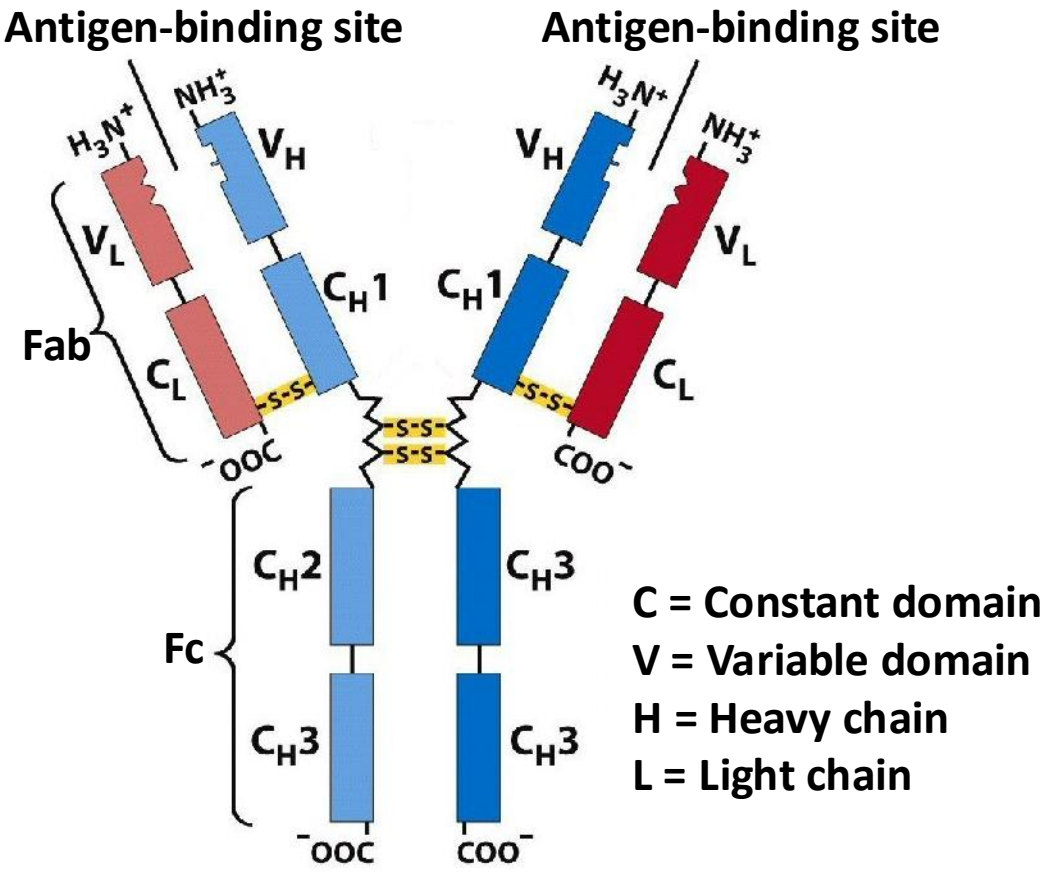
IMMUNOLÓGIAI ÉS BIOTECHNOLÓGIAI INTÉZET

4th practice: Monoclonal and polyclonal antibodies, hybridoma technology

Basic Immunology

University of Pécs, Clinical Center
Department of Immunology and Biotechnology
Pécs, 2026.

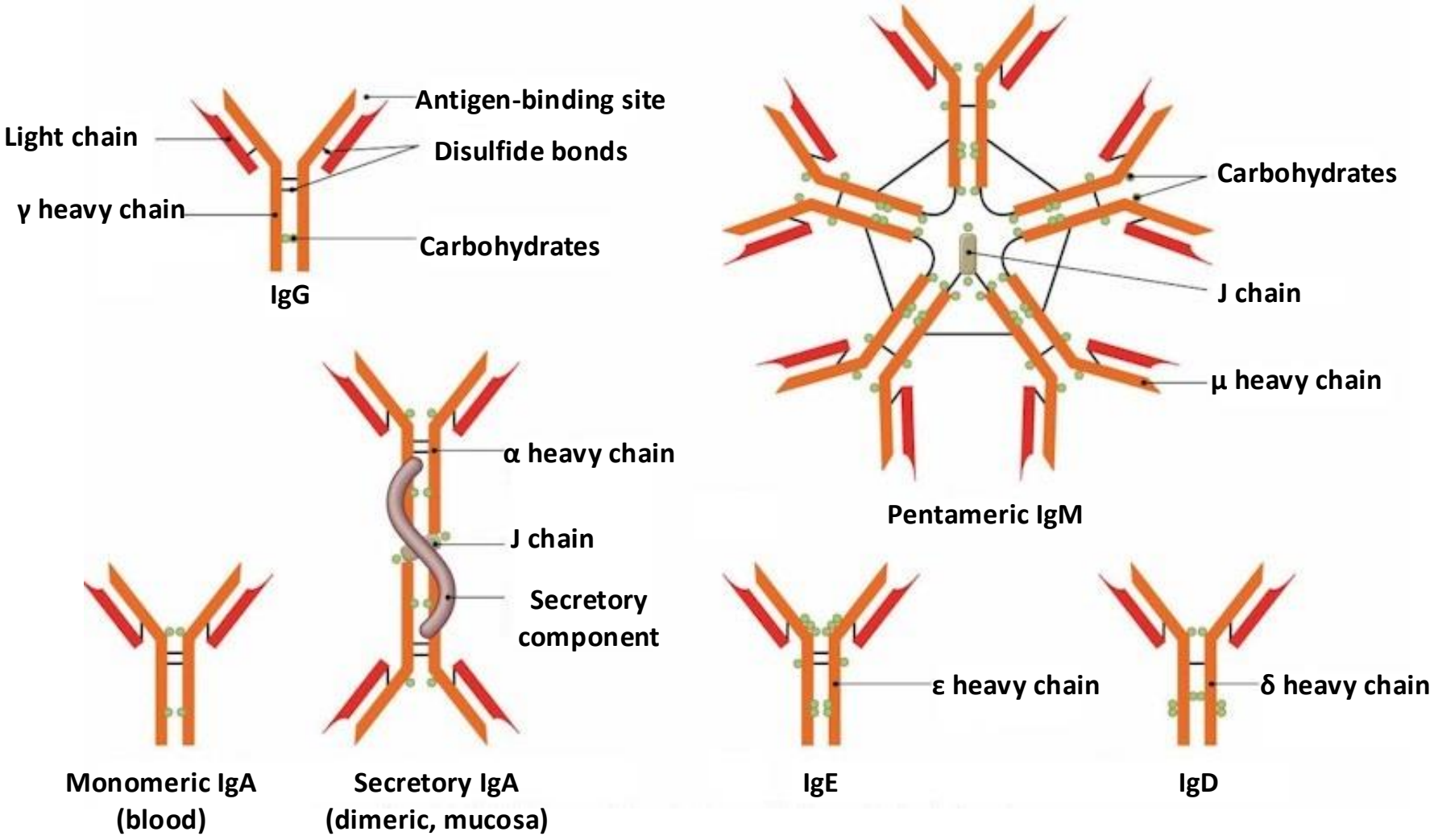
Structure of immunoglobulins



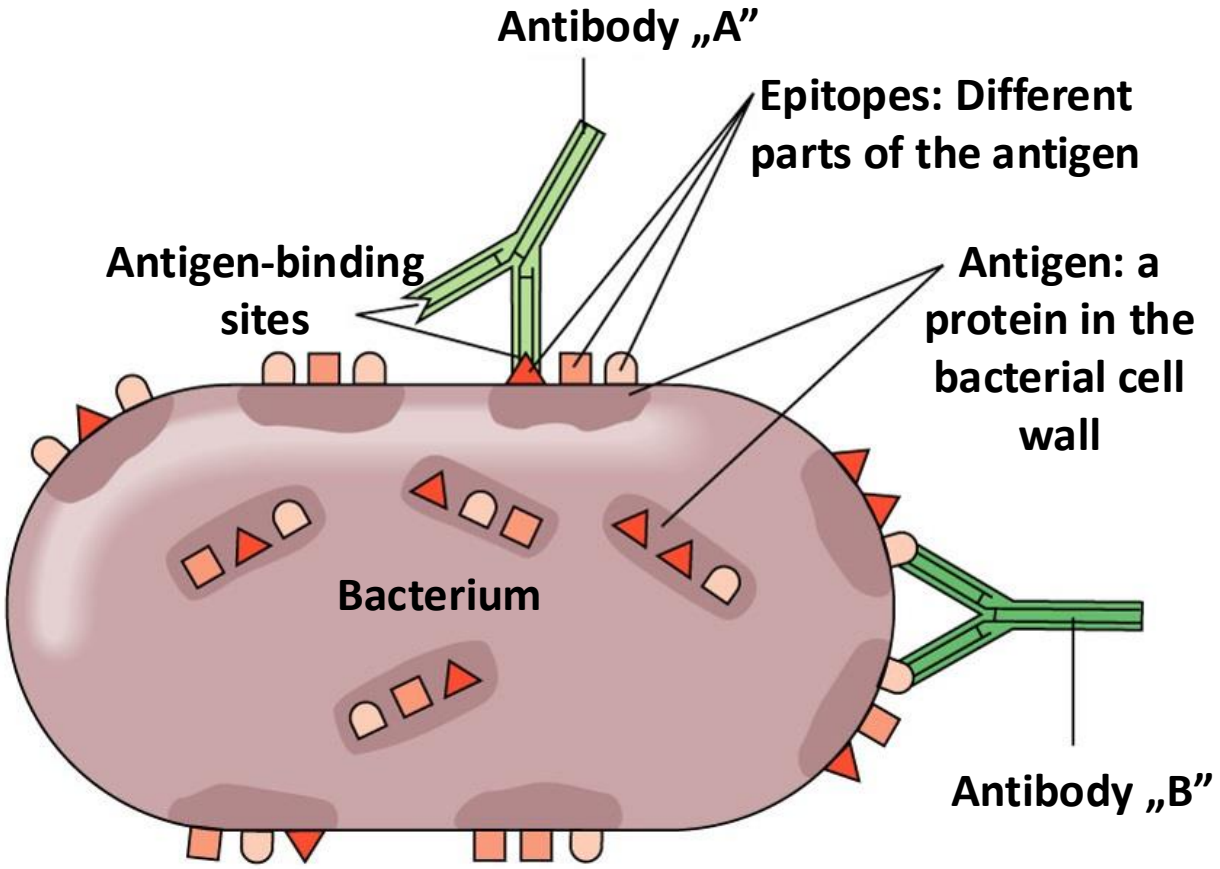
C = Constant domain
V = Variable domain
H = Heavy chain
L = Light chain

- Immunoglobulin = Antigen-binding **protein** produced by **plasma cells.**
- Made of 2 heavy and 2 light chains.
- Light chain: **κ** or **λ**
- Heavy chain: **α, γ, δ, ε** or **μ**
- **Isotype:** based on the heavy chain: IgA, IgG, IgD, IgE or IgM
- **Idiotype:** refers to the antigen-binding site
- Antigen defined by the antibody: The substance that is recognized by the antibody (e.g. a surface protein of a bacterium)
- **Epitope** (antigenic determinant)**:** The specific part of the antigen recognized by the antibody. (a smaller unit within the antigen)

