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Monoclonal Antibodies

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1.Preface

The motivation to work on this subject was, that monoclonal antibodies are very important tool in diagnostic and therapeutic matters and will have a big impact on human health.

Our questions were:

- How are monoclonal antibodies produced?
- How do they work?
- How is the technique evolving?
- What are the difficulties with this technique?

2.Introduction

The monoclonal antibody therapy consists of the use of monoclonal antibodies (clones of a unique parent cell) to bind to a chosen target cells. The patient's immune system is stimulated to attack the affected cells. Monoclonal antibody can be created from quite any cell surface target. For that reason there are a lot of ongoing research and development all over the world to create, modify and specialize monoclonal antibodies against numerous serious diseases including but not limited to arthritis, multiple sclerosis and cancer. This sort of therapy can now be applied due to the development of monoclonal antibody production using hybridoma cell technology. The first monoclonal therapies were limited and generally short-lived and the treatment had to be specialized to each individual patient and thus was unusable for the routine clinical settings. With the progress of monoclonal drug development, four major antibodies types were developed; murine, chimeric, humanized and human. Murine antibodies were the first gained by the hybridoma cell technology. But the problem was that there were too many dissimilarities between the murine and human antibodies. This led to allergic reactions and analphylactic shocks in the treated individuals. To prevent the problems of dissimilarities, human gene sequences were fused with murine parts to create chimeric and humanized antibodies which in great part are from human origin.

With the improving technologies it is possible to create human monoclonal antibodies which are hundred per cent derived from humans. In this process, blood of a patient (sane immune system) with a bacterial infection is taken. Then white blood cells are separated from the rest of the blood. The antibody producing cells are selected for further processing. Special immortal MAblgX cells are fused with the antibody producing cells. The successfully fused cells continue to grow and produce more antibodies. Then the cell lines are screened and selected for the desired characteristics of the monoclonal antibody. The selected cell lines are expanded for large scale manufacturing. The final product is a purified monoclonal antibody, which is used to treat patients with infections. It is fully human origin and it will combat the infection just like the human immune system would do. With human monoclonal antibodies the chance for severe side effects is minimized.

Since the new millennium the therapeutic market of monoclonal antibody is growing very fast, due to the progress in biotechnology. Monoclonal antibodies are used because they can be frozen and unfrozen often without changing their activity and they can also be held in cultures. This option makes monoclonal antibodies very suitable.

3. Description of engineering technique

3.0 What are monoclonal antibodies?

To understand the concept of monoclonal antibodies (mAb) we must know what antibodies are. Antibodies are proteins. They are produced by the B lymphocytes of the immune system in response to antigens. The antibodies bind to the antigen and mark it so that the antigen molecules can be destroyed by phagocytes. Each B cell synthesizes only one type of antibody which is highly specific for a single epitope (the part where the antibody binds to the antigen). But in an organism an entire population of different B cells and their respective antibodies (if the organism was exposed to the related antigen) can be found. To be a useful tool in medicine, biochemistry and molecular biology a substantial amount of a single antibody is required. Therefore a method to produce a population of B cells from a single ancestral B cell was necessary. From the population of B cells a single kind of antibodies can be harvested. The population of B cells is called monoclonal and therefore the antibodies can be described as monoclonal antibodies.

In short: Monoclonal antibodies are antibodies which are produced from a single cell line and highly specific for a single epitope.

3.1 Production

The production of monoclonal antibodies is a very complex process. The main steps are:

