Part I

Basics

1

Historical Perspective

1.1 GENE THERAPY

1.1.1 Introduction

Humankind has been plagued with disease for centuries. From the most devastating pestilences such as the ancient black death and smallpox to the modern acquired immune deficiency syndrome (AIDS) epidemic, the suffering and death toll imposed on millions of human beings have continuously challenged medicine. The majority of those great killers of the past were associated with the ecological, nutritional and lifestyle changes brought about by human progress.[1] Although not so spectacular and abundant as infectious or acquired diseases, hereditary diseases have always attracted human curiosity. Perhaps this is associated to the tragedy of parents passing on a malady to their own children. Most certainly, medical and scientific communities have been lured by the fact that the origin of many of these hereditary diseases can be traced back to single molecular defects in genes (i.e., they conform to the Mendelian rules of inheritance).[2] As written by J. E. Seegmiller, an American pioneer in human genetics, "These disorders are experiments of nature that present unique opportunities for expanding our knowledge of many biological processes. Some of our fundamental concepts of the mechanism of gene action can be traced to basic studies of human hereditary diseases."[3]

Plasmid Biopharmaceuticals: Basics, Applications, and Manufacturing, First Edition.
Duarte Miguel F. Prazeres.

Against this background, it is thus not surprising to realize that one of the holy grails of medicine e has been to cure hereditary abnormalities by eliminating or correcting the associated defective genes. This idea of "genetic correction" surfaced in the scientific literature shortly after Avery, MacLeod, and McCarty in 1944 described that genes could be transferred within nucleic acids,[4] and well before the advent of molecular biology. In an article entitled "Gene Therapy," and in what was probably one of the first times the two terms have been used together, Keeler realized that plant and animal breeding sometimes results in the permanent correction of hereditary diseases.[5] This correction, as he described it, is brought about by the crossing of the afflicted individual with a "normal one," becoming effective in their offspring and in generations thereafter. At the time, Keeler did not envisage the use of such "gene therapy" to cure human genetic diseases but concluded that the strategy could be applied to correct physical, physiological, and behaviorist gene-based deviations in plants and animals.[5] Nevertheless, the notion of correcting genetic defects through breeding could hardly be foreseen as an effective therapeutic technique to treat genetic diseases in man. And, although termed by the author as gene therapy, the process described in his article was very far from the deliberate transfer of specific genes into subjects, which is nowadays readily associated with the concept of gene therapy.

Foreign DNA and genes have been routinely introduced into humans by a number of well-established therapeutic and prophylactic procedures, although in a haphazard and unintentional way. Consider, for instance, Edward Jenner's smallpox vaccination, a technique developed in 1790 that involved the inoculation into recipients of the vaccinia virus, the agent responsible for cowpox, one of deadliest infectious diseases ever to affect humans.[6,7] As a consequence of this new procedure, patients were brought into contact with millions of viral particles, each of them harboring the 223 genes of the vaccinia virus genome. Jenner's pioneering work opened the way to the development of a steady stream of vaccines based on live or attenuated microorganisms. As a result, millions of people undergoing immunization against specific diseases are injected every year with foreign genes concealed inside bacteria, virus, and the like.[8] Although the majority of these genes are probably cleared by the recipient's immune system, their exact fate and whether they remain functional or not once inside the body is not completely clear.

Another procedure that involves the delivery of a genetic cargo into humans is the experimental use of bacteriophages to treat bacterial infections.[9,10] This type of therapy was originally developed by Felix d'Herelle in 1916, at the Pasteur Institute in Paris, and was further popularized by Georgian doctors in the former Soviet Union.[11] Again, the administration of bacteriophages in cases of infection with microbes like *Staphylococcus aureus* or *Pseudomona aeruginosa* requires patients to be loaded with phages and their genes, even though these are, in principle, expressed only in the invading bacteria and not in the cells of the human recipient.

Although genes are effectively administered to humans as a result of both traditional vaccination and bacteriophage therapy, neither procedure can be categorized as gene therapy. Rather, the conceptualization of human gene therapy as we know it today was fueled by the immense progress made in biochemistry and genetics in the 1950s and early 1960s, which included the discovery of basic concepts in bacteria and bacteriophage genetics, the elucidation of the DNA double helix structure, and the uncovering of the central dogma of molecular biology.

1.1.2 The Coming of Gene Therapy

Perhaps no other scientist contributed more to the initial development of gene therapy than Joshua Lederberg, the recipient of the Nobel Prize in Physiology in 1958 (Figure 1.1). His pioneering work and vision mark him out as one of the greatest in genetics and life sciences.[12] Lederberg is to be credited not only for his scientific discoveries in bacterial genetics and plasmid biology but also by his prescience in anticipating gene therapy. This is clear from the 1963 article "Biological Future of Man," a piece written at a time when, in his own words, molecular biology was unraveling the "mechanisms of heredity."[13] In this visionary article, and among other prospects, Lederberg discussed and hinted at the control, recognition, selection, and integration of genes in human chromosomes. The prediction of a therapy based on the "isolation or design, synthesis and introduction of new genes into defective cells or particular organs" was enunciated in more detail by Edward Tatum in 1966, who even went as far as to envision the concept of *ex vivo* gene therapy.[14] Lederberg and a number of authors elaborated further on the topic in the subsequent years, as described in two detailed accounts of the earliest writings on human

Figure 1.1 *Joshua Lederberg at work in a laboratory at the University of Wisconsin (1958). Downloaded from http://commons.wikimedia.org/wiki/File:Joshua_Lederberg_lab.jpg.*

gene therapy.[15,16] The excitement at the time was such that DNA was viewed by one of the early pioneers as "the ultimate drug."[17]

The first human gene therapy experiments, that is, those that involved the deliberate transfer of foreign genes into human recipients with a therapeutic purpose, were performed in 1970 by the American doctor Stanfield Rogers.[16,18] Earlier in 1968, and on the basis of experiments that involved the addition of polynucleotides to the RNA of tobacco mosaic virus, Rogers and his colleague Pfuderer anticipated that viruses could be potentially used as carriers of endogenous or added genetic information to control genetic deficiencies and other diseases such as cancer.[19] This belief was put to the test in a highly controversial human experiment, in which Rogers and coworkers attempted to treat three German siblings who had arginase deficiency by injecting them with the native Shope rabbit papilloma virus.[18] This attempt was based on previous studies that had apparently shown that the Shope virus codes for and induces arginase in rabbits and in man.[20] In the trial, however, and contrary to what was expected, arginase was not expressed from the gene carried by the virus, and the efforts to supplement the missing enzymatic activity failed. Although Rogers's experiments raised a number of ethical questions, no institutional or legal precepts were violated then since at the time, no specific regulations on gene therapy or institutional review boards (IRBs) existed.[21] In spite of the flawed design and consequent failure of this clinical trial, Rogers was one of the first scientists to anticipate the therapeutic potential of viruses as carriers of genetic information.[18] That such a gene therapy experiment was attempted before the establishment of recombinant DNA technology in 1973 (discussed ahead in Section 1.2.3) is a tribute to Rogers's vision.

