The use of genetic engineering in creation and manufacturing of antibodies for antineoplastic immunotherapy

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Gottfried Benn 1912

Mann und Frau gehen durch die Krebsbaracke

Der Mann:
Hier diese Reihe sind zerfallene Schöße
und diese Reihe ist zerfallene Brust.
Bett stinkt bei Bett. Die Schwestern wechseln
stündlich.

Komm, hebe ruhig diese Decke auf.
Sieh, dieser Klumpen Fett und faule Säfte,
das war einst irgendeinem Mann groß
und hieß auch Rausch und Heimat.

Komm, sieh auf diese Narbe an der Brust.
Fühlst du den Rosenkranz von weichen Knoten?
Fühl ruhig hin. Das Fleisch ist weich und schmerzt
nicht.

Hier diese blutet wie aus dreißig Leibern.
Kein Mensch hat so viel Blut.
Hier dieser schnitt man
erst noch ein Kind aus dem verkrebsten Schoß.

Man läßt sie schlafen. Tag und Nacht. - Den Neuen
sagt man: hier schläft man sich gesund. - Nur
sonntags
für den Besuch läßt man sie etwas wacher.

Nahrung wird wenig noch verzehrt. Die Rücken
sind wund. Du siehst die Fliegen. Manchmal
wäscht sie die Schwester. Wie man Bänke wäscht.

Hier schwillt der Acker schon um jedes Bett.
Fleisch ebnet sich zu Land. Glut gibt sich fort,
Saft schickt sich an zu rinnen. Erde ruft.

Table of Contents

Preface:

“The emperor of all maladies” is how Siddhartha Mukherjee in his Pulitzer Prize winning “biography of cancer” describes a disease which in the 20th century became what once was the plague or cholera. Gottfried Benn, a famous expressionistic poet, wrote at the beginning of the last century a drastic existentialistic poem about the destructive effects of cancer which was then still seen as a matter of immutable fate.

Although there are cases of cancer already detected in the antique world, only the decrease of infectious diseases due to strong improvements in hygiene in the 19th century made cancer the new challenge to focus on by medicine. Today it is fair to assume that almost everyone once in their life has witnessed a case of cancer, mostly in their own family. And even though we can look back on decades of intense research on antineoplastic therapies the mortality rate depending on the type of cancerous disease is still fairly high. But with every day the chances of survival are increasing with new discoveries and developments.

Although the idea of immunotherapy against cancer is not a new one, the development of practicable treatments with immunotherapeutic drugs created with genetic engineering sprang up recently. Today, there are multiple developments in this direction which are using genetically modified antibodies to cure cancer. With the present paper, we would like to give an inside into the use of genetic engineering in designing, creating and manufacturing of antibodies for immunotherapy of cancer.

Introduction:

According to the Global Health Observatory (GHO), 8.8 million people died of cancer in 2015. This corresponds to almost one sixth of all deaths worldwide and with that cancer is the second leading cause of death globally. Cancer however does not only affect the global demography, but it also has a tremendous impact on economy by causing costs of more than 1 trillion USD. It is fair to say that cancer represents one of the bigger challenges the health organizations and health institutions have to face right now. There are many problems concerning cancer and its treatment which lead to such high numbers of mortality. Already the detection of cancer and its type is quite difficult. But often it is the treatment itself which bears the most risks and problems. Classical antineoplastic (anti-cancer) therapeutic methods such as surgery, radiation, and chemotherapy not only fail to cure the great majority of neoplasms, but their application often leads to severe side effects associated with severe morbidity. Immunotherapy represents a new way of antineoplastic therapy and has already been proven to be quite effective. The immunophenotypic heterogeneity of cancer cells and mostly the impressive genetic ability of cancer cells to adapt still remain a great challenge in the present immunotherapy of human neoplasms. The advances in monoclonal antibodies (mAb) production have revitalized the concept of use of cancer cell specific "silver bullets." Silver bullet refers to an 80s horror movie in which only such special ammunition is capable of killing a werewolf. Antibodies represent new approaches to anti-cancer therapy: They are developed with the aim to destroy the diseased tissues and preserve the normal.