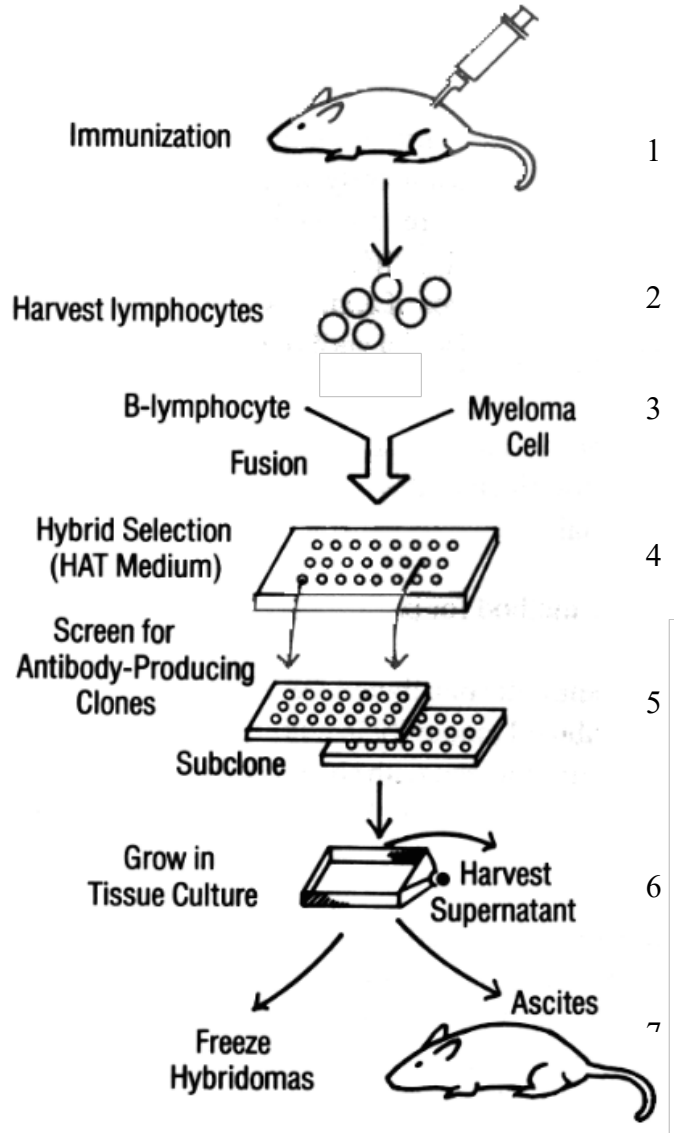
1. Immunization
2. Hybridoma cell production
3. Separation
4. Screening
5. Mass culturing

3.1.1 Immunization and test bleeds

The first step is to induce the production of antibody-producing B cells in an organism. This's done by immunizing a mouse against the antigen of interest (1). Normally the antigen is injected intraperitoneal and in two doses, an initial priming and a second booster dose (10 days later). After the immunization a blood sample of the mouse is taken (2) to check if the mouse is producing the antibodies of interest.

3.1.2 Hybridoma cell production

If the mouse produces the desired antibody, the B cells are isolated from the spleen and added to a culture of myeloma cells (cancer cells). The myeloma cells divide indefinitely but they don't produce antibodies. The B cells alone can die after 7-8 days, even though they are cultivated. The addition of polyethylene glycol (PEG) causes some of the B cells and myeloma cells to fuse together and form cells called hybridomas (hybrid = mixed). (3) The hybridoma cells provide both properties, they divide indefinitely and produce antibodies.



1: Production of mAb

3.1.3 Separation – clonal cultures

Because not all B cells fused with a myeloma cell it's necessary to separate the hybridoma cells from them. Not fused B cells die automaticly because they lack the ability to survive in culture. For the separation of myeloma cells we utilize the fact that myeloma cells are $HGPRT^-$. HGPRT (Hypoxanthine-guanine phosphoribosyltransferase) is an enzyme which is used for the synthesis of nucleotides from hyposanthine. If we place the $HGPRT^-$ myeloma cells and the $HGPRT^+$ hybridoma cells in a culture medium called HAT (hyposanthine-aminopterin-thymine) the myeloma cells die because in HAT medium only $HGPRT^+$ cells survive (4).

3.1.4 Screening

Because the collection of B cells used was heterogenous not all hybridoma cells produce the same antibody. A further problem is that the hybridoma cells are initially tetraploid (formed by the fusion of two diploid cells). The extra chromosomes are lost in the following divisions in a random manner → the hybridoma cells don't produce the desired antibody. In order to select the hybridoma cell that produces the desired antibody, each hybridoma cell is separated and individually cultured – one cell per well. All cells from each well are clonal because all of the cells derive from a single cell. - After cultivating for a few weeks the hybridoma cell colonies produced and secreted an antibody into the culture medium. Each individual culture medium is then screened for the presence of the desired antibody. The probe used for screening is the epitope of the antibody of interest. Screening is a very labour intensive process since fusion often results in thousands of hybridoma cell colonies.