Exactly a decade later, in July 1980, Martin Cline at the University of California, Los Angeles (UCLA) headed a human trial designed to treat two young women who were suffering from thalassemia. By that time, recombinant DNA technology had established itself as a powerful tool in the biological and biomedical sciences[22] (see Section 1.2.3), and a number of techniques for genetic modification of cultured mammalian cells had been crafted, including calcium phosphate transfection.[23] Cline's study was built upon experimental evidence which had shown that murine bone marrow cells could be transformed *in vitro* with plasmids harboring genes that coded for proteins like the herpes simplex virus thymidine kinase (HSVtk)[24] or dihydrofolate reductase (DHFR).[25] Once the modified cells were transplanted into recipient mice, those genes were found to be fully functional. This conferred a proliferative advantage to transformant cells when submitted to the pressure of a selective agent such as the anticancer drug methotrexate.[24] Recognizing that the techniques for inserting and selecting for expression of genes were as applicable to animals as they were to tissue culture cells, Cline and coworkers reasoned that "gene replacement" could be useful to treat patients with malignant diseases or hemoglobinopathies, such as sickle cell anemia and thalassemia.[24]

In what was judged by many as a bold leap, Cline then decided to apply these methodologies in a human study of β^0-thalassemia, a disease character-

ized by the inability of the patient cells to synthesize the β-chain of hemoglobin, as a result of mutations in the hemoglobin beta gene. The experiment involved the removal of bone marrow cells from two patients, and their subsequent transformation *in vitro* with both the β-globin and the HSVtk genes.[26] The genes were carried independently by plasmids, and the calcium phosphate methodology was used to precipitate donor DNA and to transform the recipient cells. The higher efficiency of the HSVtk when compared with its human counterpart was expected to provide a selective proliferative advantage to marrow cells once these were transplanted back into the patients. Local irradiation was administered at the site of reinjection in order to provide space for the transformed cells to settle in the bone marrow. However, neither signs of gene (HSVtk or β-globin) expression nor improvements in the patient's health were detected. Furthermore, and in what was probably the most significant outcome of the experiment, the National Institutes of Health (NIH) in the United States ruled that Cline had broken federal regulations on human experimentation, even though permission had been granted by the foreign hospitals in Jerusalem and Naples, where the two experiments took place.[26] Among the consequences suffered, Cline had to resign chairmanship of his department at UCLA, lost a couple of grants, and had all of his grant applications in the subsequent 3 years accompanied with a report of the NIH investigations into his activities of 1979–1980.[27]

Although both Rogers's and Cline's trials were heavily criticized for scientific, procedural, and ethical reasons, their pioneering actions also contributed to the establishment of ethic-scientific criteria and guidelines for prospective human gene therapy experiments, and served as catalysts for the development of the field.[15,28,29] Most notably, the Recombinant DNA Advisory Committee (RAC) of the NIH in the United States intervened and created a new group in 1984, called the Human Gene Therapy Working Group (later the Human Gene Therapy Subcommittee), specifically to deal with and regulate the human use of molecular genetics.[29–31] From then on, "RAC approval" was mandatory for any gene therapy clinical protocol sponsored by the NIH. The document *Points to Consider in the Design and Submission of Human Somatic-Cell Gene Therapy Protocols*, adopted by the RAC in 1986, constitutes one of the key regulatory documents issued to provide guidance to researchers.[32] Later in 1991, the agency responsible for the regulation of pharmaceutical products in the United States, the Food and Drug Administration (FDA), published its own "Points to Consider in Human Somatic Cell Therapy and Gene Therapy," which focused on aspects like the safety, efficacy, manufacturing, and quality control of gene therapy products.[33] In Europe, the European Medicines Evaluation Agency (EMEA*) would eventually issue similar guidelines.[34]

* Since 2004, the EMEA changed its name to European Medicines Agency and started using the acronym EMA. However, the old name and acronym is still found in documents/Web sites created prior to December 2009 (A. Laka, Document and Information Services, EMA, pers. comm.). The newest designation will be used in the text body of this book, even though the older name and acronym might appear in specific documents on the reference list.

1.1.3 Early Clinical Trials

Once the regulatory framework was in place and some of the initial obstacles and ethical controversy had subsided, a number of human experiments ensued, many of which constitute milestones in the history of gene therapy (see Table 1.1) and served as nodes from which progress grew.[35] These include, for instance, the first federally approved (in the United States) human experiment in 1989/1990, which involved 10 patients with advanced melanoma.[36] In this trial, tumor-infiltrating lymphocytes (TILs) isolated from solid tumors were first marked with the *Escherichia coli* neomycin phosphotransferase gene (*NeoR*) using a retroviral vector and then were transferred back into the cancer patients.[36,37] Among other conclusions, the trial established that gene-modified TIL cells could be detected by polymerase chain reaction (PCR), either directly in tumor biopsies or after *in vitro* expansion of the tumor cells, and that the procedure did not harm patients.[38,39] Furthermore, transduced TILs were found in peripheral blood and tumor deposits 189 and 64 days after lymphocyte transfusion. Although the study involved the deliberate introduction of a foreign gene into human subjects, it qualifies better as a gene "transfer" rather than as a gene "therapy" trial. The NeoR/TIL trial was also the very first in a whole class of gene marking protocols designed to permanently mark specific cells so that their fate, distribution, and survival could be monitored during disease progression or in response to any form of conventional therapy.[39] An advantage of genetic over traditional physical marking methods, which typically use dyes or radiochemicals, relates to the fact that integration of the marker gene ensures that the "label" is not diluted out by cell division, thus allowing the long-term follow up of the cell progeny.[39]

Shortly after the NeoR/TIL gene marking trial, in September 1990, a gene therapy protocol was initiated to treat a 4-year-old girl afflicted with adenosine deaminase (ADA) deficiency, a rare but fatal disease.[39,40] A second, 9-year-old patient, was later enrolled in the same trial.[40] The lack of ADA, a key enzyme in the metabolism of purines, results in an accumulation of deoxyadenosine, especially in the patient's T lymphocytes, with the consequent impairment of the immune system. ADA patients are thus afflicted by a severe combined immunodeficiency (SCID), which makes them highly susceptible to common infectious agents.[40] The ADA gene therapy protocol involved the isolation of lymphocytes from the patient's blood, the *in vitro* cultivation and expansion of the T-lymphocyte subset, and the introduction of the ADA gene via a retroviral vector. Following expansion, the corrected cells were infused back intravenously.[41,42] This protocol not only confirmed the safety of the different procedures implicated in the therapy but also resulted in a positive response from both patients. Specifically, an increase in the amount of ADA in the T cells was detected and the number of modified lymphocytes remained nearly constant in between treatments.[39] Furthermore, the gene therapy intervention was accompanied by an improvement in antibody responses to *Hemophilus influenzae* B and tetanus toxoid vaccines, indicating at least some immune reconstitution. In spite of these biochemical and physiological changes, doubts

TABLE 1.1. Milestones in the Development of Human Gene Therapy

Year	Target Disease	Description
1970	Argininemia	In the first human gene therapy trial, three siblings were injected with the native Shope papilloma virus in an attempt to improve arginine levels.[16,18]
1980	Thalassemia	In the second human gene therapy trial, two young women in Israel and in Italy were reinfused with their own bone marrow cells, which had received *in vitro* the β-globin and the HSVtk genes via plasmid-mediated transformation.[26]
1984	–	The NIH created the Gene Therapy subcommittee of the RAC to deal with and to regulate the human use of molecular genetics.[29] The document "Points to Consider in the Design and Submission of Human Somatic-Cell Gene Therapy Protocols" was issued 2 years later.[32]
1990	Cancer	In the first federally approved gene marking study in humans, TIL cells isolated from tumors were marked with the *E. coli NeoR* gene using a retroviral vector and were transferred back into patients.[38]
1990	ADA-SCID	The first federally approved human gene therapy trial involved the removal of white blood cells, *in vitro* cell growth, insertion of the missing gene, and infusion of the modified blood cells back into the patient's bloodstream.[40–42]
1991	–	The FDA publishes the "Points to Consider in Human Somatic Cell Therapy and Gene Therapy."[33]
1991	Malignant melanoma	First federally approved human gene therapy trial to target a complex genetic disease. TIL cells isolated from tumors were transduced with the *TNF* gene using a retroviral vector and were transferred back into patients.[36]
1993	Cystic fibrosis	First human gene therapy trial with an adenovirus vector.[143]
1999	OTC deficiency	Death of patient due to a fulminant systemic inflammatory response syndrome developed in reaction to the recombinant adenovirus vector.[47–49]
2000	SCID-X1 syndrome	The immune dysfunction of two patients is apparently cured after the reinfusion of autologous cells transduced *ex vivo* with a retroviral vector hosting the γC gene.[52]
2003	SCID-X1 syndrome	Development of leukemia-like syndrome in recipients due to retrovirus integration and proto oncogene activation.[53]
2004	Head and neck carcinoma	The first human gene therapy product, Gendicine®, received approval from the Chinese FDA.[57]
2007	Arthritis	Death of patient after receiving two injections of an AAV hosting the *TNFR:Fc* gene. The death was attributed to a prior infection and not to gene therapy.[63,64]