Immunoglobulin classes

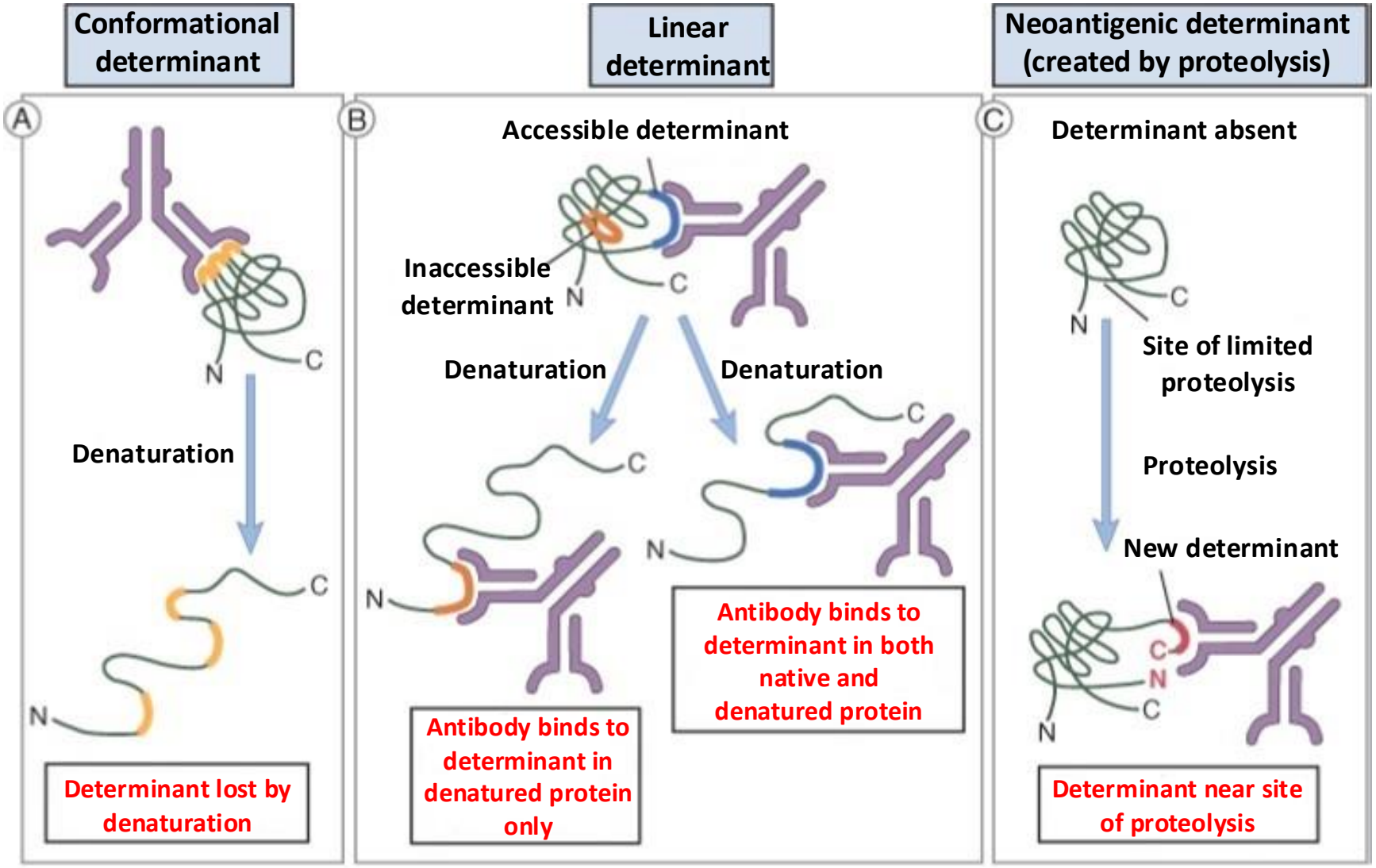


Difference between antigens and epitopes

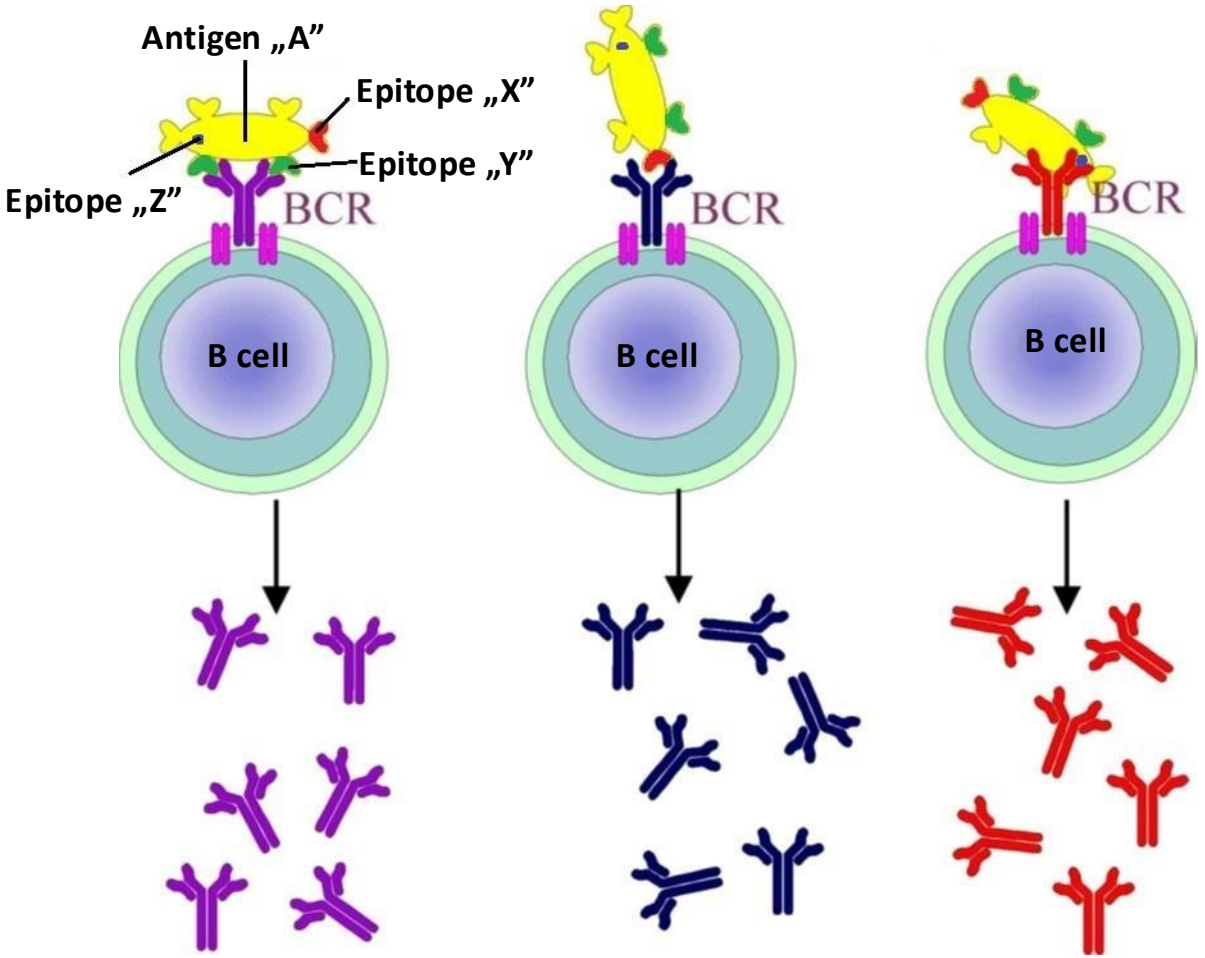


Both „A” and „B” antibodies bind the same antigen but they recognize different parts (so-called epitopes) of the target molecule.

Types of antigenic determinants



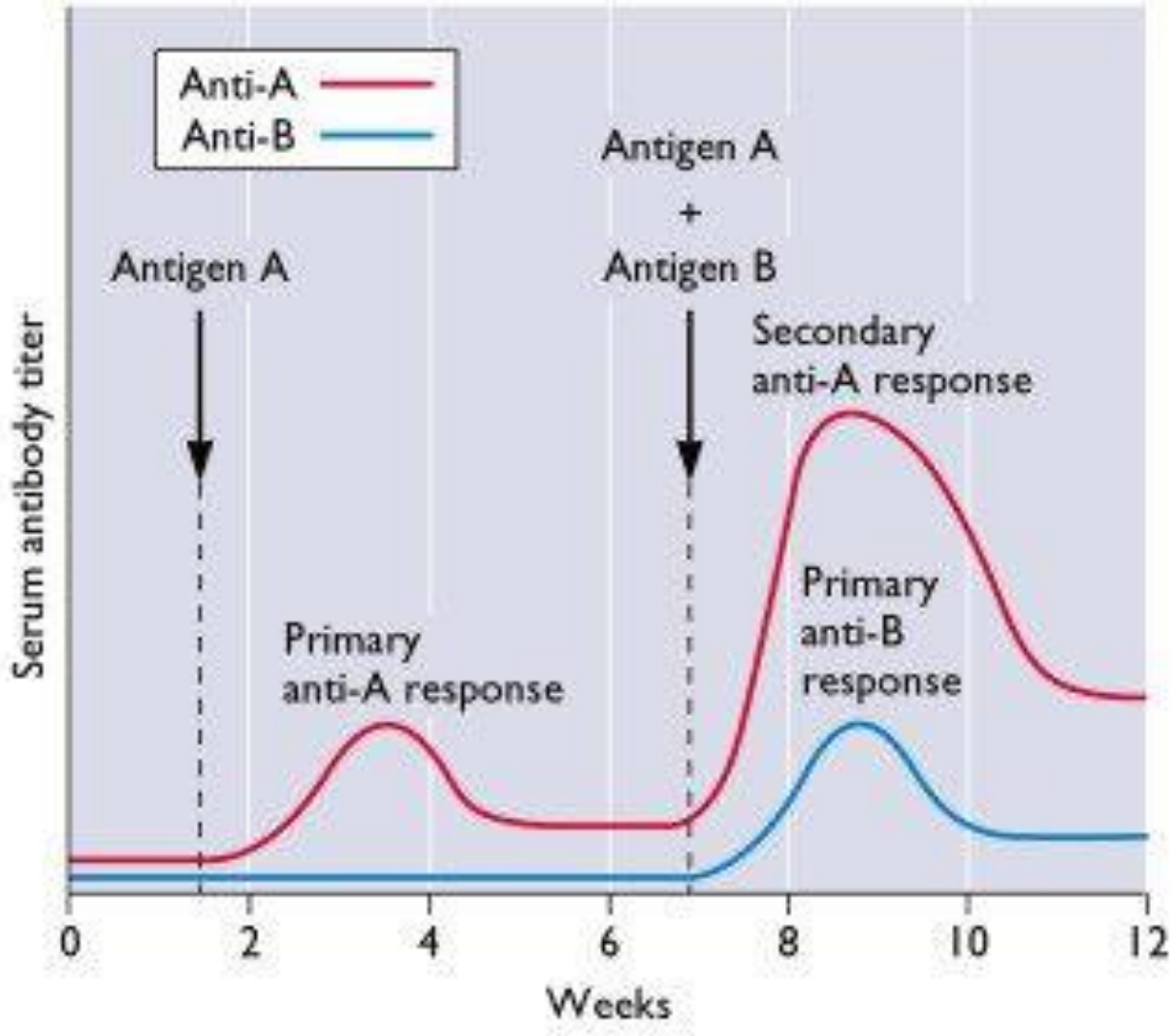
Polyclonal antibodies



Antigens cause **polyclonal cell activation** in living organisms, which always leads to the production of **polyclonal antibodies during an immune response!**

