location. Therefore, researchers generally induce strokes experimentally—for instance, by tying off a cerebral artery. Gerbils were often used for such experiments because they have an incomplete circle of Willis (a set of small vessels that interconnect the major cerebral arteries at the base of the brain), which means that one can deprive an entire cerebral hemisphere by blocking one of the carotid arteries (Graham et al., 2004). However, ischemic strokes in humans usually affect only parts of one cerebral hemisphere. Therefore, researchers nowadays prefer to block only select branches of one carotid artery (e.g., the middle cerebral artery), and they do so mostly in rats and mice (van der Worp et al., 2010).

Another relevant consideration is that the arteries running across the surface of the brain are interconnected via collateral branches, so blocking one artery may cause blood flow to be rerouted around the obstruction. Because the anatomy of this collateral system differs across species, analogous blockages in different species may lead to different outcomes (Howells et al., 2010; Sommer, 2017; Hancock & Frostig, 2017).

Given such species differences, one might expect that therapies derived from animal models of ischemic stroke would translate poorly to humans. Indeed, "in animal models of acute ischemic stroke, about 500 'neuroprotective' treatment strategies have been reported to improve outcome, but only aspirin and very early intravenous thrombolysis with alteplase (recombinant tissue-plasminogen activator) have proved effective in patients, despite numerous clinical trials of other treatment strategies" (van der Worp et al., 2010, p. 1).

One major problem, aside from the already mentioned species differences, is that treatments in animals are usually administered very soon after the induced stroke (or even before), whereas human stroke patients often remain untreated for several hours after the incident. In addition, the animal research is usually performed on young and otherwise healthy individuals, whereas human stroke victims tend to be elderly and suffer from a variety of other health problems (Hermann et al., 2019). Two additional concerns are that the existing animal and clinical studies on stroke both tend to have insufficient sample sizes (i.e., are underpowered), and that the animal research suffers from a positive publication bias and other methodological weaknesses (Philip et al., 2009; O'Collins et al., 2017). On a more positive note, recent clinical trials indicate that surgical extraction of blood clots through endovascular surgery is relatively effective against ischemic stroke in humans (Goyal et al., 2016).

5.3 CANCERS

Physicians have known about cancers since ancient times, defining them as abnormal growths that tend to grow back even if surgically removed. More recently, biologists have characterized malignant, cancerous cells as exhibiting six essential features, namely (1) the ability to proliferate in the absence of external growth signals, (2) an insensitivity to external growth-inhibiting signals, (3) the ability to evade programmed cell death, (4) a limitless proliferative potential, (5) the ability to attract new blood vessels, and (6) the potential to invade other tissues and metastasize (Hanahan & Weinberg, 2000). Although these attributes characterize most, if not all, malignant cancers, they tend to be acquired over time, with cells becoming progressively more cancerous. In a quasi-Darwinian competition among cell lineages, the cells with the greatest ability to survive will proliferate, spread, and come to predominate, which is how precancerous growths become malignant cancers. A second important point is that the details of how cancerous cells acquire their hallmark attributes can vary significantly between different types of cancer. Indeed, biologists have identified more than 100 types of cancers (National Cancer Institute, 2007). These may differ not only in the tissues where they initially arise but also in their molecular characteristics and responses to therapy.

As scientists have struggled to understand and treat cancer, they have used a variety of model systems. They started in 1915 with rabbits, smearing coal tar on their ears to prove that it is a carcinogen; this hypothesis had emerged from the much earlier observation that chimney sweeps often got cancer (Thomas et al., 2016). Along a very different track, C. C. Little (the founder of the Jackson Laboratory; see chapter 3) in the 1930s promoted the study of inbred mouse strains with varying rates of cancer incidence as the best strategy for uncovering cancer's genetic basis (Little, 1935); he considered nonheritable factors to play little or no role in cancer. It is ironic, therefore, that some of the biggest breakthroughs in cancer research came through the use of carcinogenic viruses (Vogt, 2012; Bister, 2015).