ADA, adenosine deaminase; *NeoR*, neomycin phosphotransferase gene; OTC, ornithine transcarbamylase; RAC, Recombinant DNA Advisory Committee; SCID, severe combined immunodeficiency; TIL, tumor-infiltrating lymphocyte.

about the exact role played by gene therapy in this case were raised given that the patients underwent replacement treatment with a conjugate of polyethylene glycol and ADA prior to and after the gene therapy intervention.[40]

Although gene therapy was originally thought of as a strategy to treat classical Mendelian genetic diseases like thalassemia or ADA, it soon became apparent that the concept could be extended to manage multifactorial diseases like cancer, arthritis, or cardiovascular diseases. These diseases are not directly linked to single major genetic abnormalities but are often caused by a combination of environmental factors and genetic predisposition. In many cases, more than one gene may be involved in the onset and progression of the disease.[39,43] Infectious diseases such as hepatitis B, ebola, or AIDS constitute another whole category of targets for gene therapy, both from a therapeutic and prophylactic point of view. Whether in the case of multifactorial or infectious diseases, genes can be used as purveyors of any kind of genetic information that, once expressed *in vivo* as a protein, would provide cells with a new function that would contribute to treat, cure, or prevent the target disease (see Chapter 2 for more details on the specific roles of gene products).

Cancer was the first non-Mendelian target addressed by gene therapy. Previous studies on the therapeutic use of TIL in experimental animal models had shown that secretion of tumor necrosis factor (TNF), a powerful anticancer agent, plays an important role in the regression of established lung metastases.[44] A phase 1 safety trial (see Chapter 3 for a brief description of the clinical development of medicinal agents) was designed accordingly, which aimed to immunize patients with advanced malignant melanoma against their cancers. Briefly, a retroviral vector was used to introduce the gene coding for TNF into autologous TILs[36,45] (Table 1.1). The goal of the trial, which started in January 1991, was thus to make the TNF-expressing TIL more effective against the melanoma. Given the tendency of TIL to accumulate in tumor deposits, the promoters of the study were expecting to deliver high local concentrations of TNF that could destroy the tumor without exposing patients to the high systemic toxicity associated with intravenous injections of TNF alone. An objective response was ongoing in one of the 10 patients, 2 years after treatment.[36] Another approach to improve cancer immunotherapy relies not on adding a specific cytokine gene to TIL or tumor-specific T cells but to the tumor cells themselves, in order to make them more immunogenic.[35] In one of the earlier cancer gene therapy trials of the sort, five patients with advanced cancer were immunized with live autologous tumor cells that had been genetically modified *ex vivo* to secrete either TNF or interleukin 2 (IL-2). No evidence of viable tumor cells was found when the injection sites were surgically resected 3 weeks after the therapeutic intervention.[36]

1.1.4 Failures and Successes

The first serious setback faced by gene therapy came in 1999 when Jesse Gelsinger, a young man suffering from ornithine transcarbamylase (OTC)

deficiency, an X-linked inborn error that affects urea synthesis, died after the administration of an adenovirus vector encoding OTC.[46–49] The first symptoms appeared 18 h after the recombinant adenovirus was infused into the right hepatic artery at a dose of 6×10^{11} particles/kg. The cause of the subsequent patient's death, which occurred 98 h after gene transfer, was attributed to a fulminant systemic inflammatory response syndrome developed in reaction to the adenovirus vector.[49] The effects of gene transfer in the other 17 patients who had enrolled in the trial were, on the contrary, limited to transient myalgias and fevers, and biochemical abnormalities. This seems to indicate that Gelsinger had predisposing factors to vector toxicity.[50] The direct consequences of this tragic event included the halting of several gene therapy trials by the FDA and the payment of fines amounting to more than 1 million dollars by the institutions concerned, the University of Pennsylvania, and the Children's National Medical Center in Washington, as ordered by the U.S. Department of Justice. Furthermore, the lead researchers of the study faced severe restrictions to their clinical research activities.[51]

The year 2000 saw gene therapy's first major success: a gene therapy protocol held in Paris was able to correct the phenotype of an X-linked severe combined immunodeficiency (SCID-X1) syndrome in two young patients. Specifically, the protocol involved the reinfusion of the patients with autologous CD34 bone marrow cells that had been transduced *ex vivo* with a retrovirus vector encoding the interleukin 2 receptor, gamma gene (γC).[52] These successes were later shadowed by the development of leukemia-like clonal lymphocyte proliferation in 4 of the 10 recipients of the treatment, as a consequence of the integration of the retrovirus vector in a number of sites located at or nearby genes such as *LMO2*, *BM1*, and *CCDN*2.[53,54] Although three of these four patients responded well to chemotherapy treatment, the fourth died in October 2004.[55] In spite of this unfortunate event, the follow-up of the trial clearly showed that the gene therapy procedure resulted in a direct benefit to patients, with a complete and stable restoration of the immunological phenotype extending for a number of years post-treatment.[54] A similar trial for SCID-X1 conducted in London recently yielded strikingly similar results: 1 of the 10 treated patients who had their immune dysfunction corrected was diagnosed with leukemia. Furthermore, the underlying mechanism of leukemogenesis was also associated with vector integration in a site nearby the proto-oncogene *LMO2*.[56]

In October 16, 2003, a recombinant adenovirus vector expressing the tumor suppressor gene p53 was approved by the State Food and Drug Administration (SFDA) of China for the treatment of head and neck squamous cell carcinoma.[57–59] Developed and manufactured by the Chinese firm Shenzen SiBiono GeneTech and trademarked under the name Gendicine, it became the first ever human gene therapy product to reach the market in April 2004.[57] According to SiBiono, as of October 2007, more than 400 hospitals in China had treated over 5000 patients with Gendicine, including some from several Western countries.[60] The skepticism of the international community over

Gendicine remains high, however, since accurate information regarding the design and outcome of the clinical trials that preceded approval is not available in non-Chinese, scientific journals.[55] China kept and reinforced its leading role in the development of commercial gene therapy when, in November 2005, the SFDA approved Oncorine®, a genetically modified adenovirus for head and neck cancer, which can selectively kill tumor cells with dysfunctional *p53* genes.[61,62]

In July 2007, a gene therapy patient undergoing treatment for arthritis died from massive organ failure after having her knee injected with an adeno-associated virus (AAV) vector. The vector contained the tumor necrosis factor receptor-immunoglobulin Fc fusion gene (TNFR:Fc), an anti-inflammatory protein that inhibits the cytokine tumor necrosis factor α (TNFα).[63,64] The trial, which had enrolled more than 100 patients, was put on clinical hold on cautionary grounds,* even though preliminary investigations associated the tragic outcome with a prior fungal infection. The hold was subsequently lifted after a detailed review by the RAC of the NIH ruled out the product of the transgene and its vector as the causative agent, and confirmed that the cause of death was disseminated histoplasmosis.[65]