POLYCLONAL anti-A antibody

Serum antibody titers



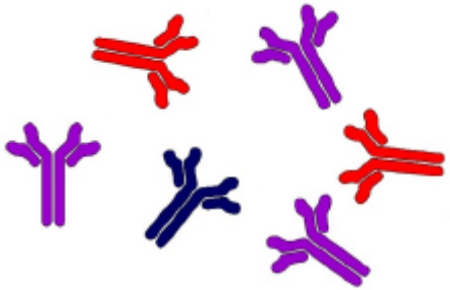
Immunization

- Immunization: Administration of an antigen to a living organism so that the organism will develop an immune response and produce antibodies against the antigen.
- Immunization to prevent infectious diseases = Vaccination (see later)
- Production of polyclonal antibodies:
  - Immunization of an animal with the antigen
  - **Polyclonal antibodies** that **recognize the antigen** can be isolated and purified **from the blood serum** of the immunized animal after the immune response.[1.]
- Problem: Monoclonal antibodies cannot be produced this way
- Solution: hybridoma technology (see later)

E.g.: Polyclonal rabbit anti-A IgG

1. Administration of antigen „A”

2. Blood serum of immunized animal with the polyclonal antibodies



3. Purification of antibodies

Administration of the antigen

- The following should be considered in order to choose the most suitable animal:[2.]
  - The amount of polyclonal antibody we need to produce
  - The ease of obtaining blood samples
  - The phylogenetic relationship between the antigen and the animal species
  - The intended use of the polyclonal antibody
- Rabbits, goats, sheep or chickens are frequently used for the production of polyclonal antibodies, whereas mice and rats are preferred for the production of monoclonal antibodies. (see later)
- The features of the administered antigen are also important:
  - Purity: In case of contamination antibodies recognizing the contaminants are also produced.
  - The form of the antigen: It is possible to give entire cells, purified antigen or fragments of the antigen. The antigen can also be attached to a carrier. (e.g. hapten)
- Route of administration: oral (per os), intracutaneous (ic.), subcutaneous (sc.) or intramuscular (im.)

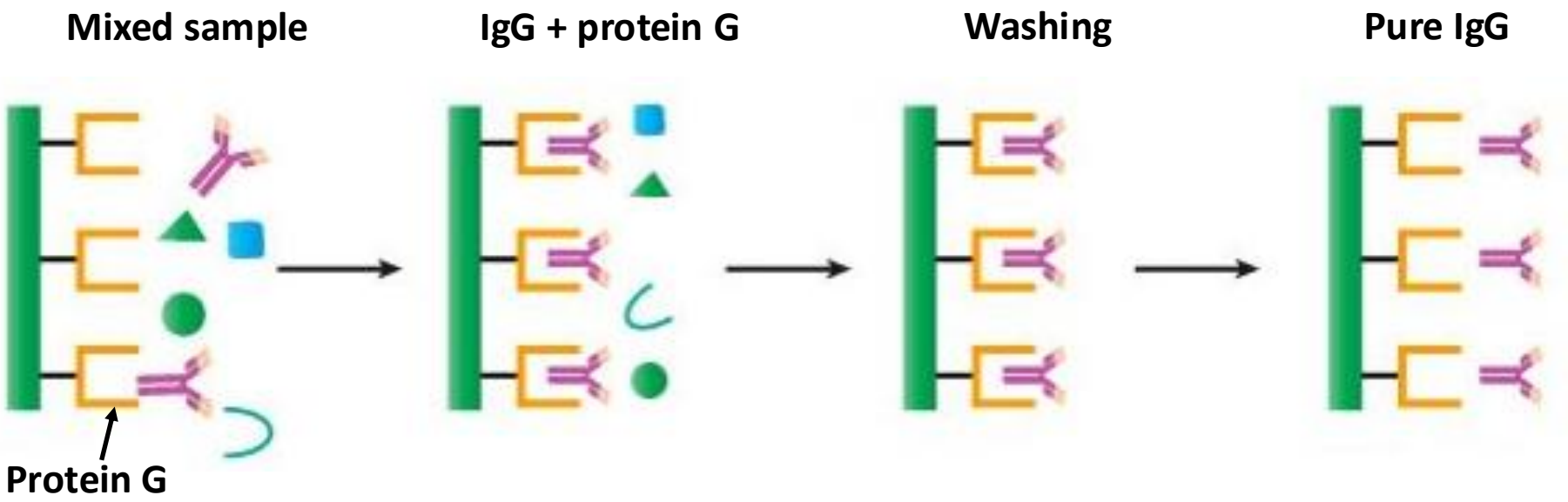
Frequently used animals

Adjuvants

- Substances that delay and **enhance the immune response** against the administered antigen resulting in increased antibody production.
- Adjuvants are also used in human vaccines. (see later)[3,4.]
- Their mechanism of action include:
  - They increase the antigen uptake
  - They activate the innate immune cells (e.g. macrophages) via PRRs
  - They enhance antigen presentation via MHC II
- Some examples of adjuvants:
  - **Aluminium salts** (e.g. aluminium phosphate, aluminium hydroxide oxide, these are the most common adjuvants in human vaccines)
  - Lipid A analogues (e.g. Cervarix© = HPV vaccine)
  - **Freund's adjuvant**: the antigen is emulsified in mineral oil
    - Complete (CFA): contains dead *Mycobacterium* bacteria (e.g. *M tuberculosis)*[5.]
    - Incomplete (IFA): contains no *Mycobacterium*
  - **Liposomes** containing viral proteins[6.]

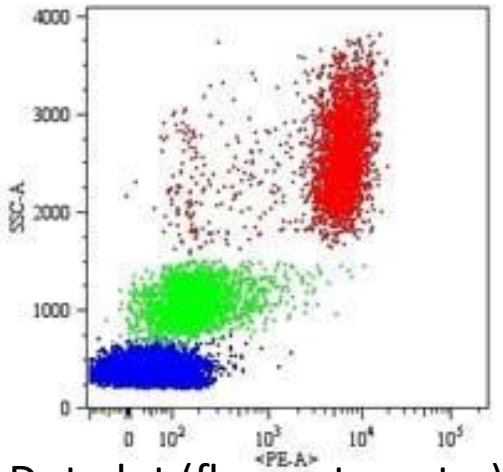
Purification of antibodies

- Antibodies are extracted from the **blood sera** of immunized animals.
- Different methods exist for the purification of antibodies of different isotypes
- IgG[7.]
  - Precipitation (e.g. with the use of ammonium sulfate)
  - **Chromatograpy**, especially affinity chromatography with the use of **protein A** (*Staphylococcus*) or **protein G** (*Streptococcus*) or ion-exchange chromatography

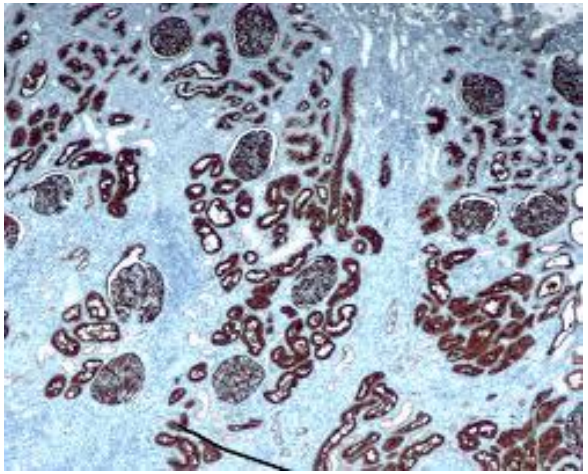


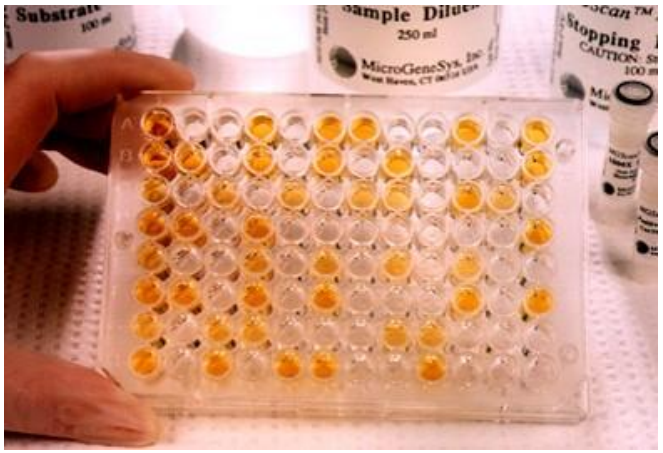
Antibody testing

- The **specificity** and **titer** (=quantity) of the purified antibody must be tested with the antigen in the very same system in which the antibody will be applied. Examples (will be discussed in more detail later):
  - Flow cytometry
  - ELISA
  - Immunohistochemistry



Dot plot (flow cytometry)


Immunohistochemistry (CD10 staining in a healthy human kidney)

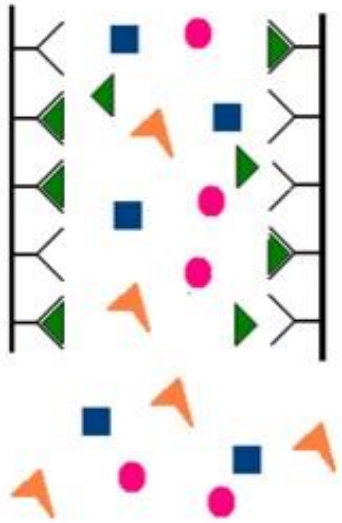

ELISA plate

Significance of monoclonal antibodies

- **Polyclonal** antibodies have **varying specificity** and **affinity** which limits their applications. (e.g. cross-reactivity, a different set of antibodies can be obtained from each immunized animal)
- The ability to produce **monoclonal** antibodies that can recognize a **single specific epitope** with **constant specificity** and **affinity** is of great importance.

APPLICATIONS OF MONOCLONAL ANTIBODIES

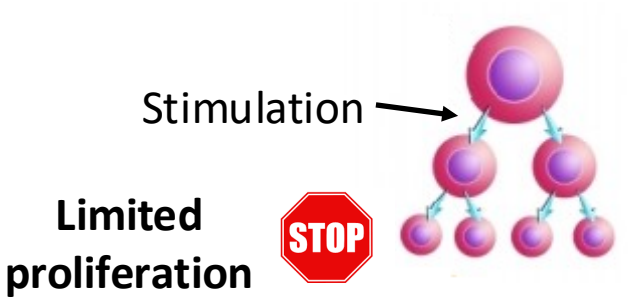
- Preparative methods:
  - **Specific purification of proteins** (e.g. immunoaffinity chromatography)
- **Analytical methods** (diagnostics and research):
  - **Serological tests** (see later)
  - **Identification and isolation of different cell populations** (e.g. identification of CD markers)
- **Therapeutic uses:**
  - Inhibiting or enhancing specific target molecules or cells (see later)



The proteins to be purified in the mixed sample **will bind to the immunoglobulins** in the column and can be obtained afterwards.