The first of these carcinogenic viruses was Rous sarcoma virus, which infects chicken cells and makes them cancerous (Rous, 1959, 1967). Studies of this and other cancer-causing viruses led to the identification of specific genes that, when inserted into the genome of host cells, transform those cells (into tumor cells). Subsequent research revealed that many of these cancer-causing genes, called oncogenes, closely resemble genes natively present in the host genomes, suggesting that the viruses had acquired them from a host at some point in the evolutionary past. Importantly, this discovery strongly suggested that animals possess potential oncogenes (aka proto-oncogenes) that when malfunctioning can cause cancer even in the absence of a viral infection. Later studies confirmed this hypothesis and showed that tumor formation usually requires a whole series of malfunctioning proto-oncogenes (as well as malfunctioning tumor repressor genes). It also showed, as Little had argued, that some individuals have heritable cancer predispositions (H. T. Lynch et al., 2004; Pomerantz & Freedman, 2011).

Complementing this research, which was largely in vitro, were studies on mice implanted with cancerous cell lines or explanted tumors (see section 5.3.4). Many recent studies of this kind have examined, for example, the microenvironment of developing tumors (Watnick, 2012; Jiang et al., 2020), focusing especially on the conditions that allow tumors to thrive (e.g., the ingrowth of blood vessels). It is impossible here to discuss these and other fundamental aspects of cancer biology in any depth. Instead, let us focus on a few key developments in cancer therapy and on the models that led to them.

5.3.1 Radiation Therapy

Because cancer cells divide more rapidly than normal cells, it makes sense to treat cancer with therapies that interfere with cell division. Historically, the first such method was X-ray irradiation. Within a few years of Wilhelm Röntgen's 1895 discovery that X-rays could be used for bioimaging, it became apparent that X-rays can also damage dividing cells. In particular, early experiments showed that irradiation of the testes in

various mammals triggered a massive degeneration of sperm progenitor cells but not of mature sperm (del Regato, 1976). Extensive studies on rams, which have conveniently large testicles, then showed in the 1920s that dividing the total dose of radiation into multiple smaller doses, given at regular intervals, made the treatment even more selective for the rapidly dividing cells. Henri Coutard and others subsequently extended this insight to the treatment of humans with cancer (Coutard, 1934; Thames, 1988).

Radiation therapy was widely regarded as a breakthrough in cancer treatment during the 1920s and 1930s, and it has been refined considerably in recent years. Especially important has been the development of computer-assisted methods for three-dimensional (3D) tumor imaging and the use of multiple X-ray beams to deliver high doses of radiation to regions where the beams intersect (Thariat et al., 2013). The "gamma knife" technique, for example, allows physicians to target tumors within the brain without damaging overlying areas. These technical advances involved extensive animal testing, much of it in laboratory mice (Verhaegen et al., 2018; Butterworth, 2019).

5.3.2 Hormone Therapy

A causal link between cancer and male sex hormones was firmly established in 1940, when Charles Huggins and his collaborators discovered that castration causes the enlarged prostate glands of old, senile dogs to shrink; injections of estrogen had similar effects (Huggins & Stevens, 1940). The following year, these scientists performed analogous experiments on dozens of men with prostate cancer and observed a similar degree of tumor regression (Huggins & Hodges, 1941). Nowadays prostate cancer can be treated in diverse ways, including surgical removal and targeted radiotherapy or chemotherapy. However androgen deprivation therapy via castration or antitestosterone drugs continues to be widely used in the treatment of prostate cancer (Gunner et al., 2016); even injections of estrogen continue to be employed in select instances (Reis et al., 2018). Not surprisingly, both treatments can have adverse effects on libido and hormone-related sexual characteristics.

Breast cancer used to be treated mainly by surgical removal of both breasts as well as the adjacent pectoral muscles and lymph nodes (Cotlar et al., 2003), but such radical mastectomies are rarely done today because other, less invasive therapies have become available. Particularly important was the realization that breast cancer, like prostate cancer, is often hormone dependent. The initial stimulus for this concept came from the 1896 discovery that removal of the ovaries causes degeneration in the mammary glands of lactating rabbits. Furthermore, ovariectomy diminished breast cancer in a small trial of three human subjects (Stockwell, 1983). However, this line of inquiry was not pursued in earnest until the 1950s, when Charles Huggins began to study breast cancer in female Wistar rats, which reliably develop mammary cancers when fed high doses of a

carcinogen (Bashyam, 2007). Using this model, Huggins et al. (1959) found that mammary cancers decrease in size when the rats have their ovaries removed or are injected with testosterone. Soon thereafter, a large clinical trial revealed that surgical removal of the ovaries (or the adrenal glands, which produce significant amounts of estrogen in older women) reduces breast cancer; importantly, it only works on tumors that express high levels of estrogen receptors (Jensen et al., 1977).

