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Review

Biotechnology and genetic engineering in the new drug development. Part II. Monoclonal antibodies, modern vaccines and gene therapy

Agnieszka Stryjewska[1], Katarzyna Kiepura[1], Tadeusz Librowski[2], Stanisław Lochyński[3]

[1]Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Technology, Wyb. Wyspiańskiego 27, PL 50-370 Wrocław, Poland

[2]Department of Radioligands, Medical College, Faculty of Pharmacy, Jagiellonian University, Medyczna 9, PL 30-688 Kraków, Poland

[3]Institute of Cosmetology, Wrocław College of Physiotherapy, Kościuszki 4, PL 50-038 Wrocław, Poland

Correspondence: Stanisław Lochyński, e-mail: s.lochynski@wsf.wroc.pl; Tadeusz Librowski, e-mail: mflibrow@cyf-kr.edu.pl

Abstract:
Monoclonal antibodies, modern vaccines and gene therapy have become a major field in modern biotechnology, especially in the area of human health and fascinating developments achieved in the past decades are impressive examples of an interdisciplinary interplay between medicine, biology and engineering. Among the classical products from cells one can find viral vaccines, monoclonal antibodies, and interferons, as well as recombinant therapeutic proteins. Gene therapy opens up challenging new areas.
In this review, a definitions of these processes are given and fields of application and products, as well as the future prospects, are discussed.

Key words:
biotechnology, vaccines, traditional vaccines, modern vaccines, monoclonal antibodies, ziagen, thalidomide, alemtuzumab

Introduction

Biotechnology can be approached from different angles. Some describe it as a field of technological activity in which biochemical, genetic, microbiological and engineering techniques are combined for the pursuit of technical and applied aspects of research into biological materials and in particular into biological processing such as the application of science and technology to living organisms, as well as part, products and models thereof to alter living or non-living materials for production of knowledge, goods and services.

Under a wide approach based on the biological nature of the products and processes involved, old techniques such as fermentation processes [36], as well as the newest ones such as genetic engineering may be

included in the range of activities falling in the failed of biotechnology [31, 40, 48]. A more comprehensive categorization of biotechnology, based on its end-use has also been proposed. In this classification products are ascribed to one of the following biotechnology thematic subsets: healthcare biotechnology, agricultural biotechnology, industrial biotechnology and environmental biotechnology [35, 49, 51].

Biocatalysis is a technique for the production of molecules using enzymes or whole cells, which allow for the obtaining of chiral products, so often present in pharmacy. Metabolic engineering is based on biocatalysis, but also largely on recombinant DNA technology. The purpose of this process is to manipulate the metabolism of the microorganism in such a way as to increase and improve its production capacity. Monoclonal antibody technology, the synthesis of antibodies targeted to a specific antigen, is mainly hybridoma technology, which involves the hybridization of mouse B cells with myeloma cells. The resulting cell hybridoma leads to cancer cell longevity and the ability to produce monoclonal antibodies used in diagnosis and therapy. Vaccine technology is the synthesis of vaccines to a large extent by recombinant DNA technology and microbial production.

A vaccine, a substance that causes an immune response without infection of the body, may be a bacterium, virus, an antigen or toxin, a recombinant vector or synthetic peptide, or DNA.

Monoclonal antibody production

Monoclonal antibodies (mAbs) are used for diagnosis, imaging, therapy and treatment. When used for diagnosis, for immunoassays, they are implemented when greater specificity is required for protein antigen than in the case of polyclonal antibodies. Thus, monoclonal antibodies are used, for example, to measure the amount of steroid hormones, or to study antigens on the surface of cancer cells. Imaging, however, indicates the location of the disease. Immunoglobulins are marked, introduced into the body of the patient, and then localized. In therapy, monoclonal antibodies have found many uses. They can be used as cancer cell receptor blockers or to deliver anticancer drugs or to remove toxins from the bloodstream. Antibodies can also be used for antigen or impurity removal, using a high protein affinity.

Polyclonal antibodies are formed by the influence of different antigens on many B cells in the body, which makes them heterogeneous. Monoclonal antibodies, however, when this technology began to be used, were created by the hybridization of murine B lymphocytes with myeloma cells of the spleen by the inactive virus. The resulting cell had cancer cell longevity and the ability to produce so-called monoclonal antibodies. They have the same specificity, or behave in a chemically similar manner. As such, this technology is also known as hybridoma technology. Nowadays, murine lymphocytes are also used, although other animal cells can be utilized. A viable method for producing mAb without the use of animal cells has not yet been developed [51] (Tab. 1).

Hybrid technology

1. Immunization – first lymphocytes must be created in response to the antigen [16]. The immunogen can be a cell membrane, a whole cell or a microorganism. The antigen used for vaccination may be in a solution with an adjuvant or in a homogenized gel. Then, the mice are vaccinated repeatedly, until the appropriate level of antigen is achieved. The animals are then killed and their spleen is removed.

2. The verification of antibody-producing mice – blood samples are taken from the animal and are measured by the content of the antibodies. For this, methods such as ELISA (enzyme-linked immunosorbent assay) and flow cytometry are applied. When their concentration is sufficient, the spleen is removed.

3. Preparation of myeloma cells – they are being prepared for fusion with mouse cells. A week before the merger they are suspended in a solution of 8-asaguanine, increasing their sensitivity to the HAT (hypoxanthine-aminopterin-thymidine) medium. This medium is used after the merger, and only hybridomic cells can survive in it [51].

4. Fusion – a process that rarely occurs spontaneously and, therefore, myeloma cells and mouse cells, viruses or chemical fusiogenes are added to the solution. Fusiogenes interact with phospholipids in the membrane, which facilitates the creation of hybridomas [29]. In this case, polyethylene glycol is most commonly used [51].