Strategies for the use of antibodies include:

1) Modulation of the immune response;

2) Obstruction of the growth and differentiation of malignant cells;

3) The use of tumor-targeted immunoconjugates, which are monoclonal antibodies that are linked to a variety of agents (e.g. radionuclides, chemotherapeutic drugs) for selective delivery to neoplastic cells (silver bullet).

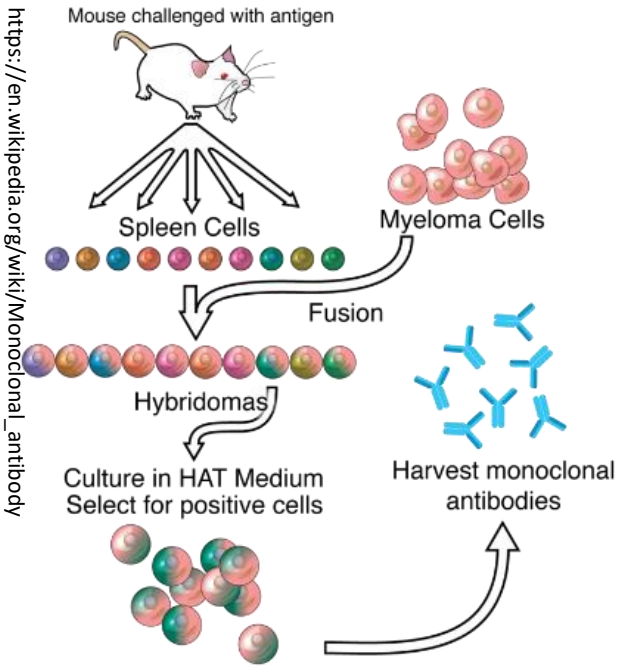
Meanwhile the field of immunotherapy is developing new approaches, which are not in the focus of the present paper. These techniques involve the reinfusion of genetically modified human T-cells.

Many steps in the development of an antibody for immunotherapy involve genetic engineering of which only a fraction is discussed in the following paper.

Essential steps are the production of monoclonal antibodies (mAbs) and the use of hybridoma technology for their reproduction, as well as the commonly used humanization method by CDR grafting.

Description of engineering technique:

Antibodies are immunoglobulins (IG) secreted by plasma cells which originate from bone marrow B-lymphocytes. Antibodies bind to antigens, *i.e.* substances foreign to the body, triggering an immune response. The body can produce more than 10^8 different antibodies. Every single can recognize and bind to one particular antigen. The so-called paratope of an antibody is specific to a particular epitope, a binding site that sits on the pathogen's antigen. In a lock and key mechanism, the antibody's paratope will lock onto the epitope of the antigen, rendering it harmless or triggering further immune responses.



Monoclonal antibodies (mAbs) have become one of the largest groups of biotherapeutic proteins (biologics) which are now being used for a wide variety of therapeutic applications. The discovery and development of hybridoma technology, a method for producing large numbers of identical antibodies, by César Milstein and Georges J. F. Köhler in 1975 created the foundation for modern day mAb discovery and development.

Figure 1. The production of monoclonal antibodies using immunization of a mouse

In a first step of the development of a therapeutic mAb a mouse is immunized. This means the mouse is injected with the antigen of interest. This triggers an immune reaction which results in multiple antibodies (polyclonal) secreted by a mixed population of B cells with each cell secreting only one specific antibody. In order to obtain the best binding antibody on its own (as a monoclonal antibody) the specific B-lymphocyte is fused with a myeloma cell (cancer cell) of the same species. The resulting hybrid (see Figure 2.) combines the capabilities of the lymphocyte and the immortality of the tumor cell. These cells can be added to a culture medium. Each cell of the grown cell colony then produces the same type of antibody. These mAbs are given off into the medium where they can be harvested in large amounts.

Although the first antibody produced by genetic engineering, *i.e.* a recombinant antibody, was produced using this technology, including the first approved therapeutic antibody muromonab-CD3 (Orthoclone OKT®3) in 1986 for preventing kidney transplant rejection, hybridoma production presented some drawbacks. Hybridomas can be labor intensive, low yielding or genetically unstable. More importantly though, the antibody sequences originated from an immunized animal and consequently had the potential of triggering an immune response in humans. Therefore, further improvements were needed to yield antibodies more human-like and safe. Today non-human mAbs for therapeutics, after their generation by immunization of animals (e.g. mice or rats), are then engineered to prevent an immune response in patients against non-human sequences.

