Immunology Review

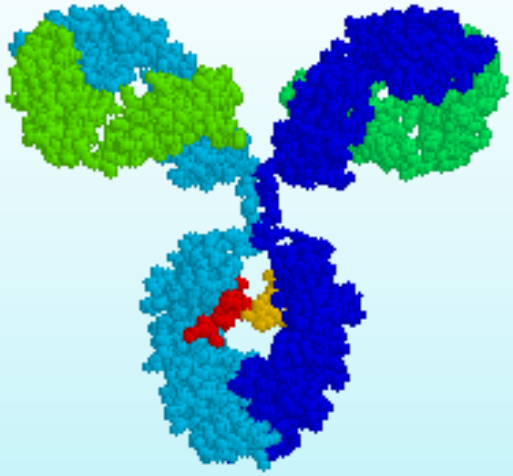
- Immunoglobulin G
- Monoclonal

vs.

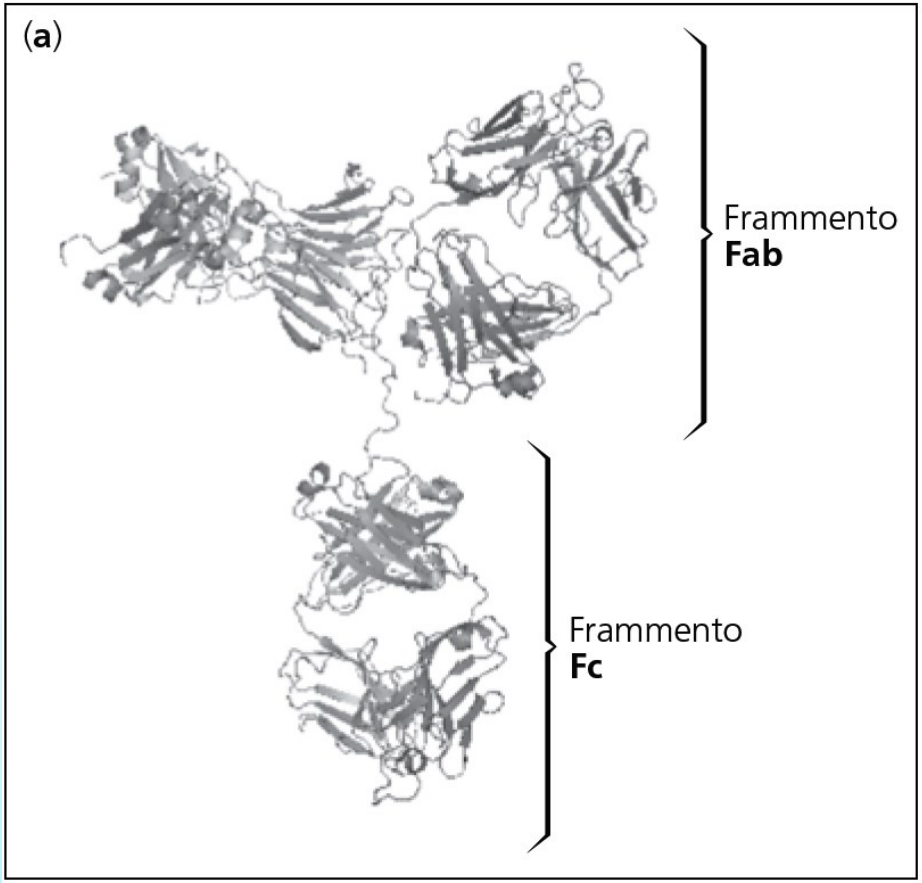
- Polyclonal

vs

- Recombinant



http://www.path.cam.ac.uk/~mrc7