5. Cloning – first, the hybridomic cells with active immunoglobulin genes must be selected. To achieve this, cloning is carried out in soft agar. The dividing cells are transferred to a tissue culture, where the rele-

Tab. 1. Comercial monoclonal antibodies

Antibody	Brand name	Target	Indication
Abciximab	ReoPro	Inhibition of glycoprotein IIb/IIIa	Cardiovascular disease
Adalimumab	Humira	Inhibition of TNF-α signaling	Several auto-immune disorders
Alemtuzumab	Campath	CD52	Chronic lymphocytic leukemia
Gemtuzumab	Mylotarg	CD33	Acute myelogenous leukemia (with calicheamicin)
Ofatumumab	Arzerra	CD20	Chronic lymphocytic leukemia
Rituximab	Rituxan, Mabthera	CD20	Non-Hodgkin lymphoma
Tositumomab	Bexxar	CD20	Non-Hodgkin lymphoma
Brentuximab vedotin	Adcetris	CD30	Anaplastic large cell lymphoma (ALCL) and Hodgkin lymphoma
Panitumumab	Vectibix	Epidermal growth factor receptor	Colorectal cancer
Cetuximab	Erbitux	Epidermal growth factor receptor	Colorectal cancer, head and neck cancer
Trastuzumab	Herceptin	ErbB2	Breast cancer
Bevacizumab	Avastin	Vascular endothelial growth factor (VEGF)	Colorectal cancer, age related macular degeneration (off-label)
Basiliximab	Simulect	L-2Rα receptor (CD25)	Transplant rejection
Daclizumab	Zenapax	IL-2Rα receptor (CD25)	Transplant rejection
Muromonab-CD3	Orthoclone OKT3	T cell CD3 receptor	Transplant rejection
Tocilizumab (or Atlizumab)	Actemra and RoActemra	Anti- IL-6R	Rheumatoid arthritis
Certolizumab pegol	Cimzia	Inhibition of TNF-α-signaling	Crohn's disease
Natalizumab	Tysabri	α-4 integrin,	Multiple sclerosis and Crohn's disease
Ranibizumab	Lucentis	Vascular endothelial growth factor A (VEGF-A)	Macular degeneration
Palivizumab	Synagis	An epitope of the RSV F protein	Respiratory syncytial virus
Omalizumab	Xolair	Immunoglobulin E (IgE)	Mainly allergy-related asthma
Pilimumab (MDX-101)	Yervoy	Blocks CTLA-4	Melanoma
Golimumab	Simponi	TNF-α inihibitor	Psoriatic arthritis, and ankylosing spondylitis
Efalizumab	Raptiva	CD11a	Psoriasis
Eculizumab	Soliris	Complement system protein C5	Paroxysmal nocturnal hemoglobinuria
Denosumab	Prolia, Xgeva	RANK Ligand inhibitor	Postmenopausal osteoporosis, solid tumor's bony metasteses
Canakinumab	Ilaris	IL-1β	Cryopyrin-associated periodic syndrome (CAPS)
Belimumab	Benlysta	Inihibition of B cell activating factor	Systemic lupus erythematosus

vant hybridoma can be located. Another method frequently used is the limited dilution method. It uses 96 plates of tissue culture and nourishing cells, supporting growth of hybridomas. The hybridomas diluted solution is distributed on the plate. After 7–10 days, the search for antigen-producing cells starts (Fig. 1).

The produced cells are then characterized and set in suitable culture conditions. Also, the properties of the antibodies are determined – whether they binds to an antigen that interacts with some other molecules, along with its isotype. Following this, the hybridomic cell culture may be started in the digester [15].

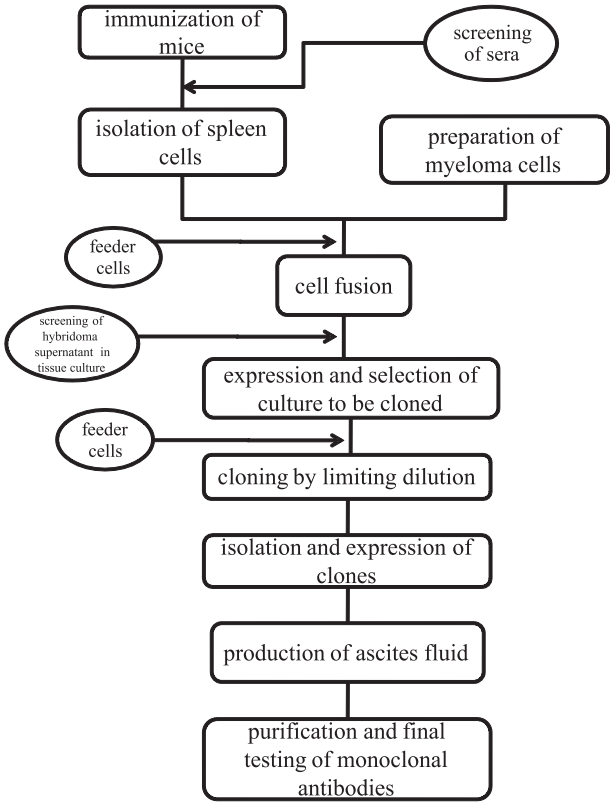

Fig. 1. Production of monoclonal antibodies by hybrid technology

Transgenic mice in the production of antibodies

Application of murine mAb in the treatment causes a human immune response, known as HAMA (human anti-mouse antibody), directed against the given immunoglobulin [30]. Thus, more appropriate therapeutic human monoclonal antibodies are also created in mice. However, the animal gene is replaced by the human gene, which creates transgenic mice [1]. Because of the large size of the coding sequence of the human antibody, minilocs are created. These short sequences contain just some of the DNA strands of the gene and are introduced into mouse embryonic stem cells, which then develop in the animal. The mice are also cultured separately, with both the heavy and light chain gene of a mouse being crossed. Then, the two types of transgenic animals are bred. The mice bearing the human gene and able to produce the correct antibody are identified by the influence of the antigen on them [9, 27].

The phage display method

Phage display technology allows for the creation of libraries of proteins and the search for properties of bonds with other molecules. First, a gene library containing the coding sequence for a protein is created. This leads also to the fusion between genes encoding the protein of the phage's shell with the library of phages. The gene library is introduced to the phage library. Phages then replicate in *E. coli*, and the encoded protein is placed on the surface of the phage, which allows for the search and finding of those clones that produce a given molecule. The affinity selection, biopanning, is used for the search in which the column is filled with immobilized molecules. The column is washed with phages. Those that produce a protein show a greater affinity for the column cells, and the particles are stopped. After washing, the right gene is cut out of the phage genome, which can then be introduced into the DNA of cells that have to produce a protein on a large scale.

The method of phage display libraries makes it possible to create antibody libraries that will bind to specific molecules, and the necessary gene libraries are taken from a human or animal. By cloning the gene responsible for expressing an antibody or its fragment of B lymphocytes, immunolibraries are created. To begin with, however, the donor cells must be immunized with an antigen. If the donor is not immunized, non-immunological libraries are created. Any further procedure is the same, because the B lymphocytes are taken and the corresponding sequences are cloned. This produces a smaller amount of antibody-producing clones, but they can be used for genetic manipulation to generate improved antibodies [49].