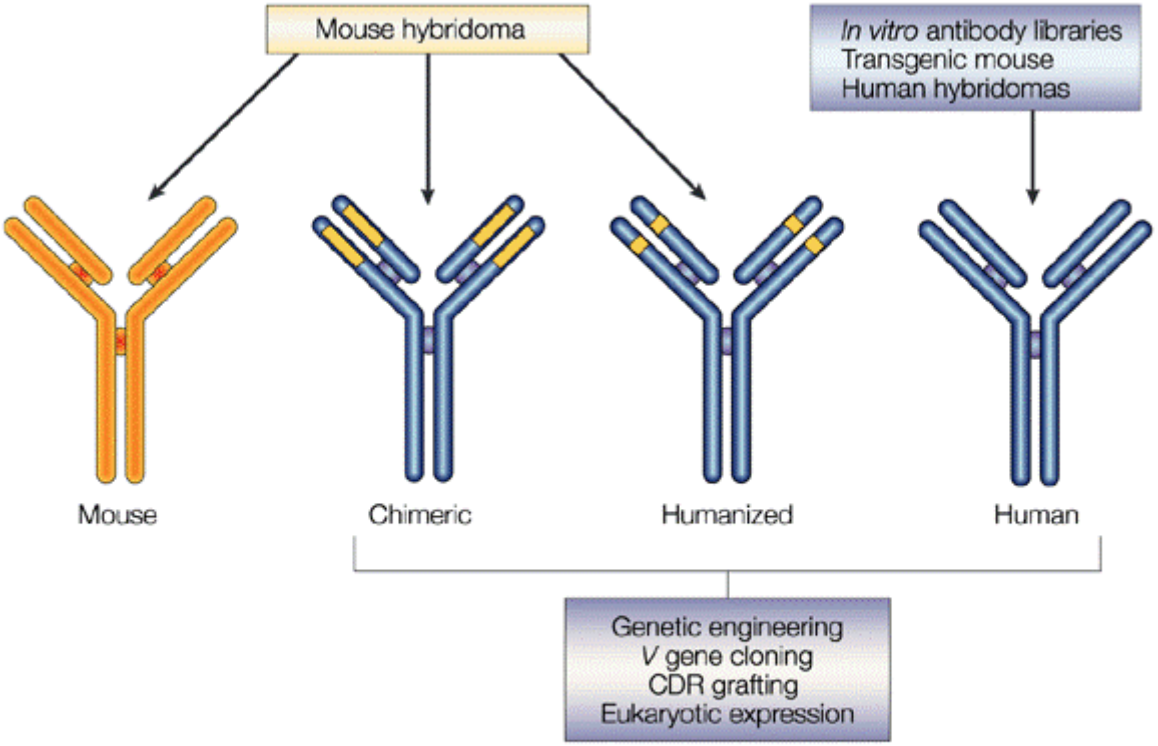
Humanization is important for reducing the immunogenicity (ability of triggering immune response) of mAbs derived commonly from rodent sources and for improving their activation of the human immune system. Initially this was achieved through the creation of chimeric mAbs by replacement of the constant regions of the rodent antibody with those of a human antibody.

Today V region humanization often uses Complementarity-determining region (CDR) grafting ("transplantation"). The complementarity determining regions (CDRs) are the hypervariable 'ends' of an antibody which are responsible for where antibodies bind to a specific antigen. CDR grafting is a humanization technique whereby humanized antibody sequences are generated by carefully selecting the CDRs of the parental antibody (typically murine) and grafting them into a human framework.

This process often reduces the mAb affinity (the binding capability), and hence, it is usually followed by affinity maturation.