Also in the 1970s, scientists discovered that the drug tamoxifen is an effective breast cancer treatment with few side effects. Tamoxifen was initially developed as an antiestrogen that might serve as a morning-after pill, and it does act as a contraceptive in rats. However, and rather paradoxically, tamoxifen induces ovulation in women (Quirke, 2017). Despite this initial disappointment, studies in 1970s showed that tamoxifen could prevent the development of mammary cancer in carcinogen-fed rats (Jordan, 1976, 2008). A series of clinical trials subsequently showed that tamoxifen is quite effective against breast cancers and has fewer side effects than the earlier therapies. Particularly important was that tamoxifen can prevent breast cancer in at-risk women and avert metastasis after surgical breast cancer removal. It was approved by the FDA in 1977, and millions of women (and some men) have benefited from tamoxifen worldwide. Some newer antiestrogen cancer drugs have been developed in the last couple of decades, but tamoxifen continues to be prescribed, often in conjunction with other therapies.

5.3.3 General Chemotherapy

In parallel with the discovery of hormone therapy, scientists in the 1940s developed drugs that selectively kill rapidly dividing cells and could, therefore, be used as a broad anticancer treatment. The first of these general chemotherapy drugs was nitrogen mustard, which is closely related to sulfur mustard (aka mustard gas). These mustard agents, which are unrelated to the mustard plant but smell like it, had been synthesized as toxins for use in chemical warfare. They were rarely used on the battlefield, but mustard gas did kill hundreds of sailors when it was accidentally released during the bombing of an Allied cargo ship carrying a secret load of this toxin during World War II (Conant, 2020b). Examination of the deceased, as well as subsequent experiments on rabbits and several other mammalian species, indicated that mustard agents selectively kill the fast-dividing bone marrow cells that generate white blood cells (DeVita & Chu, 2008; Conant, 2020a). We now know that they do so, at least in part, by forging cross-links between DNA strands, which then interfere with DNA transcription and replication.

The idea of using mustard agents to fight cancer began to be pursued in the early 1940s. Extensive early studies in rabbits and then mice showed that mustard agents, like X-rays, destroy primarily the rapidly dividing cells. More importantly, when

nitrogen mustard was given to mice that had been implanted with lymphomas, the tumors regressed. These "experiments in mice were sufficiently encouraging to consider a therapeutic trial in man" (Gilman, 1963, p. 576). Sure enough, the first clinical trial, published in 1946, showed that nitrogen mustard reduces tumors in patients with lymphosarcoma and Hodgkin's disease, both of which are cancers of the lymphatic system (Goodman & Wintrobe, 1946). It is much less effective against leukemias, both in humans and in mice. This is interesting because the entire project might have been dropped if the scientists had focused their early animal studies on leukemia rather than lymphomas (Gilman, 1963). It also shows that even general chemotherapy agents are more effective against some types of cancer than others.

Although the development of nitrogen mustard as an anticancer agent was a breakthrough in cancer therapy, the drug has a variety of adverse side effects, including a dramatic decrease in the number of white blood cells. Scientists therefore developed a variety of closely related compounds, such as cyclophosphamide, that are similarly effective but less toxic. They also developed a variety of other drugs that are not related to mustard agents but likewise interfere with various aspects of cancer cell proliferation. Particularly interesting is the history of platinum-containing drugs, which remain widely used against some cancer types. These drugs derive from a serendipitous observation made by Barnett Rosenberg in the 1960s. He had been examining the effect of electric fields on cell division in *E. coli* and discovered that passing current through the platinum electrodes he had been using created a platinum salt, later called cisplatin, that stopped the bacteria from dividing (even though they continued to grow in size). Rosenberg and colleagues (1969) then showed that cisplatin could be used to treat two types of cancer in mice. The drug was approved for use in humans in 1978 and has spawned a wide variety of other platinum-related drugs (Johnstone et al., 2014). One must note, however, that cisplatin all too frequently causes permanent hearing loss in children (Knight et al., 2005; Sheth et al., 2017), a finding that has been replicated in monkeys (Stadnicki et al., 1975) but could not have been obtained in *E. coli*.