Repertoire cloning

With this technology only fragments of antibodies can be obtained. However, it means that those parts that can be modified can be found quickly. Two discoveries have contributed to the emergence of this technology. The first is the identification of the conserved regions, flanking the coding of the sequences for protein domains. As such, it became possible to use PCR amplification of the selected antibody genes. The second step involves the use of strategy peryplasmatic secretion, which leads to the expression of antibody fragments in bacteria.

The commercially used λ-ZAP technology produces antibodies against the hemagglutinin (HA) glycoprotein located on the surface of the influenza virus. First, the mice were immunized and then the mRNA was isolated. Combinatorial libraries were created, consisting of 125 thousand clones. After screening, 10 clones were obtained that bound the antigen. λ-ZAP also produces human high-affinity mAb, directed to the tetanus toxoid. The donor was immunized with this antigen, and then the peripheral blood lymphocytes (PBL) were collected from the donor, which were the source of the mRNA. The number of positive clones is 1 in 500 [6].

Production *in vivo*

The method of *in vivo* is used on a large scale only in some countries, due to ethical and legal aspects, and the use of mAB produced that way carries the risk of viral infection. It is used also in studies of monoclonal antibodies. Hybridomic cells are propagated in the peritoneal cavity of mice or rats, which contains mineral oil. This oil makes the myeloma cells proliferate better. Hybridomic cells are introduced, and the resulting antibodies are collected in the ascites fluid. This process allows for a few milligrams of antibodies [28].

Production in mammalian cell culture

With mammalian cell culture, monoclonal antibodies are obtained on an industrial scale. Cells are grown in batches, in flasks (static) or digestion tanks [6, 53]. The tanks mix their contents, facilitating the exchange of gases and nutrients. The addition of serum and other nutritive media provides fast growth. In addition to the static method and the method of suspended cells, the production process can be carried out in conditions that mimic a continuous or semi-continuous process, where immobilized cells are applied. The cells, washed with the medium, are then behind a semi-permeable membrane. The serum is on the side of the cells and the antibodies can be eluted. The use of mammalian cells to produce mAb allows for important steps in the proper functioning of proteins, such as glycosylation. This process creates up to 500 kg of products per year [53]. Very often, the Chinese hamster ovary (CHO) is used for the production of monoclonal antibodies.

Production of bacterial culture cells

The use of bacteria for the production of monoclonal antibodies leads to large amounts of this product. To start with, the transfection of bacteria that will produce human antibodies is used. The problem, however, is in the protein post-translational processing not being carried out and, therefore, this method is mainly used for the production of antibody fragments rather than whole molecules [50].

Antibody engineering

One of the many ways to manipulate antibodies is humanization. It involves the transfer of the antigen binding place from inhuman antibodies to the human molecule. This leads to the humanization of the antibody. To accomplish this, the desirable form of protein is designed and the genes that will encode Fv, scFv, Fab or IgG are created for it. This process is repeated until the resulting molecule will bind the proper antigen. An example is superhumanization, where human FR is selected first. Two methods of *in silico* are applied. The first compares the structure of the protein not derived from a human with human immunoglobulin proteins, and the selected genes are found in humans that correspond to the first antibody. Then, among them, sequences are selected with the greatest homology to the CDR (complementarity determining region), and finally the correspondence between these passages and the FR are determined. It is also important to increase the affinity of monoclonal antibodies produced for the antigen. Frequently used for this is the error-prone PCR, which uses a DNA polymerase with a low accuracy. Taq is such a polymerase and the V gene amplification introduces errors. To increase the frequency of errors, polymerase acts in the presence of manganese ions instead of magnesium, or propanol. The greater number of mutations, the better the affinity of the molecule [50].

Production of human monoclonal antibodies IgG by BALB/c mice

This large-scale process is based on the technology described below in the text. Several weeks' old BALB/c mice had Pristan (2,6,10,14-tetramethylpentadecane) which is an isoprenoidal hydrocarbon, introduced to the peritoneal, and after ten days hybridomic cells were also injected, and the monitoring of

the quantities of ascites was conducted. After another four and ten days, the fluid was collected. The collected samples were centrifuged and the supernatant was subjected to further proceedings.

To determine the concentration of the antibody, the ELISA method was used. Ninety six plates were prepared, and IgG was imposed on it for 45 min. The samples were then alternately washed with PBS-Tween 20 and incubated with ascites fluid, with the addition of IgG derived from a rabbit. Then, the obtained solutions were analyzed by the ELISA reader at a wavelength of 450 nm. Also examined was the cross-reactivity of IgM and IgA with similar waveforms to those described for ELISA. To confirm the results, immunoblotting was used, and the resulting antibody showed no cross-reactivity with IgM and IgA, but affected the IgG heavy chain with isotyping specified classes and subclasses of antibodies in the solution. Immunoassay was used. Then, the antibody was purified by washing the ascites fluid with ammonium sulfate before centrifuging and dialysing it. This solution was subjected to ion exchange chromatography. The collected samples of antibodies were coupled with horseradish peroxidase (HRP) [1].

Production of humanized monoclonal antibodies Campath®

Alemtuzumab is a monoclonal antibody known under the name Campath®. It is produced by the Ilex Oncology, Inc., Millennium Pharmaceuticals, Inc. and Berlex Laboratories, Inc. companies. It is used to treat patients with chronic lymphocytic B-cell leukemia, when alkylates and fludarabine therapy fails to produce results. Alemtuzumab is produced by recombinant DNA technology and is humanized (Campath-1H), acting against the glycoprotein CD52. CD52 has a mass of 21–28 kD and is present on the surface of B and T lymphocytes, The NK cells and macrophages are both normal and malignant. The production of Campath-1H is based on receiving IgG1 with the basic human immunoglobulin structure and complementarity determining the regions from the rat antibody (Campath-1G). Mammalian cells (CHO cells) are used in the production. The culture is carried out in a medium containing neomycin, but the antibiotic is not present in the final product. Ready, sterile Campath has a mass of 150 kD and has the form of an isotonic solution [2].