In Europe, reference should be made to Cerepro®, an adenovirus-mediated gene-based medicine for brain cancer developed by Ark Therapeutics (Finland), which is currently being reviewed by the EMA for market authorization. The product combines the adenovirus-mediated local administration of the thymidine kinase from *herpes simplex* with the intravenous injection of the prodrug ganciclovir. The enzyme converts ganciclovir into a substance that specifically kills the dividing tumor cells without affecting the surrounding healthy cells[62] (see further details on this type of therapy in Chapter 2, Section 2.4.2.2). Although a previous request by Ark Therapeutics for marketing authorization had been refused by the EMA in 2007, on the grounds that the benefit–risk of Cerepro/ganciclovir had not been demonstrated then,[66] the expectation that the new set of phase 3 clinical data might convince the agency to turn Cerepro into the first gene therapy medicine to be marketed in the West is high.[62]

1.2 PLASMIDS

1.2.1 Introduction

The contributions of plasmids to molecular genetics and biology have been immense, as described in detail in a number of reviews by some of the most prominent scientists involved in the field.[67,68] In this section, a short summary of the most important discoveries and developments is provided, with a special emphasis on those that opened the way to the establishment of plasmid biopharmaceuticals.

* FDA Statement on Gene Therapy Clinical Trial, July 26, 2007.

1.2.2 Early Beginnings

Joshua Lederberg (Figure 1.1) devised the term plasmid in 1952[69] by joining the word cytoplasm with the Latin particle *-id*,[67] a suffix used to mean "a thing connected with or belonging to." According to the terminology used by Novick and coworkers in a nomenclature proposal made in 1976, "A plasmid is a replicon that is stably inherited (i.e., readily maintained without specific selection) in an extrachromosomal state."[70] Plasmid, which favorably contended with the earlier word plasmagene (see Lederberg[67] for further details), was thus intended to serve as a generic term for any genetic particle or element that is physically separated from the chromosome of the host cell and is able to be perpetuated in this condition.[69,71] From a functional point of view, the role of plasmids is to mediate gene flow within, and between bacterial species. They constitute a means of storing extra genetic information outside the genome of prokaryotes. Bacteria typically resort to this pool of cytoplasmic genes, which can be found dispersed across different populations, when faced with environmental changes or stresses that require adaptations for survival. The variety of plasmid-encoded genes found in nature is huge, ranging from genes that confer resistance to agents like antibiotics or heavy metals to genes that broaden the metabolic properties or confer pathogenicity to the host.[72] For instance, many of the genes that encode for restriction enzymes, the molecular tools that made recombinant DNA technology possible, are carried by antibiotic resistance plasmids. The recognition that the spread of antibiotic resistance among bacteria was frequently linked to plasmids further spurred the interest in their study.

Plasmid-mediated gene exchange among bacteria was originally described by microbial geneticists involved in the study of bacterial mating. This process was conceived for the first time as an unidirectional process involving a gene donor and a gene acceptor by Williams Hayes.[73] Soon after, the transmissible factor F (for fertility), "an ambulatory or infective hereditary factor," became one of the first plasmids to be identified and studied,[74,75] even though its exact physical and molecular nature was not readily recognized at the time. The demonstration that plasmids are made up of DNA was first presented by Marmur and coworkers in 1961 while studying the transfer of the F-factor from *E. coli* to *Serratia marcescens*.[76] This discovery was confirmed shortly after with further evidence gathered from the study of the transmission of colicinogenic factors by Silver and Ozeki.[77] The next important contribution to the understanding of the nature of plasmids was provided by Campbell, who conceptualized that episomes, a type of DNA molecule that, like plasmids, traffics in and out of cells but, unlike them, interacts with chromosomes, must exist with a circular structure.[78] This was an important departure from the established notion of DNA molecules as long, linear biopolymers. The first confirmation of Campbell's hypothesis was provided by Fiers and Sinsheimer, who demonstrated that the double-stranded DNA from the phage phi-X174 is circular.[79] Additional insights into the molecular structure of plasmids came

from the study of the circular DNA molecule of the polyomavirus. An important contribution was made in 1965 by Vinograd and coworkers, who described the presence of a "twisted circular structure" containing left-hand tertiary turns in polyoma DNA. They further demonstrated that this structure could be converted to a less compact, open circular duplex by introducing a single strand break.[80] Shortly after, Hickson et al. reported that electronic microscopic preparations of an isolated bacterial sex factor (i.e., a plasmid) showed a circular DNA molecule that similarly contained the tightly twisted and the open circular forms. The electron micrographs in Hickson's paper probably constitute the first visual record of supercoiled plasmid DNA molecules.[81] As we will see next, another report published in the same year, which would have far-reaching implications for all those involved in DNA and plasmid research, described that the intercalating agent ethidium bromide emitted an intense orange-red fluorescence when bound to DNA.[82]

1.2.3 Recombinant DNA

Three proximal scientific discoveries were at the heart of the invention and development of cloning, a pivotal technology that would radically change molecular biology and have a huge impact in the development of gene therapies. The first of these discoveries was the demonstration that bacteria treated with calcium chloride were able to uptake plasmids, and that such "transformed" bacteria could stably generate a progeny that contained replicas of the original plasmid.[83] Furthermore, the presence of antibiotic resistance genes in the plasmids made it possible to select transformed from nontransformed cells using media supplemented with the corresponding antibiotic. The combination of agarose gel electrophoresis with low concentrations of ethidium bromide was another important contribution that revolutionized the analysis of DNA fragments, which had hitherto relied on the lengthy staining and destaining of autoradiographs.[84] The third breakthrough was the isolation of the EcoRI restriction enzyme from an antibiotic resistant strain of *E. coli* and the discovery that the double-stranded DNA cut by it had cohesive termini.[85,86] This set the stage for the advent of recombinant DNA technology, as succinctly described next.

In 1973, Cohen and coworkers ingeniously combined the discoveries described above and performed the first cloning experiments.[22] In the first step of the process, a plasmid (pSC101) that contained the gene for tetracycline resistance was cut at a single site with the EcoRI restriction enzyme. Donor DNA was also treated with EcoRI, yielding multiple fragments. When these fragments were mixed with the open pSC101, complementary base pairing took place between the cohesive ends of the plasmid and of the individual fragments of the donor DNA. Subsequent ligation with DNA ligase thus generated recombined or "recombinant" DNA molecules of pSC101 with inserted DNA fragments. In the next step, these replicons were inserted into *E. coli* cells by using the calcium chloride transformation procedure.[83] Cells trans-

formed with the plasmids were then selected by cultivation in a medium containing tetracycline. This prevented the growth of nontransformed cells but fostered the proliferation of the transformed ones. An agarose gel electrophoresis/ethidium bromide analysis of the recombinant plasmid isolated from these selected clones then showed that they contained "genetic properties and DNA nucleotide sequences of both parent molecular species."[22] Reports soon appeared demonstrating that genes derived from totally unrelated bacterial (e.g., *S. aureus*[87]) and eukaryotic (e.g., *Xenopus laevis*,[88] mouse[89]) species could be replicated in *E. coli* cells. Furthermore, those genes could be expressed in the bacterium, yielding biologically active "recombinant" proteins like somatostatin[90] or insulin.[91] Once the ability to clone virtually any gene into a plasmid and subsequently to express it in a bacterial host was mastered, researchers looked into the possibility of delivering functional genes via plasmid vectors to cultured mammalian cells. Methods were rapidly developed that combined cellular transfection techniques such as calcium phosphate,[23] diethyl aminoethyl (DEAE)-dextran,[92] and liposomes with plasmids encoding genes under the control of mammalian promoters (see Scangos and Ruddle[93] for an earlier review on the mechanisms and applications of DNA-mediated gene transfer in mammalian cells).