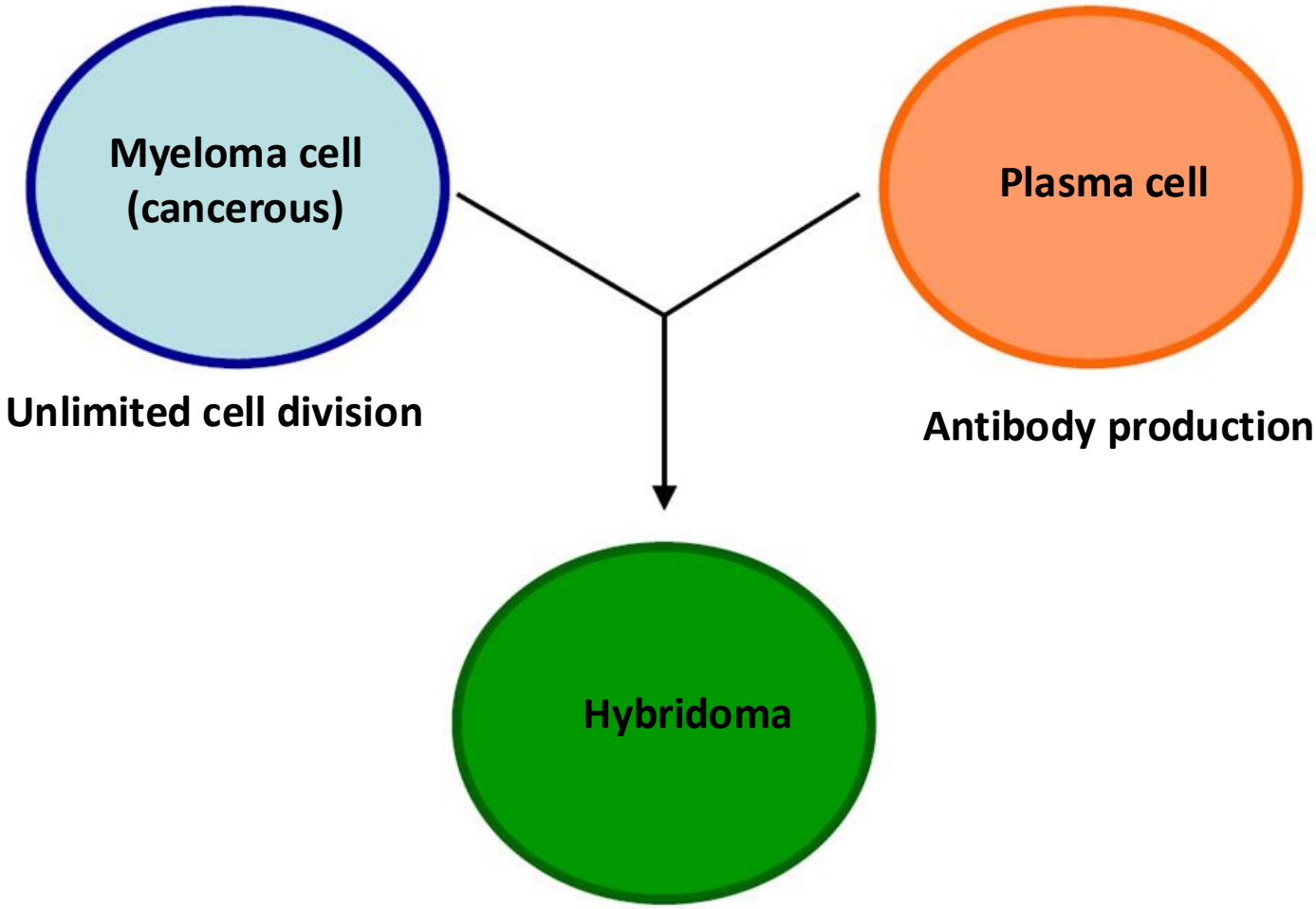
Production of monoclonal antibodies

- What is the problem?
  - One needs to produce antibodies derived from a single B cell clone. → Even if the clone is isolated and stimulated, **cell division will eventually stop** and the **cells will die**.

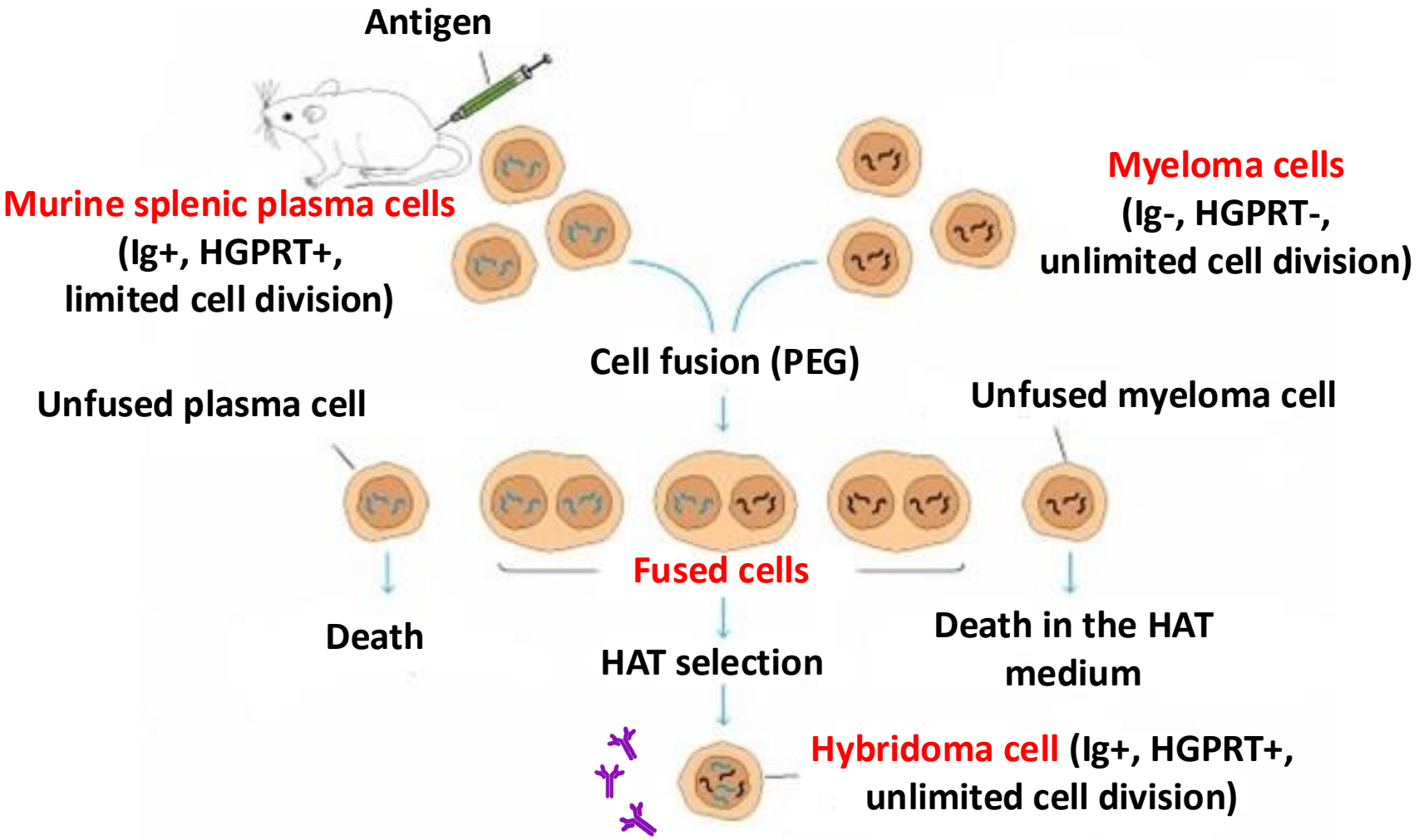


- Solution**: Immortalization of plasma cells**
  - How? → They are fused with cancerous cells
  - Why? → Cancer cell are immortal and have unlimited potential to replicate.
- Result: **Hybridoma technology**[8,9.]
  - The artificial, in vitro **fusion of plasma cells and cancer cells**
  - The resulting **hybrid cells** (=hybridoma) have the beneficial features of both cells types, namely they produce antibodies identical to those of the original B cell clones and can proliferate without limitations.

The basic principle



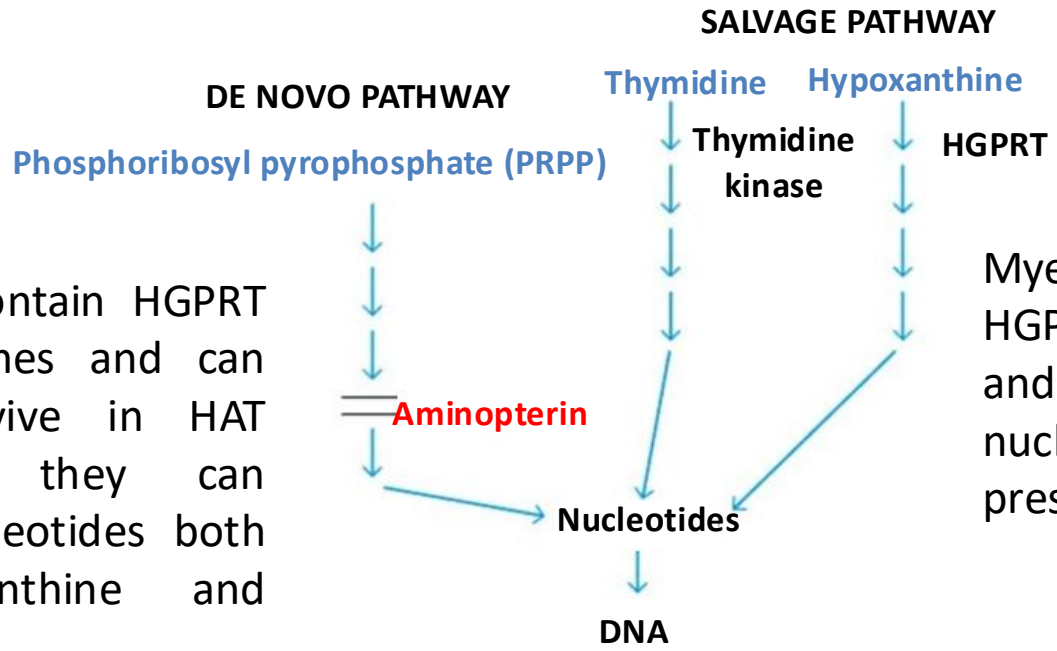
Hybridoma technology 1.