/igs/img09.jpeg



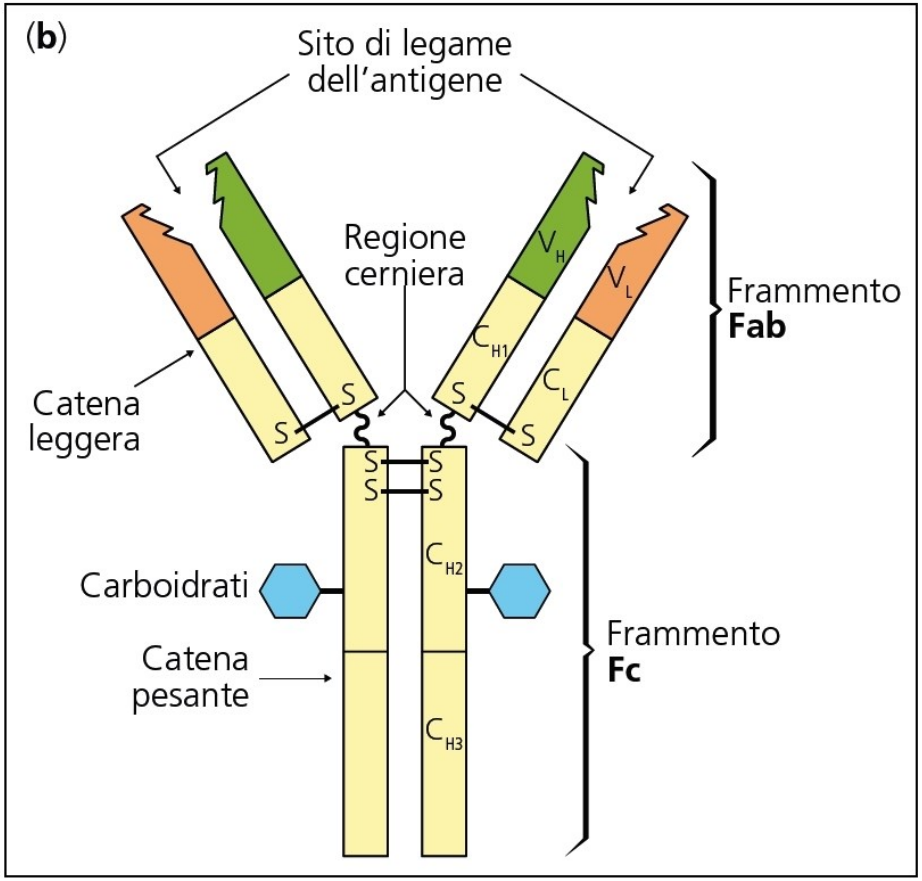
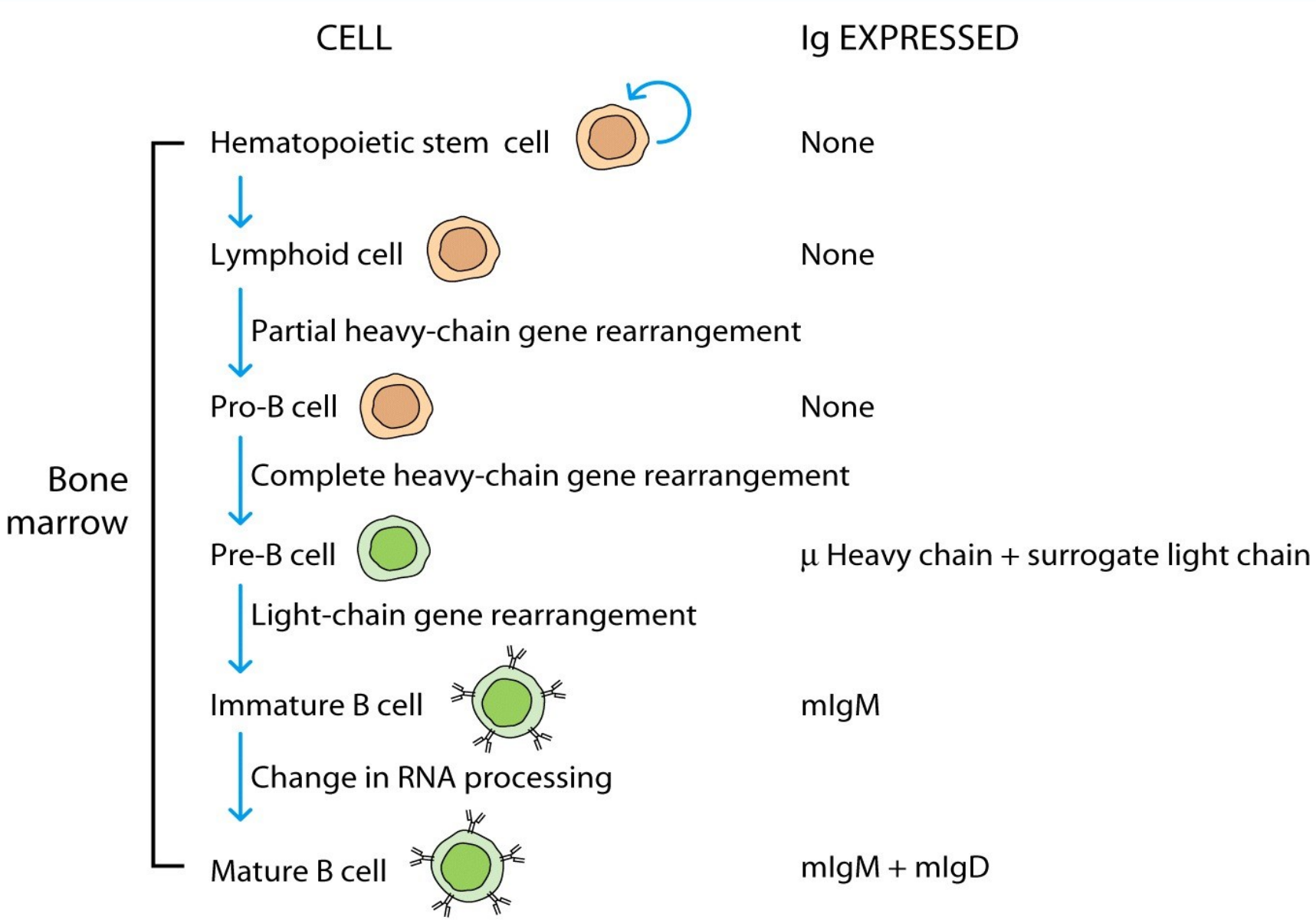