3.1.5 Mass culturing

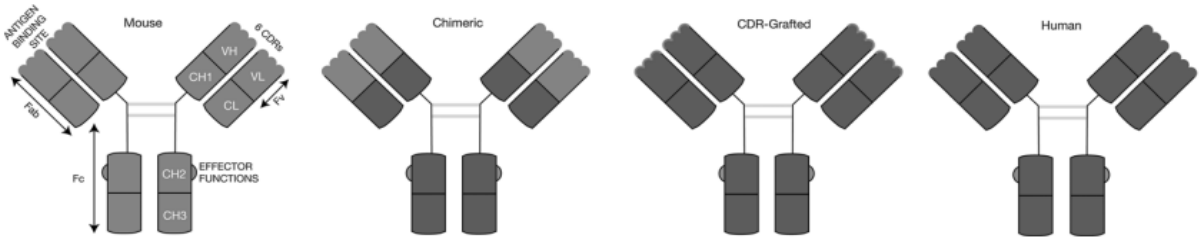
If the desired antibody was found in a hybridoma cell colony it is grown in mass culture (the hybridoma cell divides indefinitely resulting in genetically identical hybridomas). Each clone of the hybridoma cell can produce the antibody of interest. The clones can be frozen. If a hybridoma cell clone is needed he can be thawed and stored in a culture medium where the production of monoclonal antibodies is stimulated. The monoclonal antibodies then can be isolated and used. A second way to produce the monoclonal antibodies is to insert a hybridoma cell clone in an animal which then produces the specific mAb (this technique is today used very seldom because it's partially illegal.)

3.2 Recombinant monoclonal antibodies

Recombinant is a different technique to produce mAb. The production involves several technologies like repertoire cloning, phage display and yeast display. These techniques are based on the fact that immunoglobulin gene segments clone rapidly and create libraries of antibodies with slightly different amino acid sequences. Out of the library antibodies with the desired specifications can be selected. The main difference to the murine mAb is that the base of the mAb aren't mice but viruses or yeast. To produce recombinant mAb on a large scale fermentation chambers are used.

3.2 Humanizing monoclonal antibodies

The first tries to use monoclonal antibodies for therapy were not very successful because the mAb used were murine. Therefore they acted like an antigen in the human organism and caused a reaction of the immune system against the mAb. To solve this problems different techniques are used. They all have the aim to “humanize” mAb.



2: murine *chimeric* *CDR-grafted* *human*

3.2.2 Chimeric antibodies

In a chimeric antibody the variable domains (VH/VL) that contain the antigen-binding sites are from the mice and the constant domains (CH/CL) are derived from human isotypes. Chimeric antibodies are less likely to provoke a response of the immune system.

3.2.3 Humanized antibodies

In a humanized antibody only the complementarity-determining regions (main part of antigen binding site, determine to which antigen the mAb binds) and a few amino acids are murine. All other parts are from human. There are two ways to produce humanized antibodies: Humanizing via chimeric antibodies or by insertion of relevant CDRs into human antibodies (CDR grafting). Humanized antibodies are improved chimeric antibodies but more intricate in production and hence more expensive.

3.2.4 Human monoclonal antibodies

Human monoclonal antibodies are directly derived and isolated from human individuals such that no reconstruction of the antibody has to be performed. This is a huge advantage. Also, it has been shown that human monoclonal antibodies have a higher chance to successfully go through all the stages of approval by the state authorities.

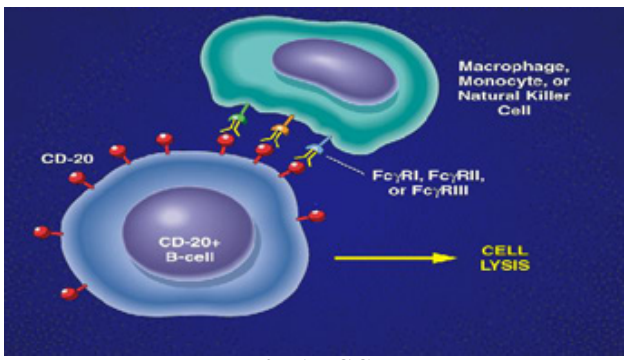
3.3 Application

3.3.1 Therapeutic treatment

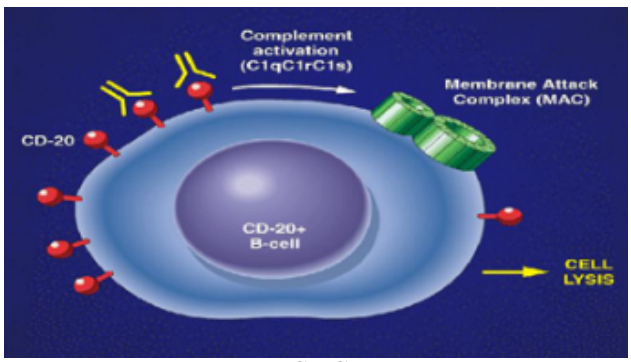
Monoclonal antibodies can be used for the treatment of B-cell malignancies. The mAb bind specifically to target cells and stimulate the patient's immune system to attack those cells. There are different ways mAb can be used. For example mAb can be used to prevent tumor growth or to destroy tumor cells. The mAb are used in different forms.