HGPRT: Hypoxanthine-guanine phosphoribosyltransferase (see on next slide)
PEG: Polyethylene glycol
HAT: Hypoxanthine-aminopterin-thymidine medium (see on next slide)

Hybridoma technology 2.

1. **Immunization** of the animals (usually mice or rats)
2. **Isolation of plasma cells** from the spleen
3. **Cell fusion**: murine plasma cell + **non-secretory myeloma cell** (cancerous myeloma cell lines, e.g. Sp2): With the use of **polyethylene glycol** (PEG) or **electric current** (electrofusion)
4. **Selection**: Desired plasma cell-myeloma hybrids are selected with the use of **HAT medium** (contains hypoxanthine, aminopterin and thymidine). Unfused cells or fused cancer cells will die.

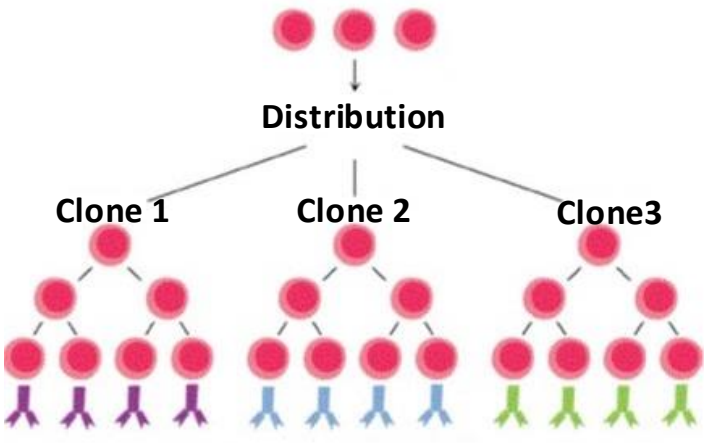


Hybrid cells contain HGPRT and TK enzymes and can therefore survive in HAT medium as they can synthetize nucleotides both from hypoxanthine and thymidine.

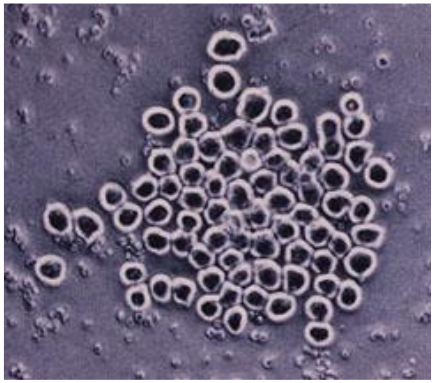
Myeloma cells lack HGPRT and TK enzymes and cannot synthetize nucleotides in the presence of aminopterin.

Hybridoma technology 3.

- **Selection of monoclones**: The cells surviving HAT selection are **transferred to a 96-well plate** with each well containing **a single cell**. Cells in the wells will proliferate creating clones that each produce the very same antibody. → Monoclonal antibody production
- **Testing** of the produced antibodies with the use of the original antigen
- Selection of the most ideal clone



96-well plate

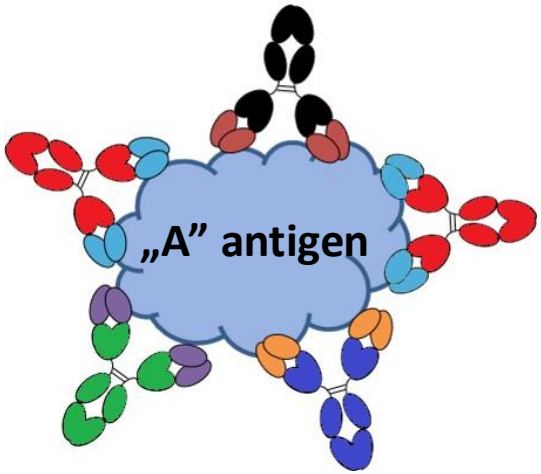


Hybridoma cells in a cell culture medium

Comparison of monoclonal and polyclonal antibodies

„You wanna play a little game?"

Polyclonal anti-"A" antibody



Monoclonal anti-"A" antibody

Polyclonal:

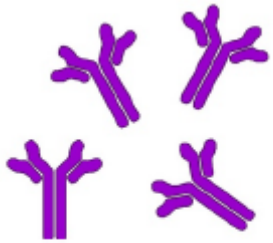
- Product of **several B cell clones**
- Recognize **different epitopes** of the target antigen
- **Varying specificity** and **affinity**
- (Consider them mixtures of different monoclonal antibodies)

Monoclonal:

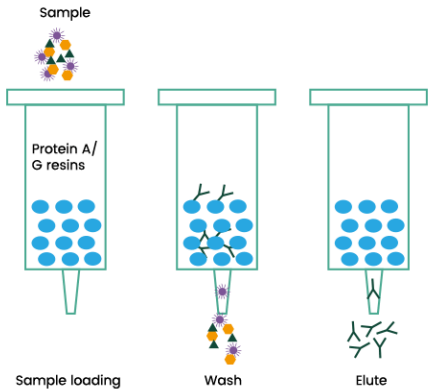
- Product of **a single B cell clone**
- Recognize a **single specific epitope** of the antigen
- Antibodies have the **same specificity** and **affinity**

Continuous antibody production

- Hybridoma cells secrete the antibodies into the medium. → They can be obtained from the **supernatant** of the cell culturing medium by:



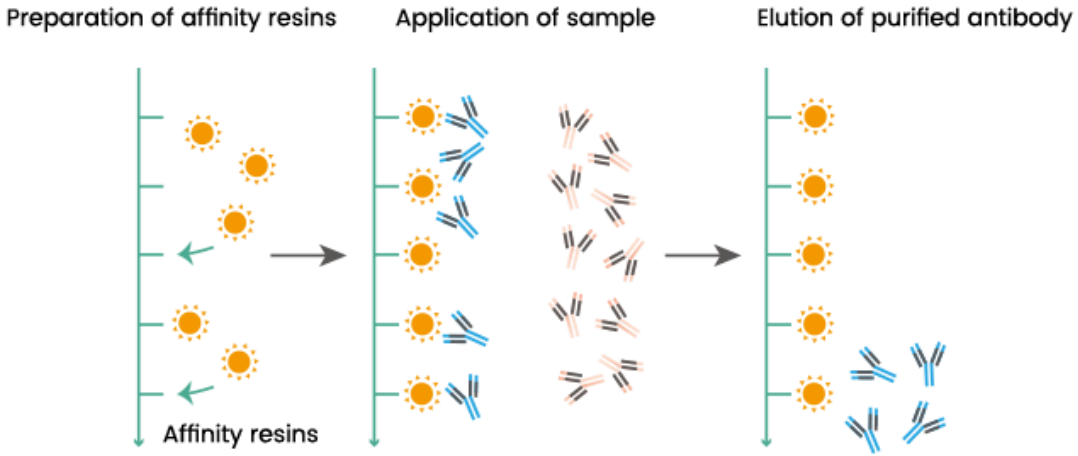
Protein-G/A affinity purification



- Industrial (mass) production:

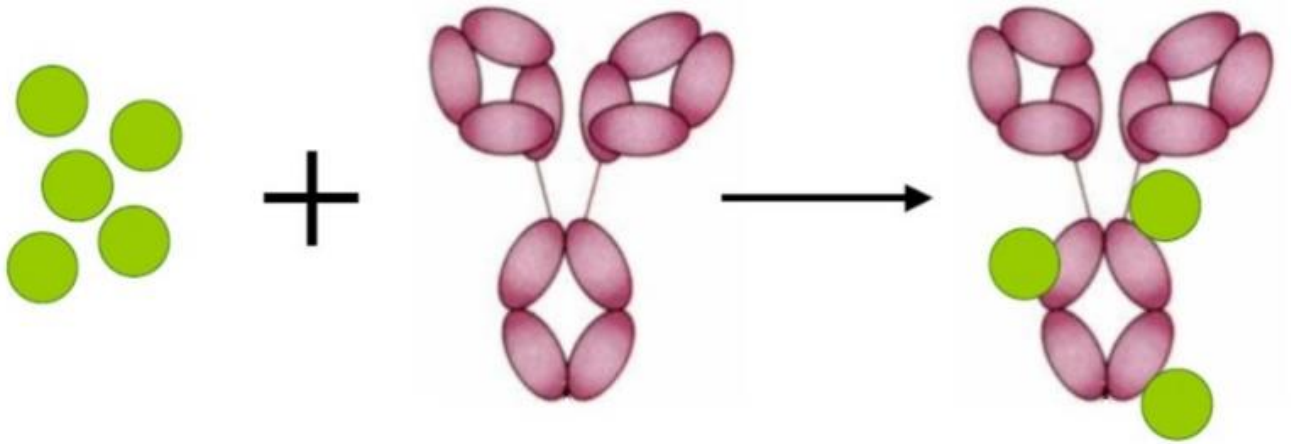
With the use of **fermenters**.

Antigen affinity ppurification

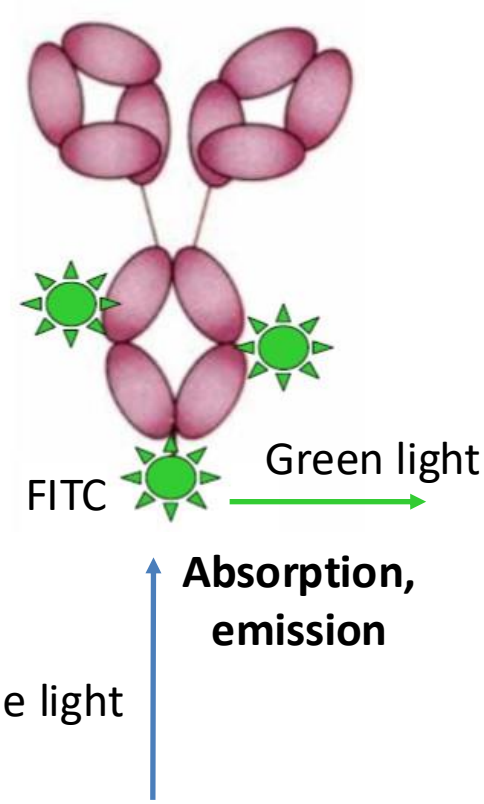


Conjugation

- The antigen-antibody reaction is not detectable on its own, but if the used antibody is conjugated with a labeling molecule then it can be detected by various methods depending on the conjugate.
- Conjugates:
  - **Fluorescent molecules** (fluorophores or fluorochromes, the are synonyms), e.g. FITC, PE, etc. (see later) → **flow cytometry, fluorescent microscopy**
  - **Enzymes** (they convert chromogens into dyes in the presence of a substrate), e.g. HRP, ALP (see later) → **immunohistochemistry, ELISA, Western blot**
  - **Radioactive isotopes:**
    - Diagnostics → γ-emitting isotopes