The crafting of recombinant DNA technology represented in many aspects a turning point for molecular biology. With the new technique and its basic tools (restriction enzymes, plasmids, and *E. coli*), scientists could now manipulate DNA and genes at will. Many consider also that the multibillion dollar biotechnology industry was born with recombinant DNA. One of the reasons for this is related to the fact that the 1984 "Cohen and Boyer patent" that protected the new discovery and granted exclusive rights to Stanford University[94] would ultimately realize around 300 million dollars in licensing deals with companies like Amgen, Eli Lilly, or Genentech. More importantly, the new technology would eventually make it possible to produce unlimited amounts of medically and industrially relevant proteins from any organism in bacteria. For example, recombinant insulin, which was marketed in 1982, became the first of a stream of commercially successful protein biopharmaceuticals that radically altered the pharmaceutical business.

1.3 PLASMID BIOPHARMACEUTICALS

1.3.1 Introduction

The majority of the earlier gene transfer and gene therapy experiments involving humans and animals resorted to viral vectors as carriers of the therapeutic genes, essentially by using cell-mediated *ex vivo* approaches. However, the ability of viral vectors to recombine, interact with endogenous viruses, and integrate raised safety concerns right from the time the first gene therapy experiments were attempted.[35] The fact that all of the severe adverse events of gene therapy reported so far have resulted from trials in which

recombinant viruses were used as vectors underscores these concerns. The use of nonviral gene carriers like plasmids, on the other hand, has been always regarded as a potentially safer alternative.[95] Plasmids had already played a prominent role in the first human experiment that resorted to recombinant DNA technologies, the thalassemia experiment headed by Martin Cline.[25,26] As seen in Section 1.1.2, the genes that were transferred *ex vivo* to the patient's bone marrow cells were hosted in a plasmid, the notorious pBR322 vector. In this case, however, plasmids were used as a tool for the genetic transfection of cells, which then constituted the therapeutic agent themselves. In the following pages, some of the milestones that marked the development of plasmid biopharmaceuticals (also shown in Table 1.2) are briefly described.

TABLE 1.2. Milestones in the Development of Plasmid Biopharmaceuticals

Year	Comments
1983	*In vivo* expression of a gene (rat preproinsulin) harbored in a plasmid is demonstrated for the first time after intravenous injection of carrier liposomes in mice.[96]
1983	The concept of targeted gene expression is demonstrated by the *in vivo* injection of plasmids complexed with liposomes carrying ligands toward cell surface receptors.[99]
1985	The uptake of a plasmid DNA vector in a phosphate buffered saline by mouse liver cells was demonstrated after *in vivo* intravenous injection in mice.[100]
1989	Integration of reporter genes into the host genome is demonstrated after the *in vivo* intravenous injection of plasmid-carrier complexes in mice.[144]
1990	Direct intramuscular injection of "naked" plasmid DNA in mice is taken up by muscle cells, and the encoded reporter gene is expressed *in vivo*.[98]
1990	Genes encoded in plasmid are delivered *in vivo* to the liver, muscle, and skin tissues of rats and mice by particle bombardment.[104]
1991	The possibility of eliciting an immune response against a foreign protein by the introduction of the corresponding gene directly into the skin of mice is demonstrated for the first time.[114]
1991	Skin cells of mice are transformed by plasmid DNA with the aid of *in vivo* electroporation.[129]
1993	The direct administration of naked plasmid DNA encoding a pathogen's (influenza virus) antigen in mice elicits a cell-mediated response and confers protection against a subsequent challenge with the pathogen.[115]
1994	In the first human gene therapy clinical protocol to use plasmid vectors, the gene encoding for the MHC protein HLA-B7 is introduced into advanced melanoma patients to improve tumor immunogenicity.[117]
1994	The name DNA vaccine is selected among others to designate plasmid-based vaccination technology.
1996	The stimulation of the immune system by CpG motifs cloned in plasmids is demonstrated.[120]
1996	Alphavirus replicon elements are added to plasmid vectors to increase the number of copies of mRNA.[127]

TABLE 1.2. ***Continued***

Year	Comments
1997	Encapsulation of plasmid DNA in PLG microparticles is described. This mode of delivery protects plasmids against degradation after administration and facilitates uptake, expression, and antigen presentation.[130]
1997	Compaction of single plasmid DNA molecules into minimally sized nanoparticles is described. The complexes can efficiently transfer and express the DNA information both *in vitro* and *in vivo*.[131]
2002	Plasmids are used to direct the intracellular synthesis of siRNA transcripts in mammalian cells, with the concomitant downregulation of the genes targeted for knockdown.[132]
2003	Californian condors become the first animals immunized with a DNA vaccine to be deliberately released into the environment in an attempt to save a local population from WNV infection.[134]
2005	Prophylactic vaccines that protect horses against WNV and farm-raised salmon against infectious hematopoietic necrosis virus become the first ever DNA vaccines to receive marketing approval.[135,137]
2007	The first therapeutic DNA vaccine is conditionally approved to treat melanoma in dogs.[138]
2008	A plasmid encoding for the growth hormone releasing hormone is approved in Australia to decrease offspring morbidity and mortality in pigs.[140]

PLG, poly(DL-lactide-co-glycolide); siRNA, small interfering RNA; WNV, West Nile virus.

1.3.2 The Initial Experiments

One of the first scientific accounts of an experiment in which a plasmid DNA molecule harboring a gene was administered directly into a live animal appeared in 1983[96] (Table 1.2). In their studies, Nicolau and coworkers used liposomes made up of phospholipids and cholesterol to encapsulate a recombinant plasmid encoding the preproinsulin gene.[96] This gene had been isolated from rat and comprised, among others, a putative promoter site for the initiation of transcription.[97] Following intravenous injection of the plasmid-loaded liposomes into rats, the authors found that blood glucose and insulin were respectively lower and higher when compared with the corresponding parameters in control animals. Further experimental evidence confirmed that the injected liposomes had been taken up essentially by the spleens and livers of the animals. These results led to the unambiguous conclusion that the insulin gene had been expressed *in vivo*, under the control of the referred putative promoter.[96] Interestingly, the researchers also described a control experiment in which free, nonencapsulated plasmid DNA solubilized in a tris(hydroxymethyl aminomethane) hydrochloride (tris–HCl)/NaCl buffer was injected intravenously in mice. In this case, however, the levels of insulin and blood glucose remained unchanged. To the best of my knowledge, this was the first time ever that plain (i.e., "naked," as it would be later referred to) plasmid DNA was injected

in vivo.[96] Seven years would have to pass before *in vivo* expression from naked plasmid DNA was described by Wolff and colleagues.[98]

Nicolau's group also attempted to increase the uptake of the preproinsulin gene by hepatocytes, by incorporating a glycolipid terminated with a β-galactose residue in the carrier liposomes. The hypothesis that β-galactosyl receptors on the surface of hepatocytes would increase the uptake of the liposomes and plasmid was confirmed, albeit this was also observed with endothelial cells, which lacked the aforementioned receptor.[99] Controls in which free plasmid was injected were also performed, but no signs of uptake were detected. Shortly after, attempts aimed at elucidating the *in vivo* intracellular fate of liposome–plasmid complexes injected intravenously in mice were reported. In these experiments, Cudd and Nicolau injected a radiolabeled pBR322 plasmid vector encapsulated in phospholipids/cholesterol liposomes in the tail vein of mice and then used electron microscope autoradiography to analyze liver tissue samples.[100] At that time, they concluded that liposome–plasmid DNA is selectively transported among organelles in the liver cells, mainly to the lysosomes, the mitochondria, and the nuclei. Again, a control experiment in which free plasmid DNA in phosphate buffered saline (PBS) was injected intravenously was described. In this case, the majority of the DNA that was taken up by the liver was found associated with the endoplasmatic reticulum and degraded 5 min after injection. Even though the model plasmid did not carry any transgene, plasmid uptake was unequivocally demonstrated for the first time after *in vivo* injection of naked plasmid DNA.[100]