3.3.1.2 ADCC/CDC mAb

ADCC stands for antibody-dependent cell-mediated cytotoxicity. ADCC means that cells are marked by antibodies and then destroyed by natural killer cells (NK). The NK cell's Fc receptor recognizes the Fc portion of the mAb which has bound to the target cell. After recognizing, the Fc receptor binds to the Fc portion and the NK cell releases cytokines (like interferon-γ; regulate the growth of cells) and cytotoxic granules (contain perforin and granzymes). The released substances enter the target cell and promote cell death by activating apoptosis (programmed death of cell).



3: ADCC



CDC

CDC stands for complement dependent cytotoxicity. In CDC the monoclonal antibody binds to the receptor and initiates the complement system. The result of it is a membrane attack complex that forms channels which disrupt the phospholid bilayer of target cells, leading to the death of the cell. The mAb used in ADCC and CDC are injected naked (lonely) into human body.

3.3.1.3 Immunoconjugates

In immunoconjugates mAb are conjugated to a second molecule. This second molecule can be a toxin (immunotoxin), a cytokine (immunocytokine), a radioisotope (radioimmunotherapy), a killer cell (cellular immunoconjugates), a liposome (immunoliposome) or an enzyme (ADEPT). If the mAb binds to a target cell the antibody conjugate is absorbed into the cell and causes the death of the cell.

- Immunotoxin: When the conjugate binds to a target cell the toxin is taken in through endocytosis and kills the cell.
- Radioimmunotherapy (RIT): The radioactively conjugated mAb deliver a lethal dose of radiation to the tumor cells. RIT requires a tumor cell with an unique antigen (an antigen that's not accessible in normal cells) so that all the radioactivity is delivered to the tumor cell.
- Immunoliposomes: Liposomes carry drugs or nucleotides and are directed against malignant cells. This technique is still in its beginning phase but delivers budding results.
- Antibody-directed enzyme prodrug therapy (ADEPT): The enzyme binds selectively to the target tumor cells. When the difference between tumor and normal tissue enzyme levels is high enough a prodrug is injected into the blood circulation which is converted to a cytotoxic drug by then enzyme only within the tumor.

3.3.2 Obstacles to successful therapy

There are some problems in mAb therapy which might take place:

- Some tumor cells may have antigens while others don't because antigen distribution is highly heterogeneous.
- The density of antigens in tumor cells varies. If it's too low mAb aren't effective.
- If mAb or prodrugs need to be delivered through the blood circulation system we have a further problem: Tumor blood flow is often not optimal so it may be difficult to reliably deliver the mAb to tumor cells.
- Sometimes tumor antigens are released → mAb binds to free-floating antigens and not to tumor cell.
- Cross-reactivity with antigens in normal tissue – normally antigens which can only be find in the target cell are chosen/if such an antigen isn't found it isn't possible to treat with mAb.

3.3.3 Examples

A lot of of antigens and corresponding mAb for the treatment of B cell malignancies are available. Some important are listed below:

Antibody	Type	Target	Use
Adalimumab	human	inhibiton of TNF-α signaling	several autoimmune diseases
Alemtuzumab	humanized	CD52	chronic lymphocytic leukemia
Rituximab	chimeric	CD20	Non-Hodgkin lymphoma
Cetuximab	chimeric	epidermal growth factor receptor	Head/neck/colorectal cancer
Infliximab	chimeric	inhibiton of TNF-α signaling	several autoimmune diseases
Trastuzumab	humanized	ErbB2	Breast cancer