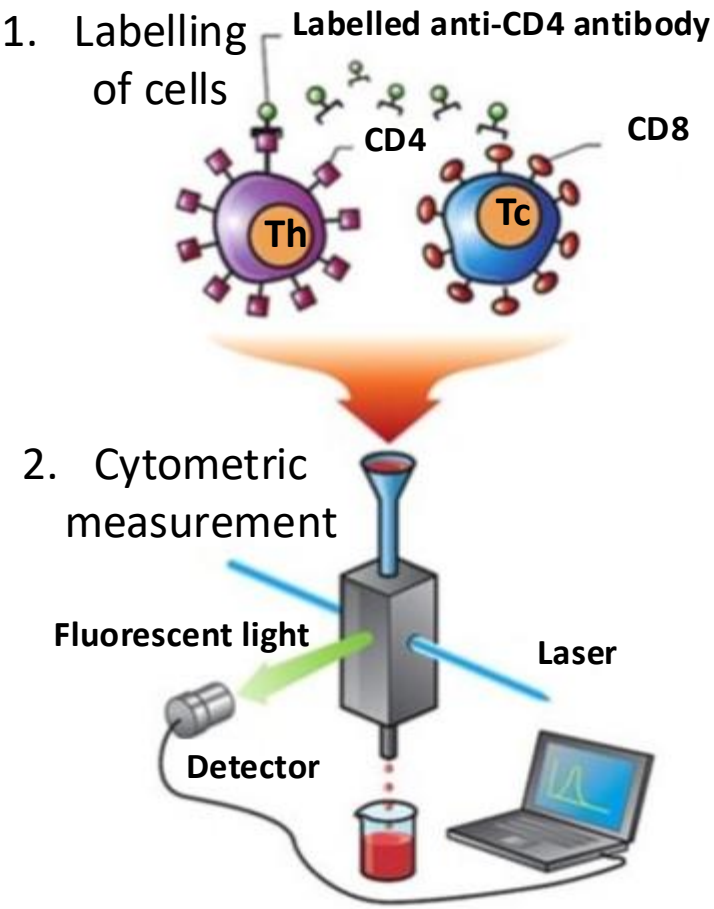
Fluorescent conjugates



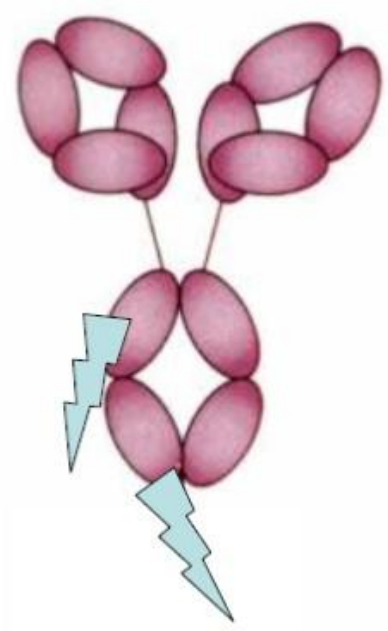
Fluorescence microscopy

Murine thymus IF[10.]:
Red: Medullary epithelial cells
Green: Cortical epithelial cells
Blue (DAPI): Cell nuclei

Flow cytometry



Enzyme conjugates



Enzyme conjugated to the antibody
+ chromogen and substrate

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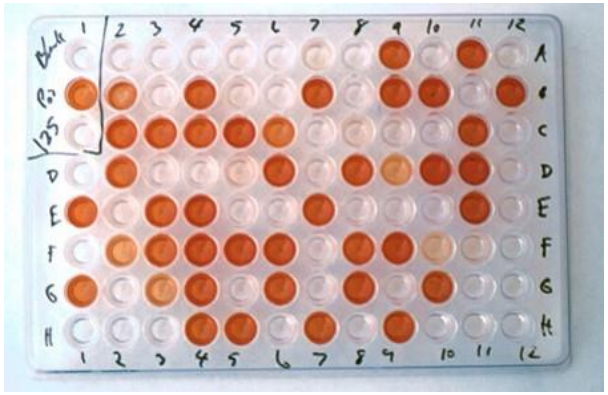
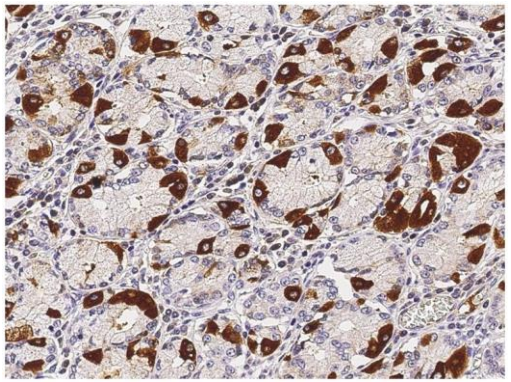
Color reaction

ELISA



Immunohistochemistry



(detection of intrinsic factor
in a human stomach)

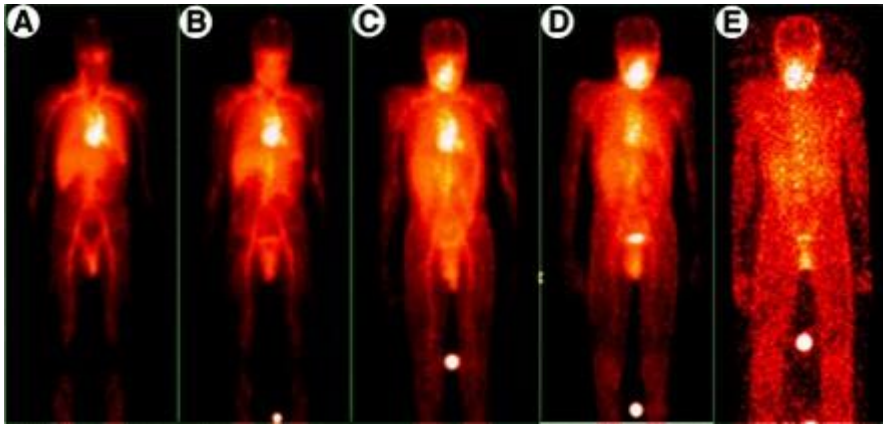
Western blot

Some frequently used enzymes: **HRP** (horseradish peroxidase), **ALP** (alkaline phosphatase)

Radioactive conjugates

Antibody + radioisotope

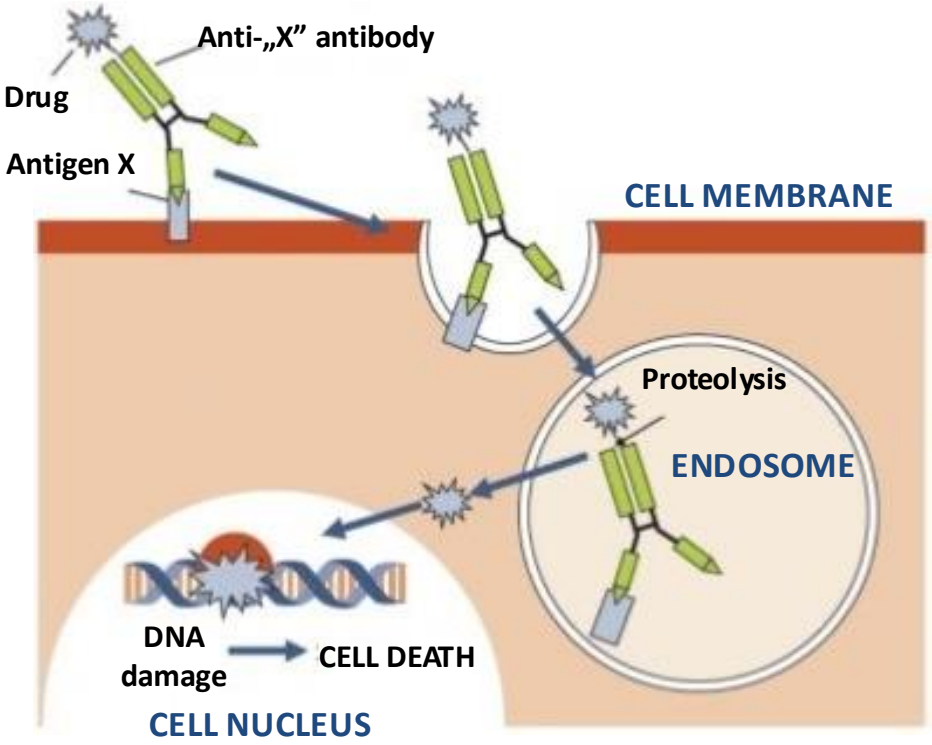
- **Diagnostic uses** (radioimmune imaging):[13.]
  - γ-emitting isotopes or positron-emitting isotopes are conjugated to the antibodies
  - The antibody will bind the target cell (e.g. cancer cell)
  - The signal can be detected with gamma cameras or PET (Positron emission tomography) which are capable of detecting even micrometastases
- **Therapeutic uses**:
  - α- or β-emitting isotopes are used → The tumor receives large doses of radiation locally



Immuno-PET images from a patient with oropharyngeal cancer, images were taken after 1 (A), 24 (B), 72 (C), 144 (D) and 312 hours (E) of the administration of labelled antibodies.[14.]

ADC (Antibody-drug conjugate)

- The antibody will **selectively** deliver the drug to the target cells then both the drug and the antibody are internalized into the cytoplasm. This therapeutic approach is mainly used against **cancers**, mostly chemotherapeutic agents are conjugated to antibodies.[11.]



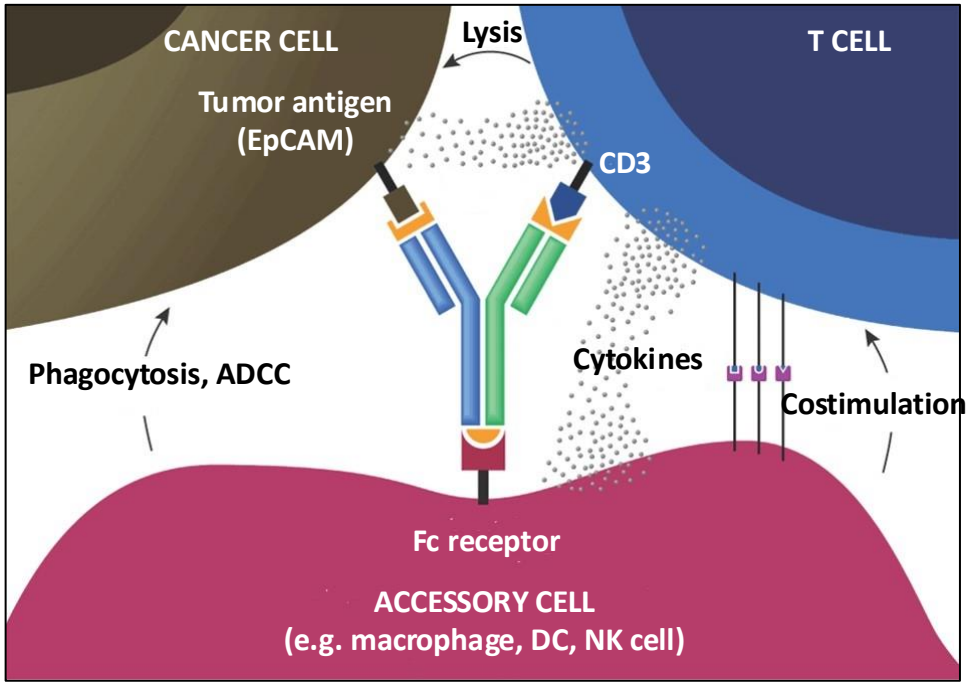
The mechanism of action of anticancer ADCs

Some examples of antibody-drug conjugates		
Drug	**Target molecule**	**Disease**
Brentuximab vedotin	**CD30**	**Hodgkin lymphoma**
Gemtuzumab ozogamicin*	**CD33**	**Acute myeloid leukemia**
Trastuzumab emtansine	**HER2**	**Breast cancer**

*Was withdrawn by Pfizer® in 2010.[12.]