Shortly after, Dubensky et al. prepared a calcium phosphate-precipitated plasmid DNA vector harboring the DNA from polyomavirus and injected it directly into the liver and spleen of mice.[101] They concluded that plasmid DNA was not stable after transfection due to degradation in the target organs. Benvenisty and Reshef also evaluated the potential of calcium phosphate to mediate the *in vivo* introduction of genes into rats. The selected chloramphenicol acetyltransferase (CAT), hepatitis B surface antigen (HBsAg), and human growth and preproinsulin genes were harbored in plasmids, precipitated with calcium phosphate, and injected intraperitoneally into newborn rats. All genes were expressed by the animal tissues, albeit transiently and with large variations among individuals. Additionally, they concluded that the injection method used favored the distribution of the genetic material in the liver and spleen.[102]

Although the calcium phosphate methodology was widely used at the time, alternative ways of delivering plasmids to target cells gradually emerged. Building up on the previous liposome work,[96,99] Felgner described the synthesis of a cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*, *N*, *N*-trimethylammonium chloride (DOTMA), the spontaneous formation of plasmid–DOTMA complexes, and their use in the transfection of cells *in vitro*.[103] The effectiveness of the technique was attributed to the formation of positively charged complexes that completely neutralize and entrap the DNA and facilitate fusion with the negatively charged surface of cells. When compared with calcium phosphate or DEAE transfection, the process was found to be from 5- to > 100-fold more

effective. The process was termed "lipofection" by the authors, a word that has since entered the vocabulary of gene therapy.[103]

Particle bombardment, a technique that would radically alter the way in which plasmids were being administered into animals was developed roughly at the same time.[104] The method was originally developed to deliver nucleic acids to plant cells in calluses and leaves[105] but was soon extended to animal cells cultured *in vitro*.[106] It relies on the coating of plasmids onto fine metallic particles of tungsten or gold, with diameters typically in the 0.1- to 5.0-μm range. The use of a device that accelerates the particles to high velocity then allows penetration of target tissues or organs. The method was used in live animals for the first time in 1990 by Yang and coworkers, who demonstrated that the *in vivo* bombardment of liver, muscle, and skin tissues of rat and mice enabled the transient expression of the reporter genes *CAT* and β-galactosidase.[104] Ever since, handheld biolistic systems or "gene guns" have been one of the favorite methods of plasmid DNA delivery. Although in the original report a gunpowder charge was used to propel the particles,[105] high-voltage electric discharge devices[104] and pressurized gases such as helium[107] can also be used for the same purpose.

1.3.3 Naked Plasmid DNA

In 1990, Wolff and coworkers pushed the boundary further and injected plain saline solutions of plasmids containing genes for CAT, luciferase, and β-galactosidase into skeletal muscle of live mice.[98] They found out that such a naked plasmid DNA molecule, devoid of any kind of adjuvant, could be taken up by the mice's cells and that the encoded reporter transgenes were expressed within the muscle cells. In the case of luciferase, substantial activity could be detected in the muscle for at least 2 months. The experimental data suggested that this persistence of activity was not related to the stability of luciferase or of its RNA transcript, but rather to the extrachromosomal lingering of the injected plasmid DNA inside the muscle cells. Nevertheless, the possibility of chromosomal integration of plasmid DNA was not excluded. The high uptake of plasmid DNA and expression levels of the reporter proteins in the muscle were attributed to structural features of this type of tissue, including its multinucleated cells, sarcoplasmic reticulum, and transverse tubule system. The expression of transgenes hosted in plasmids following their direct intramuscular injection was shortly after demonstrated to occur also in species as varied as fish,[108] chicken,[109] and cattle.[110] And transfection by direct injection of naked DNA was soon found in tissues other than the skeletal muscle, like heart,[111] liver,[112] and brain.[113]

1.3.4 DNA Vaccines

In concluding their seminal paper, Wolff and his colleagues envisaged the use of the method of direct transfer of genes via naked plasmids and into human

muscle, as a means of (1) improving the effects of genetic diseases of muscle and (2) expressing genes encoding antigens to provide alternative approaches to vaccine development.[98] The experimental demonstration of this last possibility was left to Tang, DeVit, and Johnston, who showed in 1992 that it was possible to elicit an immune response against a foreign protein by introducing the corresponding gene directly into the skin of mice.[114] The experiment involved the use of a gene gun to inoculate gold microprojectiles coated with plasmids containing the human growth hormone (hGH) or human α1-antitrypsin (hAAT) genes in the ears of mice. In either case, antibodies to both proteins were detected in the sera of the genetically immunized mice. Furthermore, secondary and tertiary inoculations of the immunized mice with the same plasmids showed conclusively that the primary response could be augmented by those subsequent DNA boosts. Overall, the data gathered led the authors to speculate that genetic vaccination of animals against pathogenic infections could be achieved by using plasmids with genes encoding for specific antigens. Natural infections could thus be mimicked by a gene-based process that involved the production of the foreign antigens in the host cells. This approach constituted a radical departure from the established immunization methodologies that required the external production and purification of the vaccinating antigens prior to their administration. The fact that the corresponding immunological response could be different in terms of antibody production and T-cell response when compared with conventional immunization did not escape the authors' attention either.[114]

The use of plasmids as carriers of antigen information for immunization purposes was validated shortly after by Ulmer and coworkers, with the quantum leap discovery that mice could generate cytotoxic T lymphocytes (CTLs) in response to the direct administration of naked plasmid DNA encoding the influenza A virus nucleoprotein (NP).[115] Following immunization by intramuscular injection, a series of assays enabled the detection of NP expression, NP-specific antibodies, and NP-specific CTLs. Although high titers of anti-NP immunoglobulin G were detected, these antibodies did not confer protection to the mice. The specificity of CLTs and their ability to detect the epitopes generated naturally were confirmed in an experiment, which showed that CTLs isolated from the immunized animals were able to recognize and lyse target cells infected with the influenza A virus. Furthermore, the cell-mediated immune response was found to be functionally significant since the immunized mice were protected from a subsequent challenge with a heterologous strain of influenza A virus, as measured by increased survival, inhibition of mass loss, and decreased viral lung titers. In the concluding remarks of their paper, the authors speculated that plasmid-based vaccination should not be restricted to the prophylaxis of infectious diseases but that it could eventually be used to elicit an immune response against tumors, given the importance of CTL response in cancer processes.[115] The findings reported by Ulmer et al. were highly relevant since the generation of CTLs *in vivo* usually requires endogenous expression of antigens and presentation of peptides processed

thereof to major histocompatibility complex (MHC) class I molecules (see Chapter 2, Section 2.5.1). And, for the first time, protection against a pathogenic infection had been obtained by plasmid immunization.