Figura 6.26

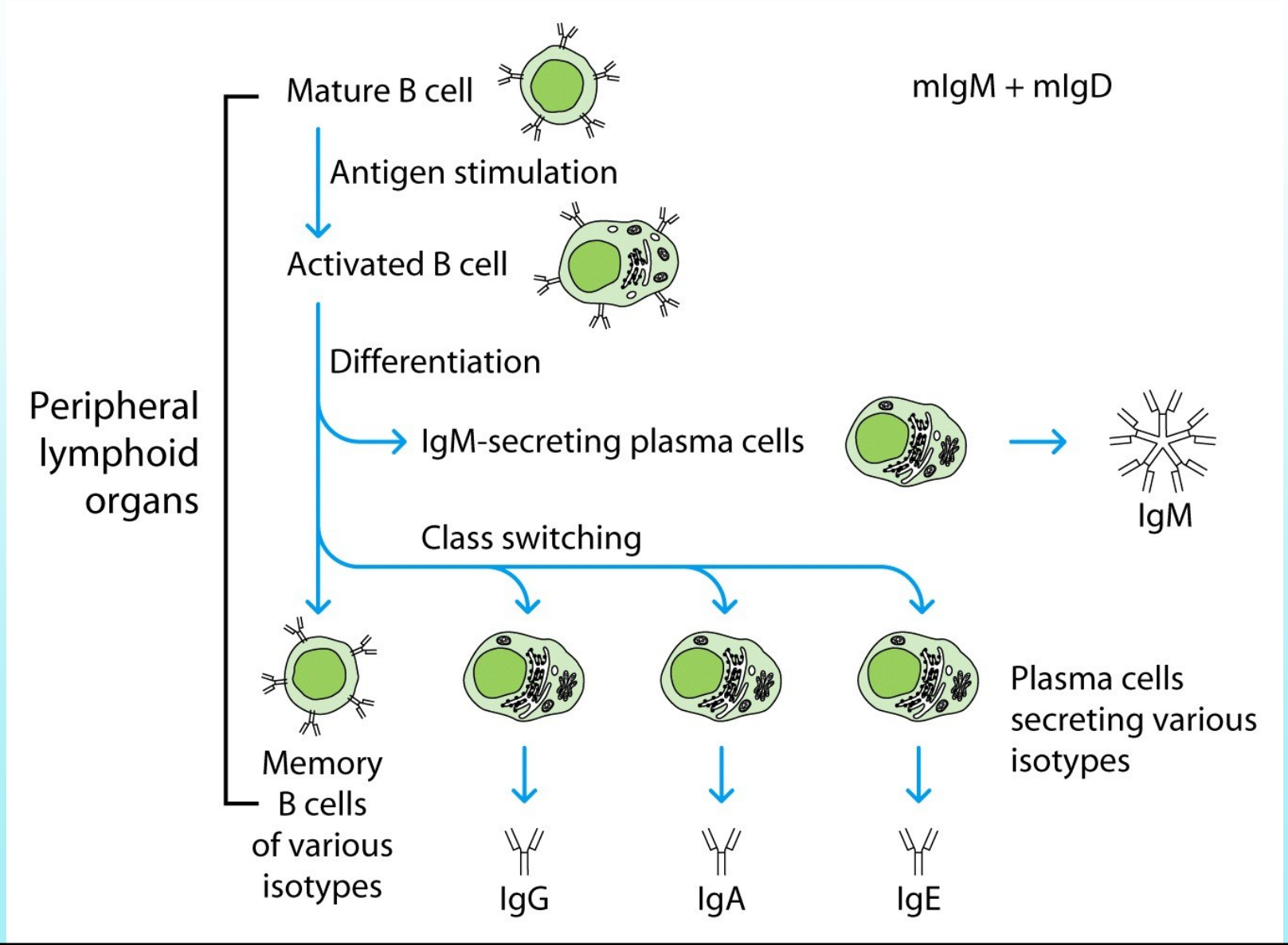
(**a**) Struttura tridimensionale di un'immunoglobulina G (codice PDB: 1HZH). La figura è stata prodotta con il programma PyMol (DeLano Scientific).

(**b**) Schematizzazione della struttura di un'immunoglobulina. Fc, frammento cristallizzabile; Fab, frammento legante l'antigene; C_H, dominio costante della catena pesante; V_H, dominio variabile della catena pesante; C_L, dominio costante della catena leggera; V_L, dominio variabile della catena leggera.

B lymphocyte development



B lymphocyte development (2)