Another class of general chemotherapy agents blocks DNA synthesis, which cancer cells require to proliferate. First to be discovered were drugs that inhibit folic acid, an enzyme that is involved in the synthesis of some DNA nucleotides. The initial experiments showing that inhibitors of folic acid can counteract cancer were performed in mice with implanted tumors (Leuchtenberger et al., 1945; Sneader, 2005). Related compounds were then synthesized and tested in humans (Farber et al., 1948). The two historically most impactful antifolate compounds are aminopterin and methotrexate, which have been used for the treatment of leukemia and several other malignancies (Goldin et al., 1955). A second class of compounds that interfere with DNA synthesis consists of modified pyrimidines, including 5-fluorouracil. This drug was created

specifically with the aim of fighting cancer, and studies in the late 1950s showed it to be effective in mice with transplanted tumors (Heidelberger et al., 1957). The first clinical trials were already promising (Curreri et al., 1958), although tumor regression was typically associated with toxic side effects. Nonetheless, 5-fluorouracil remains widely used to treat colorectal and related cancers, early-stage breast cancer, and several cancer-related skin conditions. It remains on the World Health Organization's List of Essential Medicines (as do cisplatin and methotrexate) (WHO, 2020).

The chemotherapy agents we have discussed in this section interfere with cell division generally and, therefore, kill not only cancer cells but also rapidly dividing cells in other tissues, such as hair follicles, the lining of the gastrointestinal tract, and white blood cell precursors. Because of these negative side effects, general chemotherapy drugs were widely regarded as "poisons" in the 1960s and used reluctantly by many physicians who, after all, are sworn to "do no harm." It took another decade or so before general chemotherapy became more widely accepted, mainly because, in combination with anticancer surgery, it did effectively cure some types of cancer (DeVita & Chu, 2008).

5.3.4 Targeted Chemotherapy

As discussed in section 5.3.2, the existence of hormone-sensitive cancers revealed that tumor cells may differ in the genes and proteins they express and, therefore, may require different types of therapy. The resulting quest for compounds that target specific, molecularly defined types of cancer accelerated dramatically in the 1970s, when biologists began to document the molecular genetic profiles of diverse cancers (National Cancer Institute, 2018). This work revealed that cancers vary not only between the sexes and in the tissues where they originate, but in their cancer-causing mutations.

Indeed, as mentioned earlier, scientists have by now discovered many different oncogenes and tumor repressor genes, defined as genes that cause cancer when they become abnormally active or are disrupted, respectively. To test the causal role of these genes in producing the hallmarks of cancer, researchers created hundreds of genetically modified mouse strains that express altered versions of the putative cancer-related genes. Study of these "oncomice" (Hanahan et al., 2007) revealed an enormous amount of information about the intricacies of cell cycle regulation and tumor growth, including the observation that it often takes multiple cancer-related mutations to trigger tumor formation (Hutchinson & Muller, 2000). However, using these genetically modified mice to develop anticancer therapies is difficult because one cannot know precisely when and where the cancer-prone mice will develop their tumors, which in turn makes it difficult to conduct large-scale screens for anticancer drugs.

To overcome this problem, researchers began to transplant tumor cells from mice with different types of cancer into normal mice of the same strain. Because the two sets

of mice share the same genetic background, immune rejection of the graft is unlikely. Left untreated, the transplants reliably develop into tumors. Because the transplanted tissue is typically placed just under the skin on the side of the animal, tumor growth is easy to spot and quantify. Thus, these "isograft" models are well suited to drug screening. Unfortunately, many of the drugs that work in these models have not succeeded in human clinical trials (Rangarajan & Weinberg 2003; Anisimov et al., 2005). The most likely reason for this failure is the existence of species differences in cancer biology. For example, a major type of breast cancer that expresses high levels of estrogen receptor in humans does not do so in mice (Herschkowitz et al., 2007). Similarly, disruption of the retinoblastoma gene causes retinal cancer in humans but pituitary cancer in mice (Robanus-Maandag et al., 1998). In addition, researchers have noted that it commonly takes four to six distinct mutations to trigger cancer in humans but fewer in rodents (Hahn & Weinberg, 2002).

In light of these species differences, cancer researchers increasingly focused their efforts on human cancer cells. In particular, they created large libraries of 60 or more human cancer cell lines that can be tested in vitro for their responses to diverse drugs. Because cell culture conditions differ substantially from the in vivo context (see chapter 4), the human cancer cells are often transplanted into immunodeficient mice, thus converting the aforementioned isograft models into "xenograft" models (*iso-* and *xeno-* meaning "same" and "foreign," respectively, in ancient Greek). The latter models are experimentally convenient, but the use of immunodeficient mice is potentially problematic because the immune system is known to be involved in combating cancers (see section 5.3.5). One can try to get around this problem by using mice with partially humanized immune systems (Wege, 2018), but such mice are difficult to use and expensive. Another problem is that the human cancer cell lines tend to acquire additional genetic modifications while they are maintained in vitro and adapt to those new conditions (Ben-David et al., 2018). Many cancer cell lines do retain some similarity to the original tumors, but the deviations tend to be significant (Domcke et al., 2013). Consequently, xenograft models using cancer cell lines have fallen out of favor with many cancer researchers (Auman, 2010).