Vaccine technology

The vaccine may be a bacterium (Tab. 2), virus (Tab. 3), the antigen or toxin, a recombinant vector or a synthetic peptide or DNA and all of them can be supplied in various forms [16]. The aim of vaccination is to prevent the onset of the disease by calling an early im-

Tab. 2. Bacterium vaccines

Bacterium	Diseases or conditions	Vaccine(s)	Brands
Bacillus anthracis	Anthrax	Anthrax vaccines	
Bordetella pertussis	Whooping cough	DPT vaccine	Boostrix, Adacel
Clostridium tetani	Tetanus	DPT vaccine	Boostrix, Adacel
Corynebacterium diphtheriae	Diphtheria	DPT vaccine	Boostrix, Adacel
Coxiella burnetii	Q fever		
Haemophilus influenzae type B (Hib)	Epiglottis, meningitis, pneumonia	Hib vaccine	Hiberix
Mycobacterium tuberculosis	Tuberculosis	Tuberculosis (BCG) vaccine	
Neisseria meningitidis	Meningococcal meningitis	Meningococcal vaccine	Neisvac C, Meningitec
Salmonella typhi	Typhoid fever	Typhoid vaccine	Typhim Vi, Typherix
Streptococcus pneumoniae	Pneumococcal pneumonia	Pneumococcal conjugate vaccine,	Pneumovax, Prevenar
		Pneumococcal polysaccharide vaccine	
Vibrio cholerae	Cholera	Cholera vaccine	Dukoral

Tab. 3. Viruses vaccines

Virus	Diseases or conditions	Vaccine(s)	Brands
Hepatitis A virus	Hepatitis A	Hepatitis A vaccine	Havrix, Avaxim
Hepatitis B virus	Hepatitis B	Hepatitis B vaccine	Engerix-B
Human papillomavirus	Cervical cancer, genital warts, anogenital cancers	HPV vaccine	Cervarix, Gardasil
Influenza virus	Influenza	Influenza vaccine	FluMist, Fluzone, Influvac, Vaxigrip
Japanese encephalitis virus	Japanese encephalitis	Japanese encephalitis vaccine	
Measles virus	Measles	MMR vaccine, MMRV vaccine	Priorix
Mumps virus	Mumps	MMR vaccine, MMRV vaccine	Priorix
Polio virus	Poliomyelitis	Polio vaccine	
Rotavirus	Rotaviral gastroenteritis	Rotavirus vaccine	Rotateq, Rotarix
Rubella virus	Rubella	MMR vaccine, MMRV vaccine	Priorix
Varicella zoster virus	Chickenpox, shingles	Varicella vaccine, shingles vaccine, MMRV vaccine	Varivax, Zostavax
Variola virus	Smallpox	Smallpox vaccine	

mune response to a pathogen, without detriment to health, which causes the body to be prepared for a possible infection.

Traditional vaccines

Before the recombinant DNA technology was used, the so-called vaccine was produced in a traditional way, employing natural processes. These include attenuation, killing or inactivation. Despite the development of a possible interference in the genome, to this day about 30 traditional vaccines are used, such as the one against cholera [46].

Attenuation

Attenuation is a method performed *in vitro*, often multi-stage or using random chemical mutagenesis. Therefore, it is based more on spontaneous, difficult to control processes, which may have some side effects, and sometimes, although rarely, this treated pathogen returns to its virulent state [36]. The aim is to reduce the attenuation or to even completely eliminate the pathogen's virulence. However, it does not lose its properties in generating an immune response of the host. Attenuation can be performed by chemical or thermal treatment, or culturing the pathogen under adverse conditions. Propagation in the wrong host also leads to attenuation. Bacillus Calmette-Guérin (BCG) against tuberculosis is one such a bacterial vaccine. Attenuated viral vaccines are usually set by culturing strains in eggs, chicken embryonic tissue and other animal tissues. A vaccine against inflammation parotitis, or mumps [46], is frequently created.

Killing or inactivation

As with attenuation, killing or inactivating the pathogen can be carried out by the use of chemicals or heat. However, this process must occur very precisely for the pathogens not to cease to be a vaccine and begin to cause disease. Despite the inactivation or killing, the properties of response to the antigen remain intact. Vaccination against cholera consists of an aqueous solution of a killed strain of *Vibrio cholerae*. However, the inactivated hepatitis A vaccine is created by the formaldehyde strain, HM 175th, these viruses replicate in human fibroblasts [46].

Other types of traditional vaccines

Creating a toxoid-based vaccine can be presented on an example of a commonly used vaccine against diphtheria. First, *Corynebacterium diphtheriae* are grown, and then the toxin is washed with separate formaldehyde, in order to inactivate it. The resulting toxoid is

a weak immunogen. It is absorbed by aluminum salts, which act as adjuvants. A vaccine based on an antigen is mostly produced from the antigens present on the surface of a bacteria or virus. These are mainly polysaccharides, which have much lower immunogenicity than the protein molecules. Carbohydrate vaccines are therefore often given to infants. It is also important to treat diseases caused by parasites, but not many vaccines against them have yet been created. Typhoid is an infection caused by the parasite, *Rickettsia prowazekii*, and if left untreated leads to death. To prevent it, vaccines are created which killed *R. prowazekii* [46].

Modern vaccines

Subunit vaccines

In traditional methods, these vaccines are formed by the purification of a naturally occurring product, but recombinant DNA technology allows for much more than this. The production of a molecule can be increased by introducing the coding sequence into the body that will produce it in large quantities. Mutations can then be introduced that reduce or eliminate the toxicity or alter other properties of the molecule [39], along with the use of many other tools of genetic engineering. One of the first recombinant vaccines, difficult to obtain in large quantities by traditional methods, was that against hepatitis B. It, however, used a natural antigen – the surface protein of the hepatitis B virus (HBV), produced by cloning the DNA fragment encoding this protein in the yeast genome [9]. This is one of the few examples of the application of yeast as an efficient producer of recombinant proteins.

Targeted mutagenesis also finds its application in the production of vaccines, which can be presented on an example of cellular anti-pertussis vaccine. The strongest antigen is the pertussis toxin, although its toxicity must first be eliminated before it is administered to a patient. Through the mutagenesis of amino acids, a substitution in the sequence corresponding to the harmful substance is made, and the received vaccine is then safe and effective in action [39, 45].

DNA as a vaccine

Plasmid DNA containing a sequence encoding the desired antigen is introduced into the tissue, which is capable of accepting a new piece, affording the efficient production of molecules the opportunity to begin. The resulting antigens are able to induce the correct immune response. In order to introduce the DNA, some methods are also employed in the delivery of DNA vectors in gene therapy. These are, among others, intramuscular injection and a gene gun, using DNA linked to gold particles. The advantage of using naked DNA amounts to the ease of implementation dependent on circumstances and stages of change [4]. In addition, after identifying the antigen, the route of the vaccine development is the same in all cases [39]. DNA vaccines are used in the case of HIV, malaria, tuberculosis and many other diseases [41].

Vaccines based on epitope proteins

Through the use of proteins as a vaccine, the immune response does not pose the risk of side effects. In addition, the minimization of the structure of the antigen is beneficial because during the preparation of the vaccine the parts that are unnecessary or harmful are removed, such as those that cause an autoimmune response.