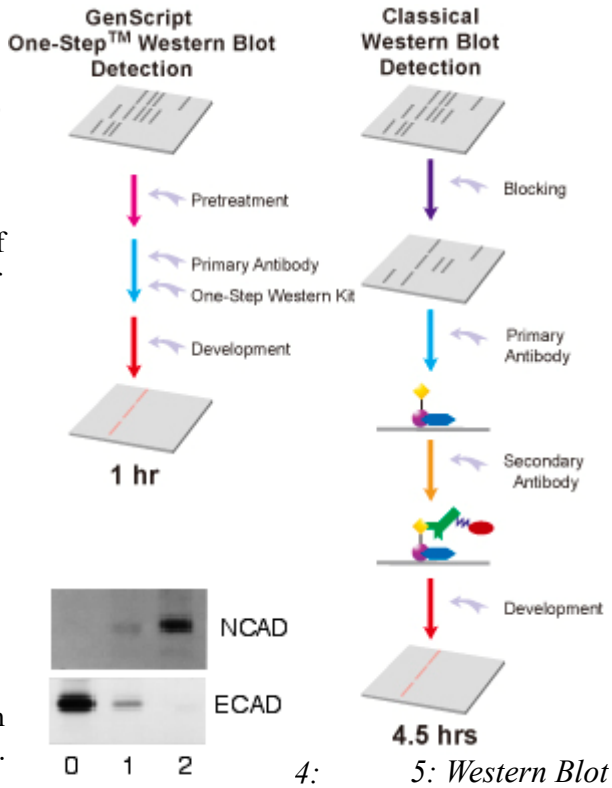
Most important is the use against cancer and autoimmune diseases. The most important target antigen is CD20 because of it's high degree of expression in b cell malignancies

3.3.4 Diagnostic and research reagents

Monoclonal antibodies are often used in diagnostic tests. With monoclonal antibodies small amounts of

drugs, hormones or toxins can be detected. There are different ways to detect the protein of interest.

3.3.4.2 Western blot test



4: Result of west. blot *5: Western Blot*

Two forms of the western blot test exist the classical western blot test is still more important than the one-step detection.
In a first step the protein of interest is separated into RNA using gel electophoresis and then transferred to a membrane. The membrane is placed in dilute solution of a protein (which binds not to the antibody of interest) for blocking non-specific binding. In a second step a dilute solution of specific (for the protein of interest) primary mAb is incubated with the membrane. The membrane is washed so that unspecific bounded mAb are removed. A secondary antibody solution is brought on the membrane. Its antibody binds specifically to the primary antibody. The secondary antibody may be bound to an enzyme or radiolabeld. Again some wash steps take place and then the western blot is ready for analysis.
Below the most important techniques are described:

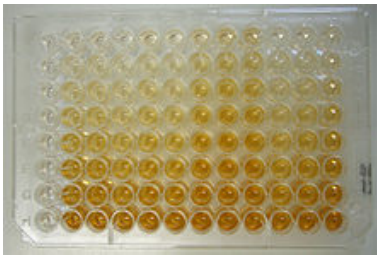
- Colorimetric detection: A substrate that reacts with the enzyme (bound to secondary antibody) and results in a change of the color of the protein bonds of interest is incubated to the western blot. Protein levels are detected with densitometry or spectrophotometry.
- Chemiluminescent detection: A substrate that luminesce when exposed to the reporter on the secondary antibody is incubated to the western blot. The light is then detected by CCD cameras or phtographic film. Further analysis of the image is done by densiometry.
- Radioactive detection: Medical X-ray film is placed directly against the western blot. The western blot creates dark regions which correspond to the protein bonds of interest.

3.3.4.3 Dot blot

Dot blot tests are used to detect biomolecules. They are a almost similar to the northern blot, Southern blot or western blot methods. The only difference is that the molecules of interest are not first separated by chromatography. Instead a mixture containing the molecule of interest is applied on a membrane as a dot. The detection is done by nucleotide probes (northern blot/Southern blot) or antibodies (western blot). The dot blot saves a lot of time (no chromatography or gel electophoresis needed) but dot blot tests only confirm the absence or presence of a biomolecule because they offer no information on the size of the biomolecule of interest.

3.3.4.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) uses specific antibodies to detect proteins, virus, antigens, antibodies, hormones, toxins or pesticides in a sample. The sample is putted in a microtiter plate. Specific antibodies (linked to a enzyme) are applied on the sample. In a last step a substance is added that the enzyme converts to a visible signal. The signal strength normally depends on the antigen concentration therefore ELISA can by used for qualitative and quantitative analysis. The ELISA test is high sensitive.



6: Typical result of ELISA

3.3.4 Examples

In the table below the most important monoclonal antibodies used for diagnosis are listed.

method	use
western blot	• diagnosis of several infectious diseases • HIV test • pregnancy test kits • Bovine spongiform encephalopathy • Lyme disease • Hepatitis B • Feline immunodeficiency virus+ status (in cats)
ELISA	• HIV test • food industry: detecting food allergens • toxicology (rapid screen for drugs)

4.Interview (with Prof. Dr. Eric Kübler)

Q: Why are only monoclonal antibodies used for therapeutic purpose?