Currently more popular are xenograft models that use patient-derived live tumor tissue rather than cancer cell lines. These patient-derived xenograft models are less convenient because patient-derived tumors can be difficult to procure. However, carefully curated "tumor banks" have been established and now offer a wide selection of different human tumors for research. A second challenge is that each patient-derived tumor can support only a limited number of transplant experiments. Researchers attempt to minimize this problem by extracting the tumors growing in previously xenografted mice and implanting pieces of those extracted tumors into additional mice

(effectively passaging the tumors). Unfortunately, some mouse cells tend to invade, and thus contaminate, the transplanted tumors, and this problem gets worse with each round of extraction-transplantation. Moreover, after several rounds of tumor passaging, the human tumor cells acquire genetic abnormalities that differ from those they would acquire in humans. Indeed, some evidence suggests that tumor cells face different selection pressures in patients versus mice, resulting in divergent tumor evolution (Ben-David et al., 2017). Yet another problem is that implanting tumor cells under the skin, rather than in the location of the original tumor, can affect how the resulting tumor spreads through the body (i.e., metastasis) (Day et al., 2015; Ireson et al., 2019). Of course, this last limitation is shared with the cell-derived xenograft models, as are the aforementioned problems associated with the use of immunodeficient mice.

Given all these limitations, it is not surprising that merely 5% to 11% of the anticancer drugs showing promise in mouse models have passed their clinical trials (Kola & Landis 2004; Ocana et al., 2011; Begley & Ellis 2012; Hay et al., 2014). Despite this high failure rate, the work has yielded major successes. Especially useful have been small molecules that inhibit one or more kinases that regulate cell proliferation. More than three dozen of these kinase inhibitors had been approved by 2018 (Bhullar et al., 2018); one of them is imatinib (aka Gleevec). This drug was strategically designed using in vitro assays but was then tested in a mouse isograft model before moving to clinical trials (Druker et al., 1996, 2001). It is used to treat chronic myelocytic leukemia, a rare type of bone marrow cancer featuring a unique chromosomal abnormality, as well as several other cancer types.

In addition to small-molecule drugs, synthetic antibodies are often used to target specific cancer-related genes. Trastuzumab (aka Herceptin), for example, is a humanized antibody that blocks a growth factor receptor called Her2, which is required for cell proliferation in breast cancers that express high levels of this particular protein (Harwerth et al., 1993; Mokbel & Hassanally, 2001; Pegram & Ngo, 2006). This very successful drug was derived from a xenograft model using a cancer cell line.

In short, extensive research on both animal and in vitro systems has led to the development of several highly specific anticancer drugs that each target a relatively small, highly specific subset of cancers. Importantly, the side effects of these treatments tend to be relatively mild, at least compared with general chemotherapy drugs.

The realization that cancers come in many different forms, each requiring a different treatment (Sánchez et al., 2017), ushered in the era of personalized medicine (aka precision medicine; see chapter 2). Of course, researchers had long realized that cancers found in different tissues require different treatments (e.g., breast cancer versus prostate cancer), but the ability to characterize cancers by their genetic mutations and expression profiles greatly expanded the number of different cancer types. This abundance,

in turn, stimulated the development of many different targeted chemotherapy drugs. It also required a shift in how clinical trials are run. Instead of testing a single drug on a heterogeneous population of cancer patients, scientists had to enroll subjects with the specific type of cancer targeted by the trial drug. As more and more drugs required testing, scientists designed very large trials that match different subject to different trial therapies based on the molecular details of their tumors. Such trials have yielded notable successes (Sánchez et al., 2017), but the vast majority of enrolled subjects end up without a match (Letai, 2017; Flaherty et al., 2020). Thus, the currently available set of targeted chemotherapy drugs addresses only a relatively small subset of cancers.