Vaccines based on epitopes do not cause a strong enough response in the general population. Therefore, a group of proteins is formed that has a wider effect, both in the population and the diversity of immune responses. A multi-epitope vaccine will be active against many pathogens and viruses. These vaccines should have a recognition place through the MHC (major histocompatibility complex), and the place of T cell recognition, to enhance the immunogenicity of the vaccine; this is introduced to a suitable carrier or in the presence of adjuvants. Further modifications can be found *via*, for example, subjecting it to epitopes, increasing their affinity to the MHC by creating chimeric peptides [4].

Recombinant epitope vaccines

Vectors, which contain the factors derived from microorganisms, are synthetic recombinant vaccines. On the basis of the genes encoding selected epitopes, artificial nucleotide sequences are created and these are then introduced into the vector through recombinant DNA technology. The vaccinia virus is the most commonly used vector, but also applicable are the adenovirus and influenza virus [4].

Adjuvants

Any substance (mineral salts, bacterial molecules, plant hormones, vitamins, etc.), which is able to enhance the immune response to an antigen may be an adjuvant. It is given in instances where the vaccine itself cannot induce a sufficient response. Thanks to adjuvants, the defence mechanisms are activated earlier and last longer, and the amount of antigen given may be smaller. Adjuvants may act selectively on the cellular or humoral immune response. For the safety of patients, only aluminium-based adjuvants are used in humans, such as aluminium hydroxide or aluminium phosphate [39].

Adjuvants can be combined with the vaccine during its production, and the adsorption occurs on the surface of aluminium compounds. In other cases, adjuvants are added to the vaccine just before it is administered. The absorption method involves mixing the antigen with saline or aluminium hydroxide. This stage occurs in certain conditions, which require the appropriate temperature, pH, etc. The resulting vaccines are called vaccines absorbed on aluminium compounds [39].

Therapeutic vaccines

The main purpose of vaccines is the prevention of diseases, but the development of biotechnology has also resulted in vaccines that cure. This is possible because the vaccines resemble pathogens. Vaccines for AIDS, cancer or tuberculosis are still in the phase of development, but for many years the so-called desensitization in the treatment of allergies has been used. Therapeutic vaccines are aimed primarily at long-term illnesses. When infectious diseases are concerned, lasting a short time, usually a prophylactic vaccine is sufficient. In the production of therapeutic vaccines, the same tools of recombinant DNA technology are used as the vaccines discussed before [4, 45].

The production of Pediarix™

Pediarix™ is a combination vaccine (polyvalent) produced by the second largest global pharmaceutical company, GlaxoSmithKline. It has been used worldwide since 2002. Its purpose is to protect against diphtheria, tetanus, pertussis, hepatitis B and polio simultaneously. The full name of this vaccine indicates the manner in which it was prepared: absorbed diphtheria and tetanus toxoids and acellular pertussis, recombinant hepatitis B and the inactivated polio virus. This preparation is safe, not infectious, and is administered by the intramuscular route. Its components are:

1. Diphtheria and tetanus toxoids.
2. Three pertussis antigenes – inactivated pertussis toxin (PT, pertussis toxin), filamentous hemagglutinin (FHA, filamentous hemagglutinin), pertactin (a membrane protein).
3. The surface protein of the hepatitis B virus (HBV).
4. Type 1 (Mahoney), type 2 (MEF-1) and the type 3 (Saukett) polio virus.

Toxoids are produced to begin with *Corynebacterium diphtheriae* grown in the Fenton medium (beef extract), which results in the diphtheria toxoid. The tetanus toxoid is obtained from cultures of *Clostridium tetani*. Here the medium is the Latham medium on bovine casein. To inactivate both toxins formaldehyde is used. *Bordetella pertussis* is grown in a modified Stainer-Scholte medium. The result is obtaining of the desired three pertussis antigens. While the PT and FHA can be directly isolated from the solution, thermal treatment is used for pertactin isolation and the protein begins to form clumps. After cleaning, antigens are inactivated – PT with glutaraldehyde, and formaldehyde, FHA and pertactin are only inactivated by formaldehyde.

HBsAg is obtained as described in the manual. The gene encoding the surface protein is introduced into the genome of *Saccharomyces cerevisiae*, which is a strain then grown in a synthetic medium. Then, the produced protein is purified.

Three types of polio virus are propagated separately in VERO cell lines (immortal cells taken from kidney epithelial tissue of African green monkeys, often used for research on vaccines) [26, 38]. The basis of the culture medium is calf serum and lactalbumin hydrolyzate. Three types of the virus are treated, and then formed them into a single solution containing each of the types.

After receiving the basic components of the vaccine, its final formation begins. Two adjuvants were used – aluminium hydroxide and aluminum phosphate. The first of these was used as an absorbent for the antigens of diphtheria, tetanus and the pertussis, one for HBV. After binding with adjuvants, the antigens are diluted and a ready vaccine is created [38].

Production of the influenza vaccine

The vaccine of the inactivated influenza virus A and B is produced under the supervision of the World Health Organization. Once every six months (February and

September) until now research on the virus and antigenic changes are presented, and on this ground the direction of a new synthesis is determined. The main influenza virus antigens are glycoproteins – hemagglutinin (HA) and neuraminidase (NA), which change frequently through mutation. After collecting samples from patients, isolation of a new strain and analysis of the antigen, there begins the production of vaccines based on the mutant strain.

For influenza A viruses, the process of mixing genetic material is carried out (reassortment), as designated by the WHO A/PR8/34 breeding and strain (H1N1), which grows at high concentrations. The resulting virus, similar in terms of antigens to newly detected strains, are grown in chicken eggs and then checked to see whether they produce glycoproteins PR8. For influenza B viruses, no strain has been recognized that would increase the growth of a culture, and therefore, the material for the production of the vaccines used are some viruses isolated from strains selected by the WHO [32].

After preparation of the production strain, the antigen's compatibility is checked with wild strains. After many tests on the virus, they are planted in the allantois of eggs and reared. The viruses are collected and inactivated by formalin or β-propiolactone [14]. β-Propiolactone interferes with viral DNA and doesn't have any impact on its immunogenicity or the structure of the protein [23]. The samples are purified by ultracentrifugation, and then with ether and a detergent the virus particles are separated. The last step is the determination of the quantities of antigens and, as such, the amount of HA is determined by the radial immunodiffusion technique using standard antigens and specific sheep anti-cheese [14].

Gene therapy

Gene therapy is aimed at treatment of the mainly genetic, but also infectious or neurological, disorders. It is used to treat cancer, AIDS, Gaucher disease, rheumatoid arthritis, α1-antitrypsin deficiency, and others. This method consists of repairing or replacing mutated genes. It also regulates the expression of genes, affects the immune system and directs the cell to be destroyed [18]. With it, the basics of the disease can also be learnt, the diagnosis improved or a form of therapy established, plus the results of treatment can be tracked. It gives great hope for an effective fight against many diseases, but it is still being researched and tested.