The drive to reduce immunogenicity by decreasing the mouse content in mAbs inevitably lead to the creation of so-called fully human antibodies. Two of the most widely used techniques developed for the production of fully human monoclonal antibodies are phage display, where a library of human antibodies is expressed on the surface of phages and then selected and

amplified in E. coli, and transgenic mice expressing a human antibody repertoire. To date, the Trianni Mouse is the only transgenic antibody discovery platform that offers the entirety of the human antibody gene diversity in a single organism. This means antibodies produced by the Trianni Mouse are a perfect match for humans.



http://nicb.ie/biotechnology/what-is-a-monoclonal-antibody/ **Nature Reviews | Drug Discovery**

Figure 2. Types of antibodies with different degrees of humanization

Interview:

Beatrice (Bea) Dolder Schlienger is a scientist working in the lab for immunology at the "Zentrum für Lehre und Forschung" of the University of Basel. As mentioned in this interview, Bea was a part of the research group which developed the method of production of hybridomas.

Aaron: "What are you researching at the moment?"

Scientist 1[1]: „We are researching on immunotherapy. A new approach of cancer treatment, where we try to strengthen the immune system to fight against tumors. There are different types of immunotherapy. The one that we're focusing on at the moment is with T-cells, the cells that kill other cells, by trying to activating them against cancer cells, but we're also working on dendritic cells, the cells that recognize tumor cells, making them visible to other cells of the immune system, which then are able to kill that cell."

Aaron: „So we're focusing on the production of genetically engineered antibodies for immunotherapy, especially on hybridomas. What do you know about this technique?"

Bea: "Hybridomas is actually already a rather old technique. I myself worked for the scientist that developed this technique in his doctoral thesis. In former times, you used to fuse B-cells with tumor cells to produce hybridomas that produce monoclonal antibodies. A monoclonal antibody is an antibody which only reacts (binds) to a very specific antigen. And through this method you can produce those antibodies."

Aaron: „You have just mentioned that this method is already outdated…"

Bea: "Well don't get me wrong, this approach isn't outdated. The method is only outdated for production. Today most monoclonal antibodies are synthesized. But we still use this method for the discovery of monoclonal antibodies that we are at the moment still using in diagnosis and therapy."

Scientist 1: "In former times you used to insert the protein that you wanted to fight into a mouse or a rabbit. This mouse therefore showed an immune reaction. The B- cells of the mouse start to produce antibodies. There are billions of different B-cells in an organism, each

[1] Scientist 1 is a colleague of Beatrice Dolder Schlienger

one reacting to something different. We as scientists hope that one of those B-cells reacts to the protein the way we would like them to. In former times they used to just isolate all antibodies. Then you have millions of different antibodies but just very few react to your protein. So they did so called limited dilution. They diluted the sample of blood so far until you could observe single cells. Then they characterized those cells, by looking at what they react to and which antibodies they produce. But today you can just look at the DNA sequences of the hybridomas that were produced and characterized before, cut those sequences out and put it in a new cell. Then you can cultivate those cells in huge tanks and therefore you'll get millions of cells producing your desired antibody. So in today's time most antibodies get discovered through the production of hybridomas or through old antibodies."

Aaron: "And what did they use to fuse the B-cells of the mouse (or rabbit) with the tumor cells? "

Bea: "Polyethylene glycol (PEG)"

Aaron: "Since hybridomas are cells from an animal, they can trigger an immune response in humans. Therefore hybridomas first need to be humanized before being able to be used on humans. Since you've said earlier that most monoclonal antibodies are synthesized today, is there still a need for humanizing them?"

Scientist 1: „Yes. You still do this today. As you know antibodies have a Y- shape and what's important for the binding is only the front part. So in former days you cut out the tail of this Y and exchanged it with a human one. But today you fully humanize the antibodies. So you only leave the front most party of the antibodies, the amino acids, and replace the whole rest of the antibody with a human (so called vector) one. (...) Most antibodies today get produced in pharma, since it is very time-consuming to produce them clinically."

Aaron: "This directly brings me to another question we wanted to ask. How long does the production usually take until you can really utilize the antibodies?"

Bea: " Mhhh... it really has been a long time since I've done that. After you've injected the protein into your mouse, it takes a while until there is an immune response. Then it takes a while until your hybridomas have grown. After you then have done limited dilution you have to characterize every different cell of your pool until you find the one you want. This is the biggest work and is very time-consuming and this for sure took about 6 weeks to two months."