A related problem is that developing novel drugs for small groups of patients makes it difficult for pharmaceutical companies to recoup their investments, which helps explain the currently high costs of chemotherapy. Yet another troubling issue is that many of the drugs targeting specific cancer-related genes appear to work in mice even when the supposed targets are deleted (Lin et al., 2019), raising doubts about their supposed specificity.

5.3.5 Cancer Immunotherapy

A recent breakthrough in oncology has been the development of immune checkpoint therapy. This form of cancer treatment is based on the discovery, made in the 1990s, that the T cells of the body's immune system can be suppressed, notably by tumor cells (Pardoll, 2012). Some degree of T cell suppression is normal and adaptive, as it prevents the immune systems from attacking its own normal cells (i.e., autoimmunity). However, reducing the level of T cell suppression with immune checkpoint inhibitors can prompt the immune system to attack cancer cells while leaving normal cells in peace. This works because cancer cells accumulate so many mutations that the disinhibited T cells can distinguish them from normal cells.

The first checkpoint inhibitors were antibodies that partially inhibit CTLA-4, a receptor on the surface of T cells. After showing that these antibodies increase T-cell activation in primary cell cultures (Krummel & Allison, 1995), researchers tested them on mice injected with mouse tumor cells. The principal finding was that anti-CTLA-4 can prevent tumor growth and cause even established tumors to regress (figure 5.4). Although high doses of anti-CTLA-4 can lead to autoimmune problems, the drug's benefits can be quite long-lasting, presumably because the body's immune system develops a "memory" for the tumor cells and quickly attacks them if tumors recur. Based on these benefits, a humanized antibody against CTLA-4, called ipilimumab, was approved in 2010 for the treatment of metastatic melanoma.

The second immune checkpoint inhibitor to be developed targets a receptor called PD-L1 (programmed death ligand 1). When molecules that bind the PD-L1 receptor

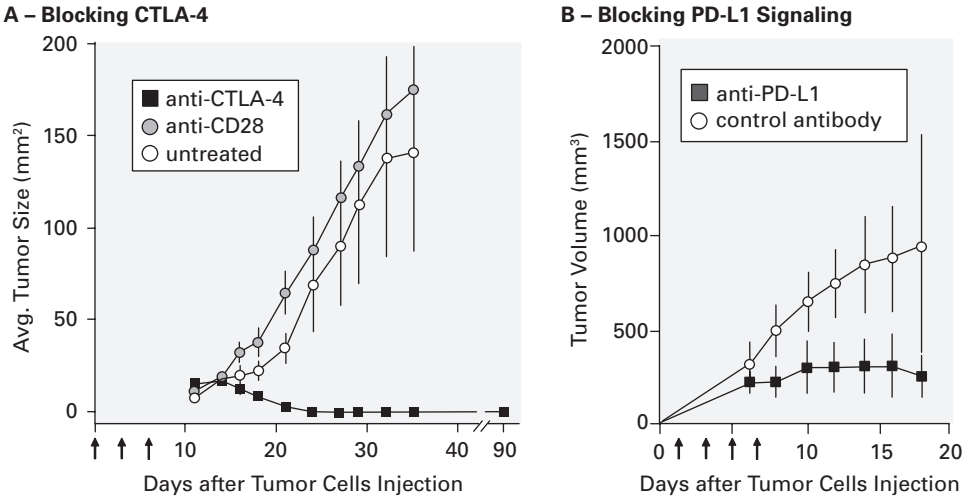

Figure 5.4

CTLA-4 and PD-L1 suppress the immune system's antitumor response. (*A*) Blocking CTLA-4. Leach et al. (1996) injected mice with cells from a mouse tumor cell line and then treated the mice with function-blocking antibodies against either CTLA-4 or CD28 (three sequential antibody injections indicated with arrows). Untreated controls and the anti-CD28 treated animals developed large tumors, whereas the anti-CTLA-4 treated mice exhibited tumor regression and then remained tumor free for at least 90 days. This finding suggests that CTLA-4 normally suppresses the immune system's ability to combat tumor growth. (*B*) Blocking PD-L1 signaling. Imai et al. (2002) injected mice with cells from a mouse tumor cell line expressing PD-L1. When these cells were co-injected with a normal rat immunoglobulin (control antibody), all the mice developed large tumors. By contrast, co-injecting anti-PD-L1 significantly inhibited tumor growth. Adapted from (A) Leach et al. (1996); (B) Iwai et al. (2002).

are expressed by cancer cells implanted into mice, tumor growth is accelerated (relative to tumor cells that lack this expression). More importantly, tumor growth is prevented when the implanted mice are treated with antibodies that block PD-L1 signaling (Fig 5.4B). Clinical trials ultimately led to the approval of several human antibodies that target PD-L1 for the treatment of several types of cancer (note that not all cancer types up-regulate CTLA-4 or PD-L1). In 2018, the Nobel Prize in Medicine and Physiology was awarded to James Allison and Tasuku Honjo for their work on CTLA-4 and PD-L1, respectively (see the appendix).