Gene therapy is performed *ex vivo*, when a gene is introduced into the cells outside the body. These cells are then introduced into the host, where the desired protein is subjected to expression. The second method, *in vivo*, is the direct injection of the gene into the body [37]. *In vitro* requires the removal of cells, and sometimes even the whole body. After transfection and the selection of cells containing a new gene, the removed fragments are introduced in place. In both methods, the gene vector is used, although this is mainly the virus, whose type depends on the delivered gene or its destination. The recombinant vector, with a new piece of DNA, is inserted into the patient, in the case of an *in vivo* process, or directly into tissue or cells *in vitro*. However, firstly, an appropriate way of supply is selected [33, 34].

Viral vectors

Most of the vectors used in gene therapy are viruses, and these are both those with DNA, as well as those with RNA. There are retroviruses, AAV (adeno-associated virus), adenoviruses, HSV (herpes simplex virus), and studies are being conducted over other lentiviruses, the cytomegalovirus (CMV) and influenza virus [7, 28]. Adenovirus type 2 (Ad-2) and murine leukemia virus are the most commonly used in this process [11]. Viruses were the first to be used in gene therapy because of their ability in introducing DNA into the targeted cells. However, for this method they have been changed so that they do not have the ability to replicate. The genes responsible for the expression of proteins harmful to the host are also removed, and the fragments meant for therapeutic use are implemented. Thus prepared, the virus is a safe vector [34].

Retroviruses

Retroviruses, like HIV or MuLV, are sized at about 100 nm. They have single-stranded RNA of 7 to 10 kb length, in two copies. The maximum length of the gene cloned into the virus is 8 kb. Before it is copied to the host genetic material, the RNA is copied by reverse transcriptase in a double-stranded DNA helix. The infection of cells by retroviruses is as follows:

1. The combination of the envelope glycoprotein with the cell surface. The coating can only connect to certain types of cells, which depends on the type of receptors on their surface.

2. Fusion of the coating and cell covers.

3. Injection of RNA into the cytoplasm. What follows is copying ribonucleotide strings into deoxyribonucleotide ones.

4. The inclusion of viral DNA in the DNA of the host. A provirus is formed.

5. Transcription initiated by the promoter of a section of the 5 'LTR (long terminal repeat). Part of the mRNA leaves the nucleus and is packed into the virion, and the remaining transcripts are translated.

6. The inclusion of RNA from virions. Next, virions are released [10].

In the case of most retroviruses, such as MuLV, should the infection occur, the attacked cells must undergo mitosis at the time a disruption of cell layers occurs. Lentiviruses do not require the cell to be divided during an infection. To insert the DNA fragment to the target cells, the provirus must have the appropriate sequences in *cis*, or LTR (at the ends of the 3' and 5'), primer binding site (PBS), polipurine (PP) and the packing sequence.

• LTR (long terminal repeat) – initiate transcription at the 5' end, poliadenylate the 3' end, include fragments of the viral genome into the genome of target cells;
• PBS – links to tRNA, which is a primer to extend one strand of DNA;
• PP – minimum 9 of purines, a starter for the polymerization of the second strand;
• a packing sequence – after binding of the signal, allowing for the packing of the viral RNA [7].

The sequences in *trans* placed in the packing cells encode the proteins necessary for packing or to infect, and the activities of which genes are cut from the virus to implement another therapeutic fragment [10]. First of all, viruses have been deprived of the possibility of replication in order to protect the tissue against uncontrolled spread of retroviruses [22]. Therefore, the cells of the *trans* sequences have gag genes (structural proteins), env (coat proteins), pro (protease) and pol (polymerase). After implementing the altered viral genome to the rest of them, an efficient vector is obtained.

Adenoviruses

In contrast to retroviruses, the adenoviral genome consists of double-stranded DNA in a single copy [20]. The length of the linear DNA is 36 kb. Adenoviruses with a diameter of 70–100 nm, have icosahedral, a protein capsid composed of capsomeres (pentons and hexons) [26]. The insert can be from 8 kb to 35 kb, depending on the deleted fragments of the genome. The advantage of adenoviruses is that they do not need the cell to be at the stage of mitosis to infect it.

To enter the cell, a connection of the envelope proteins with proper receptors on the surface of the cells must occur. This causes endocytosis. Then, the low pH damages the endosome membrane and the adenovirus enters the cytoplasm, before DNA is introduced into the nucleoplasm [18]. The life cycle begins with the transcription of early genes E, and late genes of the main MLP (major late promoters). The E1-E4 and L1 regions are transcribed. After several hours from infection, replication begins, and as it goes into further stages it stops the transcription of the host cell. The regions L2-L5 co-create structural proteins of the adenovirus. The life cycle of the virus leads to the death of the infected cell.

Adenoviral vectors (Ad) are formed with a few modifications. The first is the removal of the nonessential E3 fragment. At the same time it removes the E1, which is however necessary for viral replication and, as such, it is supplied in *trans* by the host cell. Then, in place of the E1 expression, a cassette is placed, which carries a new gene, controlled by an additional MLP. The recombinant gene can also be under the control of a naturally occurring MLP or CMV promoter. Other vectors, such as pAdBM5, were also created. In the MLP region, amplifiers were introduced to increase the transcriptional efficiency of the promoter [29, 37].

AAV has a single strand of DNA with a length of 4.7 kb. The virus itself is the size of 18 to 26 nm and has no membrane. It is able to replicate only in the presence of adenovirus. It infects non-dividing cells, and the maximum size of a cloned fragment is 4.5 kb [8]. The genome consists of two genes, the rep and the cap. The rep gene is responsible for replication, expression of structural genes and integration with cellular DNA. The cap gene produces capsid protein. Both the rep and cap are included in the *in cis* ITR (inverted terminal repeat) sequences. AAV entry into the infected cell begins by binding to the receptor; then, the virus enters the cell by endocytosis. Within the nucleoplasm, the AAV genome changes to the double-stranded DNA intermediate. The intermediate can be transcribed. It is formed by adding a second strand, or

a combination of complementary fragments of viral DNA. The particle is then incorporated in the 19th chromosome [3]. Creating the AAV vector includes the removal of *rep* and *cap* genes and replacing them with the desired genes. The *rep* and *cap* products must therefore be supplied *in trans*. Secondary gene products of adenovirus E1A, E1B, E2A, E4 and VA proteins are provided as well [42].