Scientist 1:”Today you can speed up this process through sorting. There is a machine with which you can take a mixture of cells, dye them (also with antibodies) and afterwards they have different colors on top of them and then you can shoot them one after a time through this machine and measure with a laser which color this cell has. And then you can shoot every single cell into a well, and then you can only take those that produce antibodies for instance. So you can nowadays only put those into wells that show an immune response to your protein (the ones you want). But characterizing those is still today the most time-consuming work. So to find out which antibodies show an immune response is quite easy but to find out which one of those maybe hundred antibodies that showed a response is the best one, is very time-consuming. Also because what is the best one? Is the best one, the one that binds the strongest, or the one that binds the fastest? And then you still need to look that he doesn’t bind to anything else. So for instance it could be that your antibody bind very strong to what you want it to bind to. But it also very weakly binds to a protein found on your heart. So if you would give this antibody to a patient, his heart would slowly break down. So you need to find out if there are any side effects when you characterize them and this can take years.”

(...) Scientist 1:”Antibodies can also be modified, for example that the back part doesn’t bind anymore etc. This is also used by many firms today.”

Aaron: “After producing those monoclonal antibodies. Do you then just inject them into a patient or how do you give them to a patient?”

Bea: “Yes the final antibodies are then so far developed that you can just simply inject them into a patient.” Scientist 2:” But then from the discovery of the antibodies until the clinical use there is still a long way of testing if they really to what you want them to do. This can also take quite long.”

(..) Bea: “But our job here in the lab is not to produce the antibodies. The only thing we do here right now is to test antibodies and to observe for example in animals what these antibodies trigger or what immune cells they activate.”

Scientist 1:” Many antibodies also only work for maybe 20% of patients. So this is also our job in the research to find out why this is the case. And what could we do to higher this percentage. Or how can we exclude patients from a certain therapy in beforehand. Or find out which therapy we could do on top of the immune therapy. So we do often also test things that are already on the market.”

Leon: “Antibodies mostly only protract the growing of cancer. How is the cancer able to „outwit“ the antibodies so it can continue to grow?”

Scientist 1: “A tumor for example is a dead cell that does not stop growing. (Normally if a cell gets „sick“ there is a mechanism which kills the cell, but in a tumor the dead or sick cell starts growing because that mechanism does not work anymore.) Some proteins support this growing, but if you initiate antibodies into a species, they may stop the growing of the tumor/ cancer for a short time. In that time the DNA of billions of tumor cells get destroyed. However it is possible that one of these many cells gets destroyed in an area in which the tumor does not care about losing the receptor. So it gets a second receptor. And it does not matter how many antibodies you initiate now, the second receptor will be functional and the tumor/ cancer continues to expand.
Immune system: If many cells die the immune system surrounds the cells and starts to remove the tumor cells. Many scientists hope that we are able in the future to directly promote the immune system so the tumor/ cancer gets „destroyed“ good enough, so that the immune system is able to destroy the rest on its self.”

Bea: “Most tumor cells get destroyed but some of them fight the immune system and survive longer. The tumor sometimes sends signals to the immune cells to shut down so it can continue to grow. So if we could stop this communication the T-cells could kill the tumor cells. But that is only a goal scientist want to achieve in the future.”

Aaron: “What is the advantage of the immune therapy compared to other therapies for fighting cancer?”

Scientist 1: “The immune therapy causes less side effects than the chemotherapy for example. Also the immune therapy is able to sometimes cure advanced cancer which is not curable with other therapies. The immune therapies are often used in combinations. But only 50% of the patients benefit from the immune therapy. Often the chemotherapy and the immune therapies are combined as well. They both have their advantages. The disadvantages of the immune therapies are that some patients do not benefit at all from it, and we do not know yet which patients are not appropriate for such a treatment.”

Bea: “Some patients also have very strong side effects. (For example skin, bone or stomach problems). The immune system is fighting against the cancer all the time, so you have to check that it does not „work“ too hard so you do not get any other diseases like skin or bone problems. But you also have to be sure that the immune system does not make a too little work so the cancer does not continue to grow.”

Aaron: “What are the costs for an immune therapy?”