An interesting distinction between immune checkpoint therapy and the targeted chemotherapy we discussed in section 5.3.4 is that the former targets receptors on T cells of the immune system, whereas the latter targets the cancer cells directly. Nonetheless, a major similarity is that both types of cancer therapy work only for a rather limited set of cancer subtypes (Gay & Pradad, 2017). This narrowness of scope is expected for the tumor-targeting drugs but somewhat surprising for the immune checkpoint inhibitors. One likely explanation is that cancers vary in how effectively

they can trigger an immune response, even when the brakes on T-cell activation are removed. It is also true that not all tumors suppress the immune cells in the first place. Regardless of the details, the bottom line is that all cancer therapies are limited in scope and, therefore, cancer treatment generally varies with cancer type.

5.4 PATTERNS OF MODEL USE

Despite the ongoing crisis of translation in biomedical research (see chapter 1), medical progress over the last 150 years has been remarkable. Major epidemics have been controlled, and cancer patients now have a much better chance of survival than in the past. Many deaths have been delayed by early intervention (especially in cardiovascular diseases), and modern medicines tend to have fewer negative side effects than their predecessors. An excellent example is the increased selectivity of modern anticancer drugs, compared to radiation therapy and general chemotherapy.

Thus, Paul Ehrlich's dream of discovering a magic bullet that kills the disease-causing agent but does no harm to the patient has been realized for many diseases (Strebhardt & Ullrich, 2008). However, we now realize that many diseases, notably cancer, are in fact a collection of many different diseases that each require a different treatment. Moreover, many diseases do not yet have a magic bullet of their own, and different patients may respond differently to the same therapies even when they have the same disease (see chapter 7). In short, personalized medicine is both imperative and imperfect.

Animal and in vitro models have featured prominently in virtually all the medical advances we have reviewed (although it is hard to prove that they were *necessary* for the advances; see Matthews, 2008). In general, biomedical researchers have managed to discover or create model systems that reliably develop the disease of interest, or at least some symptoms reminiscent of the human disease (for more discussion of "partial models," see chapter 6). The scientists then expose their model to a variety of compounds or other manipulations (e.g., radiation) to test whether any of these treatments can prevent or reverse one or more of the disease-related attributes.

Historically, researchers have tested relatively large and semirandom sets of compounds that were either synthesized by chemists or isolated from microbes and plants. Beginning in the mid-1980s, the pattern changed. Biologists began to gather enormous amounts of information about the genetic and molecular aspects of the various diseases and then targeted specific disease-linked molecules and pathways for therapy development. Often they were able to design potential therapies "rationally" by creating antibodies against specific molecules or designing compounds likely to interact with putative binding sites.

Some observers have suggested that the adoption of more target-based screens has contributed to the current translational crisis, but this remains debatable (Swinney & Anthony, 2011). In any case, even target-based drug development typically requires multiple rounds of trial and error. As Paul Ehrlich used to say, success in medical research requires patience, skill, money, and luck (i.e., Geduld, Geschick, Geld, und Glück)—that is still true.

In vitro models have made significant contributions to many medical advances. The development of polio vaccines, for example, progressed much faster after scientists discovered how to grow the virus in cultured cells (see chapter 4). The discovery of statins and ACE inhibitors also owes much to in vitro systems, ranging from cell-free assays to isolated animal organs. Major developments in cancer therapy likewise built on knowledge gained from in vitro systems, including the carcinogenic viruses, the microbial systems that provided early insights into cell cycle control (i.e., yeast and *E. coli*), and the cultured T cells used to develop immune checkpoint therapy. Discoveries made in these in vitro models were always followed by in vivo animal studies, except in cases of exceptional urgency (i.e., the AIDS and COVID-19 pandemics).