The herpes virus type I (HSV-1)

The double-stranded DNA of the herpes simplex virus (HSV) is surrounded by a triple-layer lipid containing the glycoprotein. The linear genome is approximately 152 kb long, and the virus has a diameter of 180–200 nm [42]. It is possible to clone DNA up to 30 kb into HSV-1.

The virus attacks the epithelial cells of the skin and mucous membranes. Enveloped glycoproteins bind to heparin sulfate, a cellular receptor. After the merging, the genome with the viral protein goes into the cells. One of these proteins is a transcription factor VP16, which initiates the transcription of genetic material after it gets to the nucleus. To start with, five early genes are transcribed (IE – immediate early, α). The mRNA early (E, β) encodes proteins necessary for DNA synthesis. Late genes (L, γ) are responsible for the synthesis of the virion structural proteins as well as the particles necessary for infection. The casing is formed from the host cell nuclear membrane.

To minimize the HSV-1 vector's harmfulness, IE genes are removed [42]. HSV vectors are also produced in cell lines and the missing proteins are given in *trans*. For proper replication and packing, the virus needs only one area of replication start and a packing signal in sequences in *cis*.

Another type of vector HSV-1 is amplicon, containing the origin areas of bacteria and herpes virus. It also has packing sequences. Amplicon replicates in a cell in the presence of a wild virus.

Vaccinia virus

1. Belonging to poxviruses, the vaccinia virus has double-stranded DNA with a length of 185 kb. The entire virus has a diameter of 300 to 400 nm, and the size of the insert that makes it possible to introduce is up to 50 kb [33]. In contrast to previously described viruses, this one does not make its genome in the nucleus. The entire life cycle occurs in the cytoplasm. After the merger of viral and host cell covers, the genome enters the cytoplasm, the necessary enzymes are released and transcription begins [21]. Early proteins are formed to begin with, participating in replication. They are also responsible for the initiation of subsequent transient gene transcription [33]. Then, the resulting mRNA produces transactivators of the late gene transcription. The proteins constructed at the end build the casing; they are also early transcription factors, intended for another line of viruses [21]. For the production of vectors, homologous recombination is used. The advantage is that the recombination occurs naturally during replication. There are four methods of vaccinia virus vectors synthesis.

2. The use of homologous recombination that occurs in the cell. Transfection of the virus and plasmids containing the desired gene is evoked. Between these two particles, recombination occurs and the recombinant containing the proper gene is formed.

3. Ligation of the gene from the viral DNA by *in vitro*.

4. The use of viral DNA as part of the BAC (bacterial artificial chromosome). Vectors are produced in bacterial cells.

5. Catalysis by rabbit fibroma virus (SFV – Shope fibroma virus). This allows recombination and replication with a high frequency [12].

Non-viral vectors

Non-viral vectors include RNA, DNA or synthetic oligonucleotides containing a given gene; their transport to the cell is not associated with the use of the virus [54]. The gene produces a therapeutic protein or encoded antisense RNA, blocking the expression of any of the naturally occurring genes. Therefore, it is possible to insert a plasmid, bacteriophage, cosmid, BAC or YAC to the bacteria. There is also research on the use of artificial mini-chromosomes as vectors. Each of the molecules is limited to a maximum length of the inserted fragment. Therefore, for the plasmid it is 15 kb, for bacteriophage – 20 kb, cosmid – 45 kb, BAC – 100 kb, and YAC – 1000 kb. The most commonly used are plasmids, due to their easy amplification and introduction to the cells. In comparison to the viral vectors, non-viral ones have much lower transfection efficiency.

In addition to the DNA used as a vector, its variants are also synthesized, linking it with the liposomes (lipoplex), polymers (polyplex) and peptides [12]

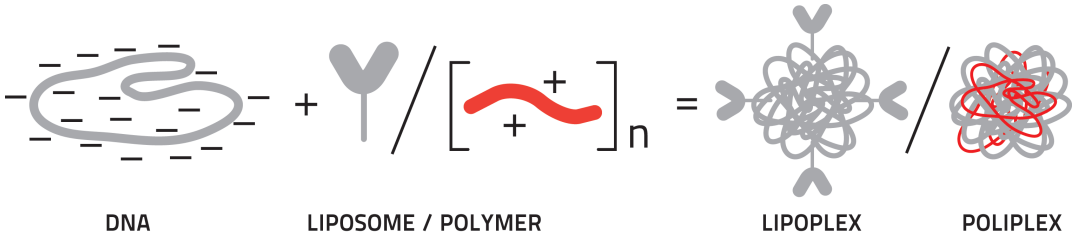

Fig. 2. The development of the DNA complex liposomes and polymers

(Fig. 2). Because of the positively charged liposomes or polymers, the negative DNA molecule receives a positive charge, which facilitates the introduction of the created complex to target cells whose surface is of an anionic character. Moreover, this way the nucleic acid is protected against nuclease. Ligands are attached to the complexes to prevent the interaction of vectors with undesired cells. The complexes are absorbed into the cell by endocytosis, phagocytosis, or pinocytosis.

Polymers used for the synthesis of non-viral vectors can be degradable and non-degradable. Degradable polymers are less toxic to cells because they have a lot of easily hydrolyzable bonds. Their flaw is the low efficiency of transfection. These substances include poly-L-lysine and poly[α-(4-aminobutyl)-L--glycolic acid]. Non-degradable polymers are characterized by higher transfection efficiency, while at the same time they have increased toxicity due to their large positive charge, resulting in aggregation of blood components during *in vivo* applications. The most commonly used non-degradable polymer is polyethyleneimine (PEI). Synthesized polyplexes are stabilized by the method of cross-linking. It involves the formation on a system of networks on the surface of the complex. They can construct various bonds, e.g., disulfide bonds [17].

Methods for introducing non-viral vectors into the cell

Direct injection – the simplest method is to use a syringe and needle, but it is not effective for all tissues. It is used *in vitro* and *in vivo*, mostly for the introduction of naked DNA. There are many varieties of direct injection:

1. Gene gun – carriers are tiny metal balls with DNA on their surface. They are shot from a gas pistol directly into the cell. However, the depth to which the balls can get is limited and most often this method is applied to the superficial tissues.

2. Electroporation – short and intense electric impulses destabilize the membrane, which enables DNA to be more easily absorbed. DNA is introduced into the tissue, and then the impulses are applied.

3. Ultrasound – this method is not completely understood. However, ultrasound is likely to increase the permeability of the membrane.

4. Jet injection – The injected DNA is in a solution. The high pressure is created when the solution passes through a small hole enabling the DNA to break through the tissue.