Still in 1993, and concurrently with Ulmer's paper, a number of reports described the development of "gene vaccines" as a means to generate immune responses against infectious agents such as influenza, human immunodeficiency virus (HIV), and bovine herpes virus. The first study was focused on the effect of the route of inoculation on the ability of plasmid vaccines expressing influenza virus hemagglutinin glycoproteins to raise protective immunity both in mice and in chicken.[109] Experiments were set up in which DNA was inoculated via the intramuscular, intranasal, intradermal, intravenous, subcutaneous, and intraperitoneal routes. The results showed that by bombarding plasmid-coated gold particles to the epidermis of the test animals with a gene gun, 250–2500 times less DNA was required to obtain protection when compared with direct injection in saline. Though less effective, the mucosal, intravenous, and intramuscular routes could also be used to raise protective immunity. An important conclusion was that the higher efficiency of transfection obtained with intramuscular injections did not necessarily correlate with a higher efficiency of vaccination.[109] The second study, by Wang and coworkers, was the first of many to come in the subsequent years, which attempted to develop vaccine prototypes based on the use of HIV genes hosted in plasmids. The experimental evidence accumulated in this report proved that direct injection of a plasmid DNA construct harboring the HIV-1 gp160 envelope protein in mice muscle could elicit both cellular and immune responses.[116] Serological responses of mice and cattle immunized with plasmids encoding bovine herpes virus-1 glycoproteins were also detected following intramuscular injection.[110]

Besides conferring protection against a plethora of phatogens, the immune system contributes to the surveillance and destruction of neoplastic cells. However, the fact that most tumor cells escape normal defenses in immunocompetent hosts suggests that an appropriate stimulation is required to augment the response of the immune system. As described in Section 1.1.3, the delivery of specific genes via viral vectors had already been attempted in humans by a cell-mediated *ex vivo* approach with the objective of obtaining an immune therapeutic effect against malignancy.[36] In the early 1990s, Gary Nabel's group rather focused on the direct use of plasmids encapsulated in liposomes as carriers of MHC genes to tumor cells.[117,118] In what was probably the first human gene therapy clinical protocol to use plasmid vectors, the gene encoding for the MHC protein HLA-B7 was introduced into advanced melanoma patients, with the expectation that expression of HLA-B7 would stimulate the local release of cytokines, thus inducing a T-cell response against the tumor.[117,118] Lactated Ringer's solution containing the plasmid/HLA-B7 constructs complexed with liposomes were typically injected directly into the patient's melanoma nodules. Both plasmid DNA and HLA-B7 protein were detected in tumors biopsies. Most importantly, immune responses to HLA-B7 and tumor were detected and tumor regression was even observed in one of

the five patients enrolled.[117] This study provided the first evidence on the safety and effectiveness of intratumoral gene transfer in cancer.

The immunization experiments reported in 1992 and 1993 gave birth to a new generation of vaccines. On a meeting convened by the World Health Organization (WHO) in May 1994, the name DNA vaccine was selected among others (genetic immunization, gene vaccines, and polynucleotide vaccines) to designate the new technology.

1.3.5 Further Developments

The few experiments carried out in the decade that run from 1983 to 1993 and described above (Table 1.2) constituted the seeding ground from which a whole new class of medicinal products, plasmid biopharmaceuticals, would emerge. Researchers worldwide swiftly built upon the findings, hints, and speculations of the pioneers, and as a consequence, the research devoted to the potential application of plasmids as biopharmaceuticals for gene therapy or vaccination virtually exploded. Entrepreneurs and investors were also lured by the potential of plasmids as therapeutic and prophylactic agents, and not surprisingly, a significant number of research publications and clinical trials involving plasmids have been sponsored by commercial ventures and companies. The growth is exemplified, for instance, in Figure 1.2, which shows the cumulative number of scientific articles published between 1994 and 2009 that had the words DNA vaccine in the title. The evolution of the number of gene therapy clinical trials in which the target genes were carried by naked plasmid

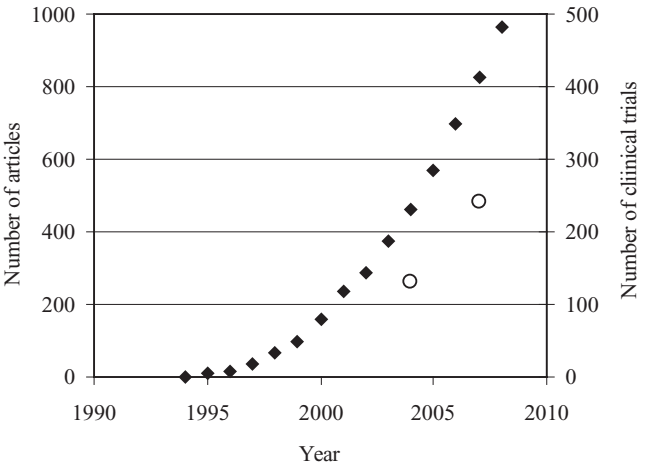

Figure 1.2 *Evolution of the number of DNA vaccine scientific publications (◆) and human clinical trials, which used naked plasmid DNA as a gene delivery vector (○). The number of publications (reviews, articles, and proceeding papers) with the words "DNA vaccine" in the title was obtained from the ISI Web of knowledge (http://isiwebofknowledge.com, accessed February 18, 2010). Clinical trial data were extracted from Edelstein et al.*[55,142]

DNA is also shown in the figure. The popularity of plasmids among the cohort of gene therapy vectors available can be attributed to a number of tangible and perceived characteristics, which include the short time and simplicity associated with their manipulation, production, and purification, and their potential safety, as justified by the lack of severe adverse reactions and events observed so far.

Some events, scientific breakthroughs, and a few papers among the huge numbers that have been published in the last 15 years deserve to be mentioned due to their incremental contributions to the development of plasmid biopharmaceuticals (see Table 1.2). This includes, for instance, the use of cytosine–phosphate–guanine (CpG) motifs, either cloned in the plasmids themselves or added as adjuvants to DNA vaccine formulations, as a means to stimulate the immune system. The search for ways to improve the immunogenicity of DNA vaccines was prompted by the recognition that expression of an antigen hosted in a DNA vaccine does not always and necessarily translate into an immune response. Building upon the finding that bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides activate the immune system,[119] Sato and colleagues included CpG dinucleotides in precise locations of a plasmid backbone.[120] Their subsequent experiments showed that human monocytes transfected with plasmid DNA containing those short immunostimulatory DNA sequences (ISSs) transcribed large amounts of cytokines like interferon-alpha, interferon-beta, and interleukin 12 (IL-12).[120] Other ways to manipulate the immunogenicity of DNA vaccines have been devised, which include, for example, the fusion of specific targeting or signal sequences to antigens. According to the sequence that is added, different pathways can be used by the host cells to process the antigen, and thus the concomitant recognition by the immune system can be modulated and controlled to some extent[121] (see Chapter 2, Section 2.5.2 for further details).

Further realization of the potential of DNA vaccines came with the discovery that the immune response generated by DNA vaccination could be manipulated through the coexpression of immunologically relevant proteins such as cytokines. One of the first experiments was reported by Xiang and Ertl, who showed that coimmunization of mice with a plasmid coding for the G protein of the rabies virus and with a plasmid expressing the granulocyte macrophage colony-stimulating factor (GM-CSF), a hematopoietic growth factor that enhances the antigen-presenting capacity of dendritic cells, clearly improves T-helper and B-cell responses.[122] On the contrary, coinoculation of a plasmid expressing interferon γ (IFNγ), a cytokine that regulates responses by upregulating the expression of MHC determinants in different cells, led to a slight decrease in the T-helper and B-cell response.[122] Subsequently, several laboratories published reports describing the effects of codelivering other cytokine genes such as *IL-2*[123] or *IL-12*.[124] In many cases, plasmid DNA vaccines are only capable of eliciting weak immune responses. The so-called prime-boost vaccination has been developed to take advantage of this characteristic. The strategy consists in priming with a first DNA vaccine inoculation and then

boosting the immune system with either recombinant antigen or with attenuated recombinant viral vectors. This leads to a more effective recognition of antigens and thus to an augmentation of immune responses (e.g., antibody titers) to pathogens.[125,126] The incorporation of alphavirus replicons in plasmids was a further contribution in the struggle to ameliorate the efficacy of DNA vaccines and plasmid vectors.[127] The approach relies on putting the transgenes under the control of replicase genes used by members of the alphavirus genes, such as the Sinbis virus. Once the plasmid DNA reaches the cell nucleus, the encoded replicase complex is expressed and an antisense RNA is synthesized. This self-amplifying RNA, which encodes the transgene of interest, is then directly replicated in the cytoplasm by the replicase complex, resulting in high-level expression of the transgene.[128]