For the therapies we reviewed here, the in vivo results were generally concordant with the in vitro data, but this is not surprising, given our focus on the most successful therapies. Even so, a few compounds were effective in vivo but not in vitro because they had to be metabolized by liver cells before they could exert the desired function (e.g., Prontosil). In addition, immortalized cell lines often produced dubious results because those cells tend to harbor serious chromosomal aberrations and, like all extensively cultured cells, acquire mutations that adapt them to the in vitro environment. Therefore, abandoning a research avenue after negative in vitro results might sometimes be ill-advised.

A major challenge of working with in vivo animal models is the existence of species differences. It is unfortunate, for example, that the viruses responsible for AIDS and hepatitis C naturally infect only a limited number of nonhuman primate species, or that the antiviral drug remdesivir is metabolized differently in rodents and primates. Similarly, the observation that penicillin is far more toxic to guinea pigs than other species had long been puzzling. Another odd aspect of rodent biology is that rats do not respond to compactin, which lowers cholesterol in many other species, including humans (Endo, 2010). In reaction to all these species differences, some observers have concluded that animal experiments are inherently misleading and should be abandoned (Knight, 2007; Langley et al., 2015).

In my view, a more productive response is to work with multiple species so that the species differences become more apparent and research avenues are not abandoned too early. In the words of Abraham Kaplan (1973), "The dangers are not in working

with models, but in working with too few, and those too much alike" (p. 263; quoted in van der Staay, 2006). Of course, this more comparative approach raises new questions of its own: for instance, how many species is enough? We will return to such questions in chapter 7. For now, it is useful merely to note that regulatory agencies have traditionally required preclinical results to be presented for at least two species, one of which should be a nonrodent. In toxicology, this dual-species approach seems to optimize the prediction of human results (Monticello et al., 2017) (see also chapter 4, section 4.3).

As we discussed in chapter 2, biologists often select their model systems for experimental convenience (i.e., Krogh's principle). In biomedical research, it is common to think that one should start with the most convenient model, which is often in vitro, and then work one's way up through "lower animals" (e.g., fruit flies) to "higher animals" (e.g., dogs or nonhuman primates) before beginning clinical trials. Indeed, many successful therapies began with in vitro research, and nonhuman primates are usually tested only after other models have been examined.

However, the model sequence is rarely linear. This is most apparent when in vitro models are literally combined with in vivo models, as in the transplant models for cancer and hepatitis C research. It is also unclear where human cultured cells fit into the supposed sequence because they are high on the species spectrum but lack in vivo complexity. Even more important is that "convenience" has many dimensions (e.g., cost, ease of breeding or experimental manipulation, ethical concerns), and what is most convenient depends on the experimental aims. For the last few decades, mice have been regarded as most convenient for nearly all in vivo experiments, while rabbits, guinea pigs, and other mammals have receded in popularity (see chapter 3). However, nonhuman primates have remained critical for determining safe and effective doses for human use.

All of which raises an interesting question: What about the non-mammalian in vivo models that, according to the 3Rs of Russell and Burch (1959), should replace mammalian models whenever possible (see chapter 2)? Although fruit flies, *Caenorhabditis elegans,* and zebrafish are all embraced by sizeable research communities and regularly used for high-throughput screening of drugs (see chapter 3), their contributions to medical breakthroughs have thus far been meager.

One possible explanation for the strong focus of the biomedical research community on mammalian models—and within mammals, the persistence of nonhuman primate models—is that even broadly conserved biological principles are often implemented differently in different species. That is, the principles may be conserved, but the molecular details often vary. Crucially, those species differences can impact how a system reacts to pathogens, toxins, genetic mutations, and potential therapies.

Biomedical researchers therefore seek (at least subconsciously) to study systems that tend to minimize those species differences; some even experiment on themselves, as in the case of Hering and Murrell tasting the nitroglycerin (see section 5.2.1). Although species differences do not always increase predictably with phylogenetic distance, they do increase on average (section 2.3.1; Striedter, 2019). Therefore, nonhuman primates tend to be more similar to humans than rodents or other mammals—so long as one averages across a multitude of traits. Similarly, nonprimate mammals tend to be more similar to humans than non-mammalian vertebrates or, for that matter, invertebrates.

We will return to these ideas in chapter 7. For now, it is interesting simply to point out that the two organ systems that have diverged most dramatically in evolution are the immune system, which featured heavily in this chapter, and the nervous system, which shall be our focus in the next.

Model Systems in Biology

History, Philosophy, and Practical Concerns

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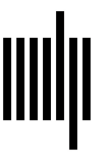

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