A: To treat a disease with monoclonal antibodies, a huge amount of it is necessary. Only monoclonal antibodies can be produced in the required amounts

Q: Do you think the future of monoclonal antibodies lies in therapeutic uses or in diagnostic purposes? Why?

Q: What do you think will be used in the future for diagnostic purposes and what for therapeutic purposes? Monoclonal or polyclonal antibodies? Why?

A: In both. Although polyclonal antibodies often have a better therapeutic effect, it is still very difficult to produce them in high amounts. In addition, certain monoclonal antibodies may still have a better effect. The future will favour the polyclonal antibodies for therapy but the monoclonals will not disappear. For diagnostic purposes, the monoclonal antibodies will never disappear for their unsurpassed specificity. Monoclonal antibodies recognize only one epitope and this epitope most of the time appears only in the protein to be diagnosed. With polyclonal antibodies, the probability that other proteins are also bound by them is increased. This is bad as it could falsify the results depending on the assay format.

Q: Which factors have to be given so that polyclonal antibodies have a chance of being used for therapeutic purpose?

A: The ability to identify and clone all the single antibodies of the polyclonal antibody mixture. This would allow to produce them independently in host cells in vast amounts.

Q: How long will it take, until polyclonal antibodies will be used for therapeutic purpose?

A: Difficult to say, Maybe ten years.

Q: Which is the most important use of monoclonal antibodies in therapeutic purpose?

A: Presently, the treatment of cancer and inflammatory diseases is predominant. One of the best examples is the use of avastin against angiogenic cancer. It blocks the the growth of new blood vessels by binding the protein vascular endothelial growth factor (VEGF).

5.Discussion

Advantages: The use of monoclonal antibodies offers many advantages compared to polyclonal antibodies. Monoclonals can be held in cultures and can also be frozen and unfrozen several times often without changing their antigen-binding characteristics. This enables to retain the same charge of antibodies for different therapies. If not they would have to be produced again in vivo from scratch. This would lead to different antibodies. Another great advantage is that monoclonal is their high specificity. For example, very specific antibodies can be produced which recognise only the affected target cell without making damage to sane cells. Other applications of monoclonal antibody therapy have already been performed with success. Monoclonals can be used to combat diseases but the natural role of antibodies is to prevent diseases, they protect people against infections. Since a long time antisera (a sort of vaccination) from immunized donors are used to pass immunity to vulnerable people. Monoclonal antibodies now start to substitute antisera, providing advantages in reproducibility, freedom from contaminants and potency.

Disadvantages: It is difficult to say if the monoclonal antibody therapy is beneficial because it’s hard to

measure an immediate effect. Hundreds of patients have to be involved to see how the medicine works. It takes a very long time till a new pharmaceutical is on the market. The therapy is individual to the different patients and has to be approved every new time. A single antibody probably won't recognise all sick cells. Combinations of different antibodies may increase the rate of effect. This needs also a lot of detecting and measuring time. Every sort of disease requires a specific product. The investments and research which are made to create a new medicine are enormous. The market of the monoclonal antibody therapy is becoming very big and the prizes are very high also due to the laborious production process. On the other side for the production of monoclonal antibodies animals, mainly mice, are used which are held in large quantities, often in small cages. The animals don't have any chance to live a normal life. For ecologists this is a thorn in their flesh. Monoclonal antibodies change the immune balance more frequently than other medicines. In this case patients can get very serious diseases. Often there are side effects like stomach pain, diarrhea, fever, weakness and irritation.

Future steps of monoclonal antibody therapy: The recent developmental steps made in immunology are opening a large range of new therapies. New options include improved monoclonal antibody vaccination, therapies with whole cells and natural molecules which work in concert with the immune system. One of the main goals is to better understand how the whole human immune system works so new research will be carried out to make an antibody therapy more effective. Other important goals are to increase the productivity and decrease the production costs for the pharmaceutical and therapies to enable more people to be treated against the diseases. The medicines have also to be improved to prevent severe side-effects.

6.0 Summary

MAb play a very important role in diagnostics and research because they bind many different molecules with high specificity. In addition mAb are effective drugs (against cancer, autoimmune diseases and others). But in order to use the great capability of mAb to treat other serious diseases a lot of research has still to be done. Hence it's not remarkable that many companies invest a huge amount of money and spend a lot of effort on the development of new mAb. Therefore the market of mAb is growing very fast. Probably the future of disease control belongs at least partially to monoclonal antibodies.

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