Scientist 1: “An immune therapy is very expensive compared to a chemotherapy for example. There are hundreds of companies which produce chemotherapies, while the immune therapy is very new and more complicated. But the production of the antibodies is not even the most expensive part. If you discover or create a new antibody you have to be able to prove that it actually works. There are different phases for that. In the first phase you have to show e.g.

with 50 “normal” patients that it’s safe to use. In the second phase you have to prove that it works in cancer patients. In the third and final phase you have to compare between for example 1000 patients that used your therapy or maybe didn’t and what impacts different doses of your therapy have on them etc. If you proved that your therapy is actually working good enough, you can make an application to the state so they can spread your new or advanced therapy. All these expenditures make it very expensive.
But still the success rate of most immune therapies is relative low and every patient is different. So one goal of doctors is to be able to analyze every patient one by one, so everybody gets the best treatment for their well-being.”

Aaron: “How do you produce these antibodies for immune therapies?”

Scientist 1: “Antibodies are produced in cells. In the industry they often use CHO (Chinese hamster ovary) cells. These cells grow extremely fast, are very stable and produce many proteins.
There are also proteins that we can produce in bacteria. This way is much cheaper but the problem is that antibodies are very complex and they have structures that bacteria do not know and that’s why you can only produce them in mammal cells. But there are also molecules which you can produce in bacteria and which are similar to antibodies. A company in Zürich (Molecular partners) for example produces such molecules.”

Aaron: “What things are very important in the production of antibodies?”

Scientist 1: “Everything must be sterile, that can be very difficult. There’s a list called GMP (Goods manufacturing practice) on which it says all the important things you have to consider. (For example there must be prove that every substance used in the production is sterile and working.) If you do something wrong it can be very expensive.”

Aaron: “Since when are scientists using the immune therapy in Switzerland?”

Scientist 1: “Treatments which are immune therapy-like already exist for quite a while. But they were not that successful. Some treatments even exist over 50 years. But the „good“ immune therapies were first used approximately 5 years ago.”

The following pictures were taken in the laboratories we visited:

- Biozentrum Basel
- Zentrum für Lehre und Forschung Basel

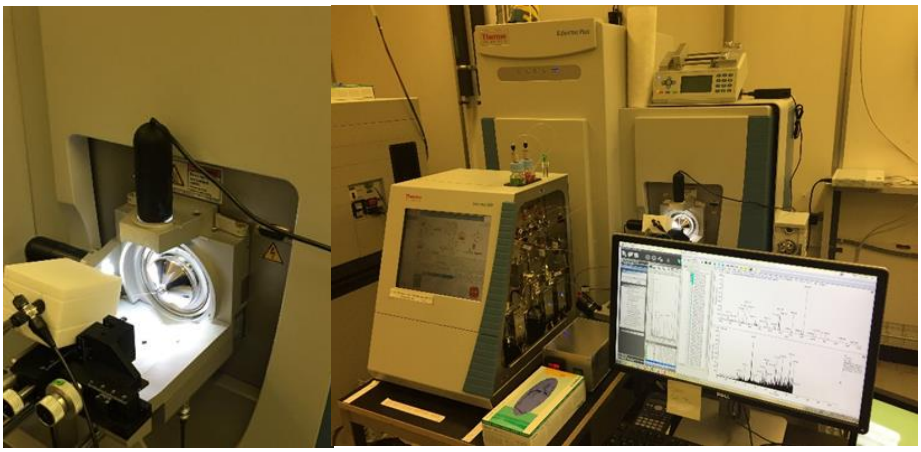


Figure 3. Mass spectrometer. These instruments are broadly used in chemical analysis.

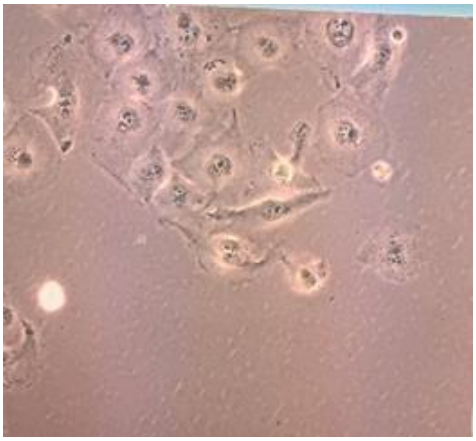


Figure 4. Tumor cells under the microscope.

Figure 5. An exhaust hood in which experiments are performed.

Figure 6. An incubator. It is used to give cell cultures the ideal temperatures to grow.