5. Magnetofection – DNA is associated with paramagnetic particles. The magnetic field can introduce a gene into a cell.

Transfer with the use of carriers – liposomes, peptides, or polymers, which form a complex with the DNA, are designed to reduce the size and charge of DNA. This facilitates the introduction of genetic material into cells. It also protects nucleotide chains against nucleases. These methods are used *in vitro* and *in vivo* [37].

1. Lipoplexes – a complex of DNA/liposome. There are cationic, anionic and neutral liposomes. Transfer to the nucleus begins by binding with the cell surface; then endocytosis comes, coming through the endosome and liposome range, and finally the DNA is transported into the nucleus [12].

2. Polyplexes – a complex of DNA/polymer, and it is possible to use natural proteins (such as histones) as well as artificial (polyamino) and other polymers. The fact that polyplexes have no charge facilitates their transport in the blood [42].

3. Lipopolyplexes – a complex of DNA/polymer/liposome, LPD; this complex performs the same function of packing and protecting the DNA in order to deliver it to the nucleus.

Peptide-DNA complex – the forming of, for example, short peptides from human histones and binding with DNA [12].

siRNA technology

The RNA interference (RNAi) represents a natural mechanism to protect the genome. In the past few years, the field has emerged at a surprisingly high pace. The underlying molecular mechanism of gene silencing provides with short interfering RNAs (siRNAs), allowing for the targeting of any gene with high specificity and efficiency. siRNAs can now be obtained in various ways that allow for numerous *in vitro* and *in vivo* applications. Successful knock-downs of disease-related genes indicate that siRNAs open the door for novel therapeutic procedures [44]. Control of disease-associated genes makes RNAi an attractive choice for future therapeutics. Basically, every human disease caused by activity from one or a few genes should be amenable to RNAi-based intervention. This list includes cancer, autoimmune diseases, dominant genetic disorders, and viral infections. RNAi can be triggered by two different pathways: (*i*) an RNA-based approach where synthetic effector siRNAs are delivered by various carriers to target cells as preformed by 21 base duplexes; or (*ii*) *via* DNA-based strategies in which the siRNA effectors are produced by intracellular processing of longer RNA hairpin transcripts. The latter approach is primarily based on nuclear synthesis of short-hairpin RNAs (shRNAs), which are transported to the cytoplasm *via* the miRNA export pathway and are processed into siRNAs by Dicer. While direct use of synthetic siRNA effectors is simple and usually results in potent gene silencing, the effect is transient. DNA-based RNAi drugs, on the other hand, have the potential of being stably introduced when used in a gene therapy setting and permit, in principle, a single treatment of viral vector-delivered shRNA genes [24].

Gene therapy in the treatment of SCID (severe combined immunodeficiency)

Severe combined immune deficiency (immunodeficiency) is a genetic disorder manifesting itself in the first 5 months of life. Symptoms include constant infections and delayed physical development [8]. There is a smaller amount of T and B lymphocytes or their damage. The condition can be caused by mutations in a gene on chromosome X or adenosine deaminase deficiency (ADA), and it is in the treatment of this second case that gene therapy was first applied [47]. ADA enzyme deficiency, decomposing deoxyadenosine, causes the accumulation of purines, which are converted to toxic adenosine triphosphate in T cells. It destroys the immunological system [20, 25]. In 1990, a retroviral vector was used in such gene therapy. It was a LASN vector based on the murine leukemia virus (MuLV). A gene was then introduced to it as a cDNA with a length of 1.5 kb, encoding the ADA. It was under the control of a retroviral promoter in the LTR sequence. The vector also contained the *neo* gene b, controlled by the simian virus promoter (simian virus 40 – SV40). PA317 packing cells were used for LASN packing. T cells were collected from 2 patients and bred with interleukin-2. This breed has undergone transfection by prepared vectors. The transfection efficiency ranged from 0.1 to 10%. Along with the observation of the whole process, more vectors were added. Before introducing the modified cells to the patient's body, they were washed with 0.5% albumin solution. After two years of therapy, the new gene expression was preserved [13, 19]. Today, gene therapy is still used for SCID treatment.

Gene therapy familial hypercholesterolemia

Familial hypercholesterolemia is an inherited genetic disease, caused by a mutation in chromosome 19. It is characterized by elevated levels of LDL (low-density lipoprotein), leading to coronary artery disease, even at a very early age. The basis of the disease is a defect in the LDL receptor gene. The malfunctioning receptor lowers cholesterol metabolism. Heterozygotes have half the normal receptors, while homozygotes have from 0 to 20% of them [5].

The first patient subjected to this therapy was homozygous about the mutation of the LDL receptor gene. Treatment was performed *ex vivo*. A fragment of the patient's liver was taken and hepatocytes were isolated from it. The retroviral vector was prepared by introducing the gene encoding the normal human LDL receptor. This gene was under the control of the β-actin promoter from chickens and the cytomegalovirus amplifier. The vector was introduced into liver cells, which were then selected for the cloned insert, testing their ability to adopt the fluorescently labelled LDL. Modified hepatocytes were introduced to the body. The LDL level was decreased, while the HDL level increased. Furthermore, the efficacy of lovasta-

tin, which had not occurred before using gene therapy, was observed. Lovastatin inhibits 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMGR), which prevents the synthesis of mevalonate, a precursor of cholesterol [23]. The effect of gene therapy lasted for 2.5 years, without any immunological response to the similar use of a recombinant gene. Using the therapy *ex vivo* with other patients also yielded satisfactory results.

Conclusions

The presented processes are dominating in biotechnological production methods, therapeutics, and recombinant DNA technology as a basic tool for the majority of them, allowing for their constant development and improvement. Genomics, the study of the genomes of organisms, and proteomics, studying proteins normally synthesized in the cell, produce a lot of information then used as the basis for the design of new biopharmaceuticals [6, 16, 27, 46, 49]. Drugs produced by biotechnology assume an increasing share among therapeutics. Most biopharmaceuticals are produced using *E. coli*, *S. cerevisiae* and animal cells (as CHO).

With the development of biotechnological processes comes an increase of the hopes of discovering drugs for diseases that have been the biggest challenge for researchers so far, such as cancer or AIDS. In cancer therapy, the research is carried out using monoclonal antibodies or vaccines. Technology is also used to produce vaccines to be used in the treatment of Alzheimer's disease. Gene therapy is also a promising tool to fight cancer, genetic and infectious diseases, and although it is still at an early stage there are increasingly new and encouraging results. Biotechnology is thus a rapidly expanding field, showing the possibility of the effective treatment of diseases that today may seem incurable.

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