The development of novel and more efficient delivery strategies has always been at the forefront of research on nonviral gene therapy. For instance, in 1991, Titomirov and colleagues injected plasmid DNA sub-cutaneously in mice and then used a special device to deliver two high-voltage pulses in opposite polarities to the corresponding skin area.[129] These experiments showed that this type of *in vivo* electroporation was a feasible method to transform cells. Subsequent efforts have developed the *in vivo* electroporation technology up to a point where it is probably one of the most effective ways to deliver plasmid DNA. The encapsulation of plasmid DNA in poly(DL-lactide-co-glycolide) (PLG) microparticles, a methodology first described in 1996, constitutes another example of an incremental innovation in the delivery area. Key features of the technology are the ability of the particles to protect plasmid DNA against degradation after administration, and to promote uptake, expression and antigen presentation in appropriate cells in such a way as to elicit both systemic and mucosal antibody responses.[130] A third milestone in plasmid delivery was reached in 1997 with the description of a process to compact single molecules of DNA into unimolecular nanoparticles with sizes small enough to enable nuclear pore entry.[131]

The range of applications of plasmid biopharmaceuticals was further expanded in 2002 with the description that plasmids can direct the intracellular synthesis of small interfering RNA (siRNA) transcripts in mammalian cells, with the concomitant downregulation of the genes targeted for knockdown.[132] This constituted a credible alternative to the specific suppression of gene expression by RNA interference, which until then was typically accomplished by the direct administration of synthetic siRNAs.

At the same time that progress was being made in the area of plasmid biopharmaceuticals, governmental agencies in the United States, Europe, and elsewhere started intervening in an attempt to regulate the experimentation performed with human subjects. Relevant documents issued earlier by the FDA include the Points to Consider (PTC) documents "Points to Consider in Human Somatic Cell Therapy and Gene Therapy" of 1991[33] and "Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications" of 1996.[133] These have since then been superseded by guidance to

industry documents (see Chapter 11 for more information on regulatory issues).

1.3.6 The Road to the Market

One of the ultimate goals of the scientific and technological advances described in the previous pages has been to introduce safe and efficacious plasmid biopharmaceuticals in the market for the benefit of society. Although this goal remains elusive, a number of successes can be described that are contributing to the paving of a road to the market. In 2003, condors were the first animals immunized with a DNA vaccine to be deliberately released into the environment. The high risk faced by a small colony of California condors in face of the West Nile virus (WNV) epidemic, which reached New York City in 1999 and subsequently spread westward, prompted the U.S. Centers for Disease Control and Prevention (CDC) to expedite the inoculation of the entire population of the endangered animal, both in captivity and in the wild, with a DNA vaccine containing genes coding for WNV proteins. This prospective vaccination is believed to have saved the endangered population of condors from subsequent infections with the natural WNV, which circulated during the 2004 season.[134] The immunization of the Californian condor with DNA vaccines is one of the veterinary case studies described in more detail in Chapter 10.

The year 2005 was marked by two historical events, the full licensing of two veterinary DNA vaccines. The first vaccine, licensed by the Center for Veterinary Biologics of the United States Department of Agriculture (USDA) to Fort Dodge Animal Health (Fort Dodge, Iowa), a division of Pfizer Animal Health (New York), became the first DNA vaccine for any species to be registered with a governmental regulatory body.[135] The vaccine was designed and developed jointly by the CDC and Fort Dodge to protect horses against WNV, and was the predecessor of the WNV vaccine used experimentally in condors referred above and described in detail in Chapter 10. Although the license was issued in 2005, Fort Dodge only launched the vaccine in December 2008, under the trade name West Nile-Innovator® DNA.[136] The second DNA vaccine licensed in 2005 was developed by Aqua Health, Ltd., an affiliate of Novartis Animal Health (Victoria, Canada), to protect farm-raised salmon against infectious hematopoietic necrosis virus.[137] Commercialized under the trade name Apex®-IHN, the DNA vaccine is supplied in sterile saline with the recommendation to be administered intramuscularly into anesthetized fish (Apex-IHN information, Novartis Animal Health Canada, Inc.—Aqua Health Business). The circumstances surrounding the development of the Apex-IHN DNA vaccine, together with the underlying rationale and the major findings regarding efficacy and safety, are described in detail in Chapter 10.

More recently, in early 2007, the USDA conditionally approved a therapeutic DNA vaccine to treat melanoma in dogs.[138] The vaccine was designed to deliver an MHC gene to dog tumors on the basis of the underlying principle developed in the early 1990s by Nabel and coworkers.[139] This case study is also

examined in more detail in Chapter 10. Finally, in 2008, the Australian Pesticides and Veterinary Medicines Authority approved the market entrance of an injectable plasmid DNA encoding for porcine growth hormone releasing hormone (GHRH), a protein that plays an important role in the growth and development of mammals.[140] Manufactured and sold by VGX Animal Health, Inc. (The Woodlands, Texas) under the trade name LifeTide™ SW 5, this veterinary product is used with the specific goal of decreasing perinatal mortality and morbidity in pigs, and therefore improving sow productivity.[140] According to the company's description, the plasmid is delivered to muscle cells by intramuscular injection followed by electroporation with the portable electrokinetic device, cellectra™. The GHRH, which is produced by the transfected cells, then induces the production and secretion of the endogenous growth hormone. The net result is a decrease in offspring morbidity and mortality and an increase in body weight.[141] LifeTide SW 5 thus became the first plasmid biopharmaceutical approved for the gene therapy of animals produced with the intent of supplying the human food chain.

The events described in the last paragraphs constitute a remarkable record if one considers that only 13–16 years had gone by since an immune response was demonstrated following the injection of an antigen encoded in a plasmid.[114] This extraordinary speed in bringing a product from a totally new class to the marketplace, together with the large number of plasmid biopharmaceuticals undergoing preclinical development and clinical trials (more than 300), suggests that other products are likely to hit the market in the near future. At the current stage of development, it is very hard to anticipate the potential value of the plasmid biopharmaceutical market (see Chapter 8). The number of disease targets (infectious diseases, cancer, cardiovascular disease, etc.) and potential consumers (humans and domestic and farm-raised animals) is certainly huge (see Chapters 2 and 8). However, the field is in much need of a commercial success in the human arena, which, once arrived, could provide the necessary momentum for further progress.

1.4 CONCLUSIONS

Major developments have taken place in the past 30 years, which have contributed significantly to the establishment of plasmid biopharmaceuticals as a whole new category of medicinal agents. The profound progress made in this area owes much to the breakthrough discoveries and insights of gene therapy pioneers like Joshua Lederberg and Stanley Rogers, and plasmid scientists like Stanley Cohen, Jon Wolff, and Gary Nabel, but also to the incremental advances described by so many researchers. Now that this collective effort is on the verge of producing tangible benefits for society, it is only fit to recall how it all started and progressed. Although this has been the major purpose of this introductory chapter, I am aware that the brief account given in the preceding pages may have missed influential discoveries and scientists. I hope neverthe-

less that I have provided the reader with a factually correct and stimulating historical perspective of the development of the plasmid biopharmaceuticals field.

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