Figure 7. Microwell plate. The different wells contain tumor cells. By adding different antibodies to the different vessels their effects can be examined and compared.

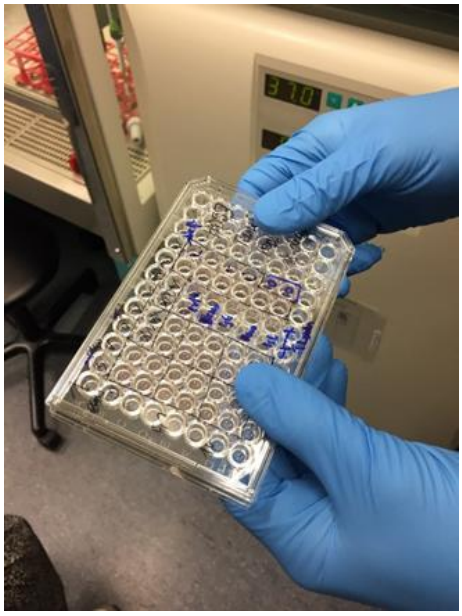


Figure 8. Ultra centrifuge. Centrifuges are used to separate samples into their different components.

Discussion

The production of monoclonal antibodies brought up a new way of cancer treatment, which for example causes less side effects than the chemotherapy, as the body uses its own immune system to fight against cancer cells. (Immunotherapy) Through this some types of cancer that weren't curable in the past have now gotten curable. Cancer cells are different from patient to patient therefore the therapy which a patient needs is different from person to person. So the more possible ways of curing cancer are known, the higher is the chance that one is very effective for a patient. So every new cancer treatment that is developed increases the chances of patients being cured from cancer.

Apart from the progress in cancer treatment, the production of monoclonal antibodies resulted in progress in many other fields:

1. Diagnostic tests

   Monoclonal antibodies for a given substance can be used to detect the presence of this substance. They can detect protein on a membrane. (through Western blot- and immune dot blot tests) They are also very useful in detecting antigen in fixed tissue sections (immunohistochemistry) and detecting substances in a frozen tissue section or in live cells.

2. Analytic and chemical uses

   Through the method of immunoprecipitation, antibodies can be used to purify their target compounds from mixtures.

3. Other therapeutic treatments

   Monoclonal antibodies can be used to fight against autoimmune diseases such as rheumatoid arthritis, asthma and Crohn's disease, but also other diseases such as hepatitis C or RSV infections in children.

The production of monoclonal antibodies brought up many advantages in medicine. Patients with various diseases that weren't able to be cured 40 years ago do now have new perspectives due to monoclonal antibodies. The biggest problem/ disadvantage of therapies using monoclonal antibodies are their costs: A therapy normally costs at least 100'000 francs, since it is a very new therapy (most drugs have patents) that are produced by few companies (In contrast to chemotherapy for example) and because during the creation of a new drug you need to prove that it really works and doesn't cause to many or to harmful side effects. All these reasons cause the price to go up drastically. This brings up the question if such an expensive drug is even reasonable, especially for elders. Expensive therapies like therapies using monoclonal antibodies increases the expenditures of health insurances thus increasing the contribution payed by an individual and therefore increasing the pressure on the working class. The other disadvantage is the success rate that is very low in comparison to other drugs (success rate of immune therapy is at around 50%)

What are future research steps?

Research does not stop. Being able to cure cancer would probably be one of the most outstanding achievements in decades. At the moment dying of cancer can only be delayed. But there is still a lack in perfection of the modern therapies. So scientists try every day to optimize the treatments of cancer and they all have similar goals for the future.

One important goal for the near future is for example to increase the success rate of immune therapies. Only 50% of all patients using an immune therapy really benefit from it. The problem is that we are not able yet to find out which patients are appropriate for the different therapies and which are not. In our interview they told us that it could be very useful to analyze every single cancer, because every patient is different. So if we could examine every single patient, his disease and what he exactly needs the success rate probably could increase. But that is way too much work. First we must invent some robots doing that for us. Increasing the success rate could help many people and if the success rate increases the demand of immune therapies increases as well.