Other modifications

- **Bispecific antibodies**:[15.]
  - Recombinant antibodies capable of binding two different antigens simultaneously.
  - Application: They are used in the treatment of **cancers** by cross-binding immune cells and cancer cells.
- **Fusion proteins:**[17.]
  - Recombinant proteins usually attached to the Fc fragment of IgG. Some examples (see later):
    - Abatacept (CTLA-4 + IgG1)
    - Etanercept (TNFαR + IgG1)
    - Romiplostim (TPO + IgG1)

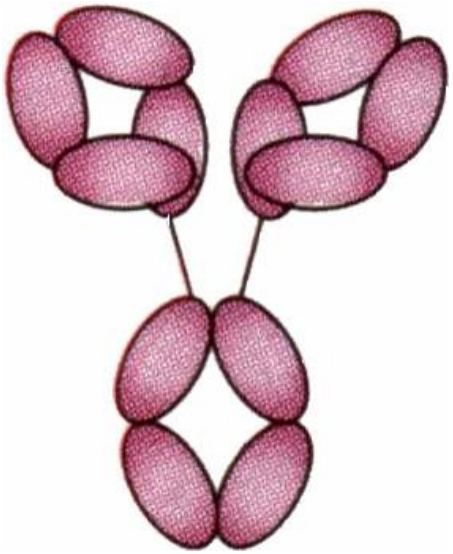


Mechanism of action of a bispecific antibody (catumaxomab)[16.]

Abatacept (CTLA-4 + IgG1), Etanercept (TNFαR + IgG1) → Rheumatoid arthritis (RA)

Romiplostim (TPO + IgG1) → Immune thrombocytopenia (ITP)

Murine antibodies



- The first therapeutic monoclonal antibody (muromonab) was a murine immunoglobulin.
- It was used after solid organ transplantations to prevent and treat rejection. (see later)
- Disadvantage: This is a **foreign protein** for the human immune system!

↓

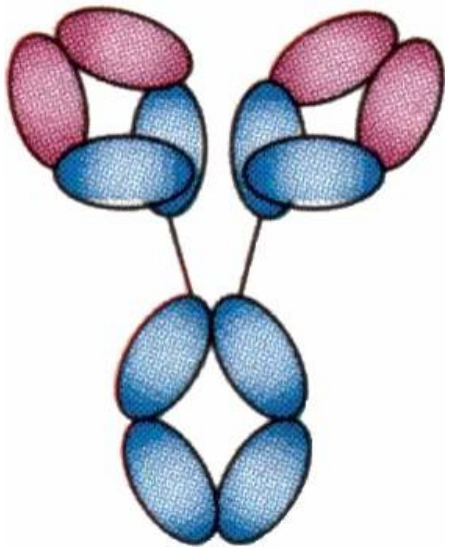
Many patients produced antibodies against the drug and some developed severe anaphylaxis (see later):[18.]

HAMA (human anti-mouse antibody)

Although the constant domains are evolutionary conserved structures, they are not identical in different species.

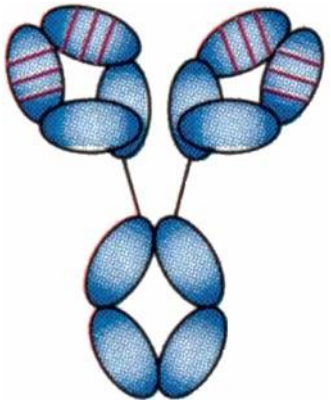
Muromonab is the only murine therapeutic monoclonal antibody. It is still used in otherwise untreatable acute rejections but it is no longer administered for prophylaxis.[19.]

Chimeric antibodies



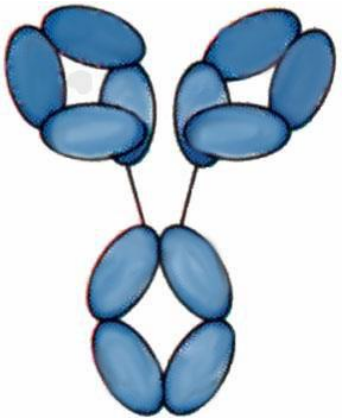
- The gene segment coding the variable region (Fv) of the chosen murine antibody is fused with the segment encoding the human constant region (Fc).
- The resulting chimeric immunoglobulin will have the **same specificity** as the original murine antibody but the constant region will be identical to human immunoglobulins.
- Roughly 75% of the molecule is of human origin.
- Advantage compared to murine antibodies: Contains less murine-derived sequences therefore it has **lower risk of being recognized as foreign** by the human immune system. The human Fc region also increases the **half-life** and the **effector functions**.
- Disadvantage: Some patients produce antibodies against these drugs.[20.] → **HACA: human anti-chimeric antibody**
- Chimeric antibodies are still widely used to treat diseases (see in table at the of the presentation)

Humanized and human antibodies



HUMANIZED:

- The gene segments encoding the hypervariable regions (CDRs) of the murine antibody are implanted to the genes of human immunoglobulins.
- > 90 % of the molecule is of human origin.
- The specificity of the antibody is similar to the original murine antibody while the half-life and effector functions are similar to human immunoglobulins.



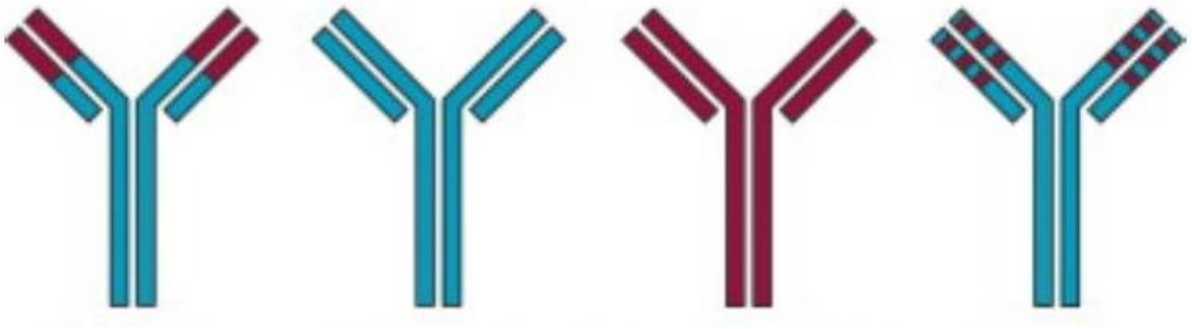
HUMAN:

- Genes of the human immunoglobulins are inserted to mice, then hybridomas producing human immunoglobulins are created after immunization.[21.]

↓

Fully human immunoglobulin

Nomenclature



Inflximab
Rituximab
Abcixmab

Adalimumab
Ipilimumab

Muromonab

Daclizumab
Trastuzumab

mab = monoclonal antibody
xi = chimeric antibody
zu = humanized antibody
mu = fully human antibody
li = has immunomodulatory effect
tu = has antitumor effect
ci = can be used to treat cardiovascular diseases

The WHO developed a **standardized nomenclature** for monoclonal therapeutic antibodies.[22.]
Muromonab is an exception as it was the first therapeutic monoclonal antibody:

Mur – o – mon – ab

murine monoclonal antibody

Some FDA-approved antibodies 1.

Year	Drug	Type	Trade name	Target	Application
1986	muromonab	murine	Orthoclone-OKT-3	CD3	Rejection
1994	abciximab	chimeric	ReoPro	Gp IIb/IIIa	PCI
1997	daclizumab	humanized	Zenapax	CD25	Rejection
1997	rituximab	chimeric	Rituxan, Mabthera	CD20	B cell NHL
1998	infliximab	chimeric	Remicade	TNFα	RA, Crohn's disease, Psoriasis
1998	trastuzumab	humanized	Herceptin	HER2	Breast cancer
1998	basiliximab	chimeric	Simulect	CD25	Rejection
2001	alemtuzumab	humanized	Campath	CD52	CLL
2002	adalimumab	human	Humira	TNFα	RA
2004	bevacizumab	humanized	Avastin	VEGF-A	Colorectal cancer

Some FDA-approved antibodies 2.

Year	Drug	Type	Trade name	Target	Application
2004	cetuximab	chimeric	Erbitux	EGF-R	Colorectal cancer
2006	natalizumab	humanized	Tysabri	α4 integrin	SM, Crohn's disease
2006	panitumumab	human	Vectibix	EGF-R	Colorectal cancer
2006	ranibizumab	humanized	Lucentis	VEGF-A	Macular degeneration
2009	golimumab	human	Simponi	TNFα	RA
2010	denosumab	human	Amgen	RANK-L	Osteoporosis
2010	tocilizumab	humanized	Actemra	IL-6 R	RA
2011	ipilimumab	human	Yervoy	CTLA-4	Melanoma
2014	nivolumab	human	Obdivo	PD-1	Melanoma, non-small cell lung cancer
2015	secukinumab	human	Cosentyx	IL-17A	Psoriasis