Another goal is to minimize the side effects of the therapies. If the immune system is overstrained with fighting cancer the patient may get problems with his skin, bones, stomach etc. Most people with bad cancer logically use the immune therapies although they sometimes cause strong side effects. But some people rather renounce the side effects and try another therapy. Minimizing the side effects also would increase the demand for immune therapies and if the demand increases the industry can sell and produce more antibodies. So the market brings more profit and more companies would invest in the advancing of the therapies. And finally if enough antibodies get produced it will hopefully be as with the chemotherapy, the costs will shrink. As you can probably imagine immune therapies are very expensive. For some treatments you have to pay more than 100'000 Swiss francs. Many people are not able to pay that much money. So it is very important that every patient has an opportunity to get a reasonable treatment.

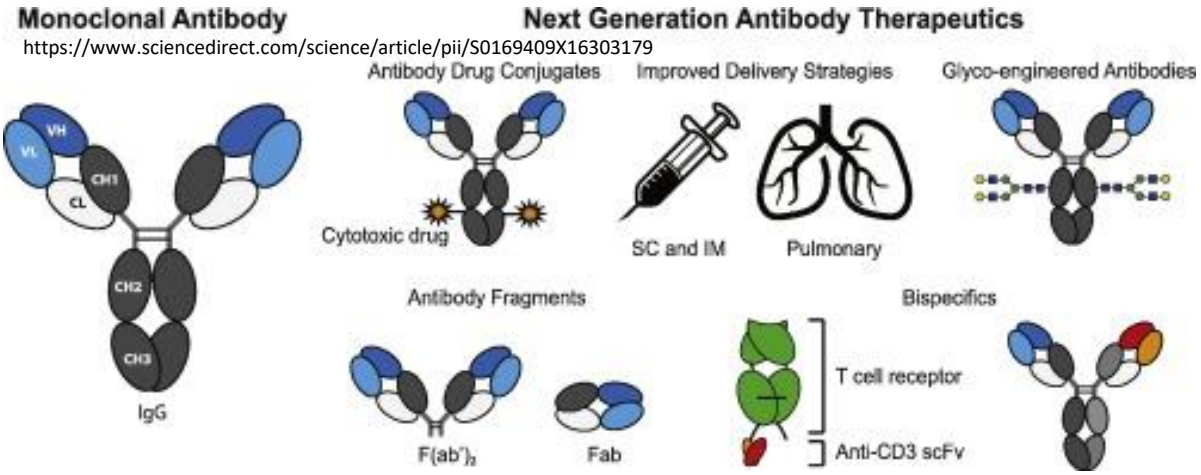


Figure 9. Future developments of new engineered antibodies

Future outlook

Today, five of the top 20 bestselling oncology drugs are already immune therapeutics. In fact with the immune therapeutic oncological drugs Rituxan, Avastin, and Herceptin Roche reached 21$ billion in sales. On the other hand, we are standing only at the beginning of the

immunotherapeutic treatment of cancer and we can observe that a lot of progress was made in this field. Novel more efficient, more precise and less harmful treatments are presently appearing. Fig. 9 illustrates the most important next-generation antibody therapeutics. These innovative treatments can only be realized by ever more involved tools of genetic engineering, helping in both, creating new molecular entities, and ever more efficient manufacturing technologies.

For the sake of brevity, the present paper did not dwell on the important trend towards personalized treatment, a mega trend of modern medicine, to the appearance of which immune therapies are delivering a major contribution.

In view of these promising developments, there is realistic hope that the present generation will see that cancer, “the emperor of all maladies”, will be turned into a chronic disease or even became a curable condition.

Summary:

Cancer affects millions of people each year, and as the world population continues to grow, the rate of new cancer cases will likely increase, too. The evolving techniques of genetic engineering are a major driver of the rapid development of the rising star in the field of oncological treatments, the immunotherapy. The present paper describes the different methods of genetic engineering used to find and improve antibodies for antineoplastic immunotherapeutic drugs. The processes mentioned in this paper involve the search for antibodies by immunizing animals with the targeted antigen and the production of a hybridoma for the reproduction of the needed monoclonal antibodies. Furthermore humanization methods are discussed which are needed in order to decrease the immunogenicity of the specific mAb. Unfortunately, treatments using monoclonal antibodies are still very expensive. More research is needed to minimize the side effects and to lower the costs for immune therapies, so that every patient gets the best treatment for his or her condition for an affordable price.

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