Therapeutic monoclonal antibodies for immune response modification

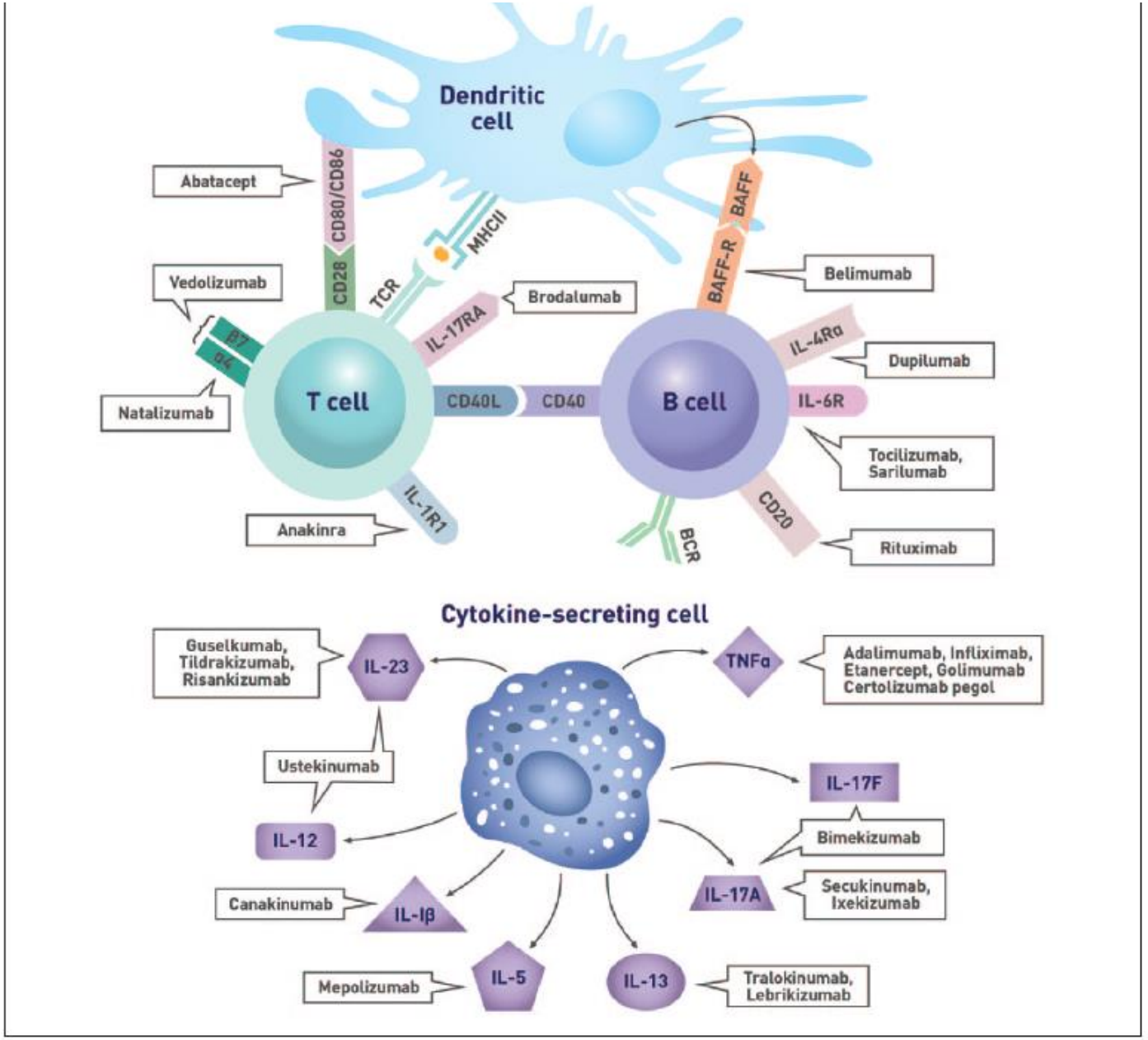
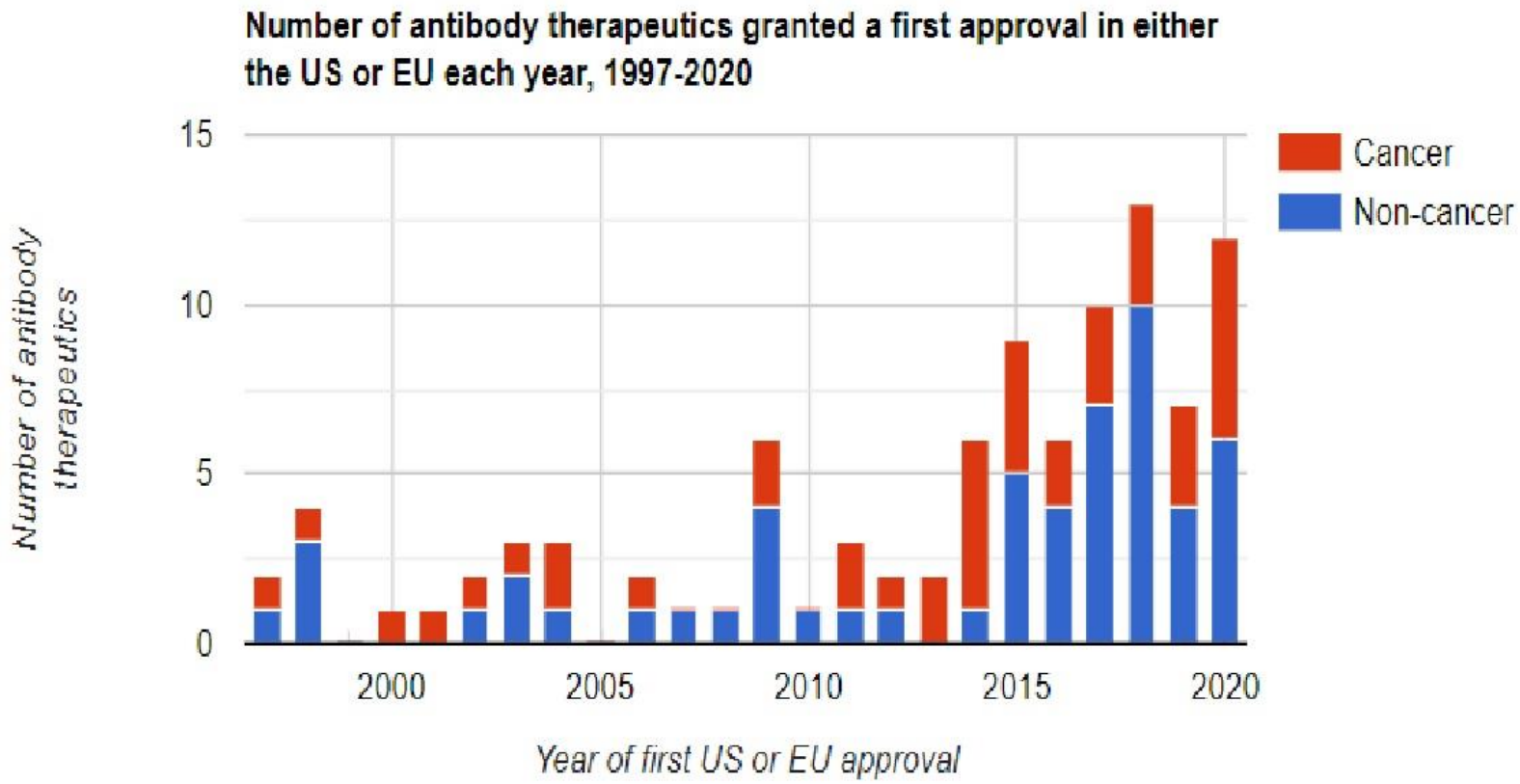


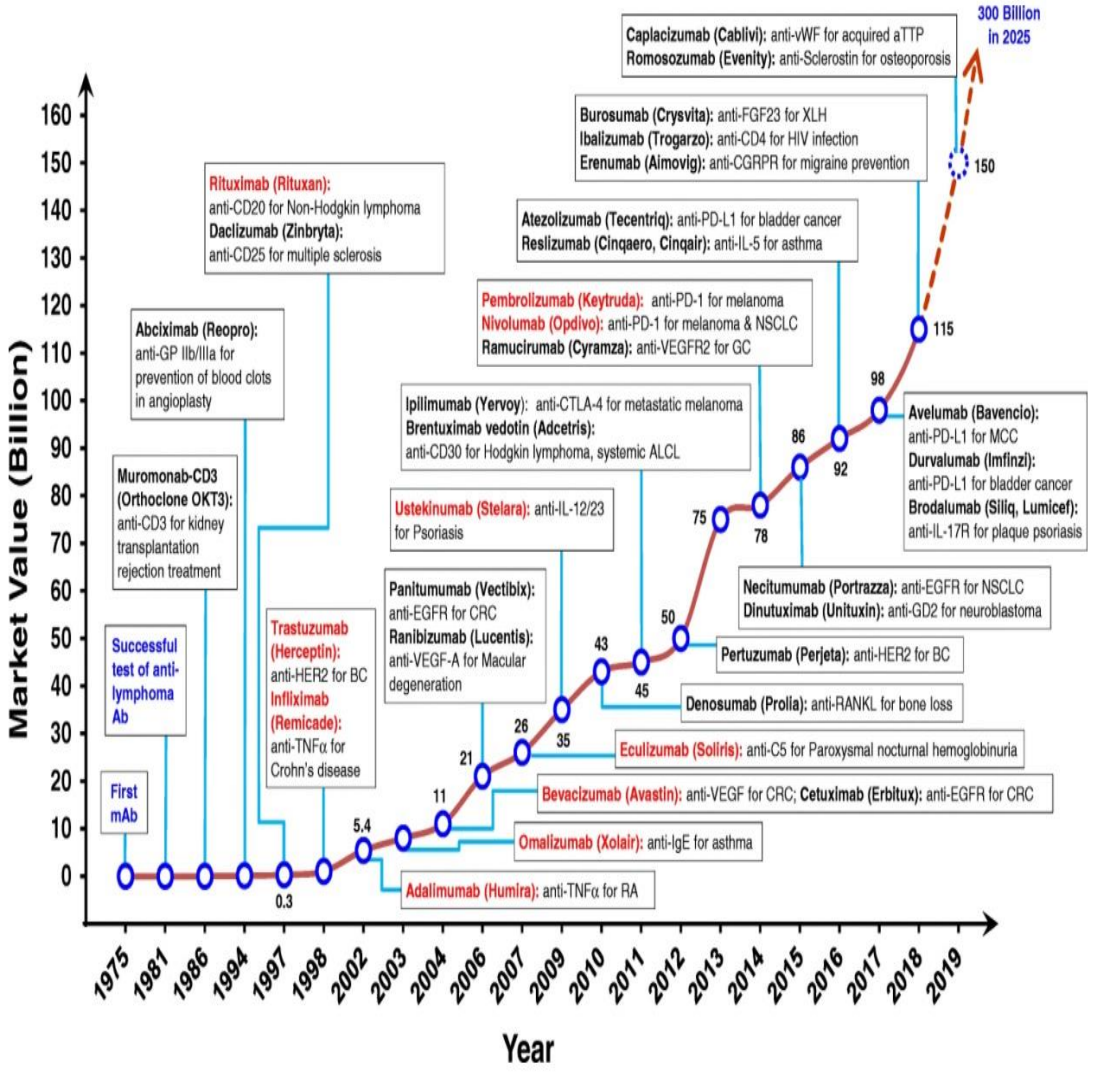
Figure 1. Immunological targets of biologic agents.

FDA-approved antibodies



2021 https://www.antibodysociety.org/resources/approved-antibodies/

Market of therapeutic antibodies[23, 26.]



1 g gold vs 100 mg infliximab

57.55 $ 517.8 $ (in Hungary)

(2021.02.24.)

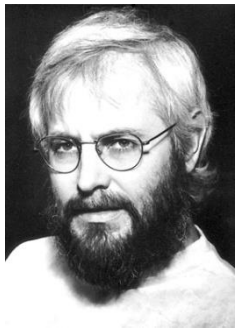
- **Recombinant proteins produced in mammalian cell cultures (133 Kg in 2013)**
- **Monoclonal antibodies produced in mammalian cell cultures (8182 Kg in 2013)**
- **Monoclonal antibody fragments, conjugates or fusion proteins produced in mammalian cell cultures (1677 Kg in 2013)**
- **Recombinant proteins (insulin too) produced in microbial fermentation (8497 Kg in 2013)**
- **Monoclonal antibodies produced in microbial fermentation (102 Kg in 2013)**
- **Products produced in plant cell cultures (189 g in 2013)**

Thank you for your attention!

Gerald M. Edelman

Rodney R. Porter

Niels K. Jerne



Georges J.F. Köhler

César Milstein

Were awarded the 1972 Nobel Prize in Physiology or Medicine:
„For their discoveries concerning the chemical structure of antibodies”.[24.]

Were awarded the 1984 Nobel Prize in Physiology or Medicine:
„For theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies”.[25.]

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