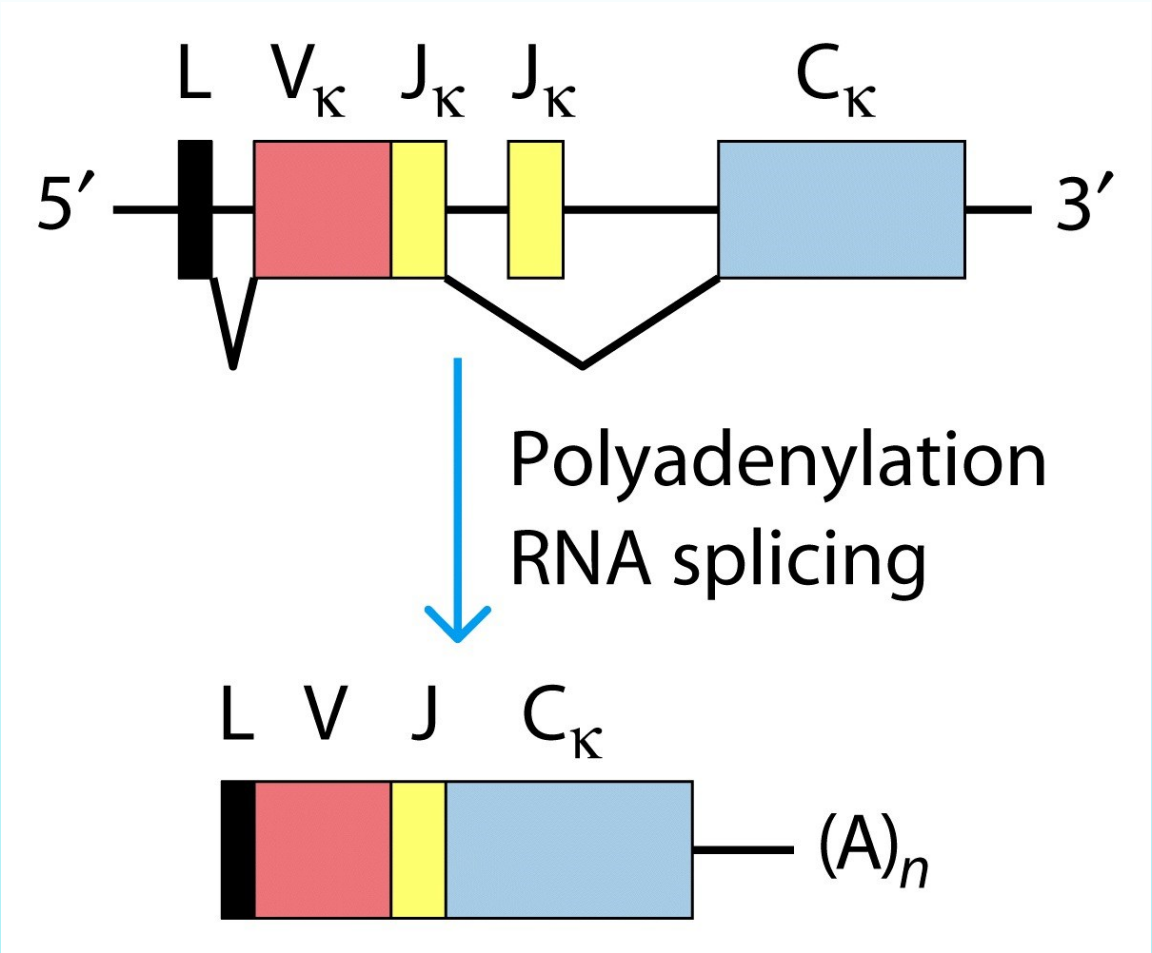
Sottoclassi immunoglobuline

Table 1: Immunoglobulin Subclasses

Class/Subclass	Heavy Chain	Light Chain	MW (kDa)	Structure	Function
IgA1 IgA2	α1 α2	λ or κ	150 to 600	Monomer to tetramer	Most produced Ig; protects mucosal surfaces; resistant to digestion; secreted in milk
IgD	δ	λ or κ	150	Monomer	Function unclear; works with IgM in B-cell development; mostly B cell bound
IgE	ε	λ or κ	190	Monomer	Defends against parasites; causes allergic reactions
IgG_1 IgG_{2a} IgG_{2b} IgG_3 IgG_4	γ1 γ2 γ2 γ3 γ4	λ or κ	150	Monomer	Major Ig in serum; good opsonizer; moderate complement fixer (IgG_3); can cross placenta
IgM	μ	λ or κ	900	Pentamer	First response antibody; strong complement fixer; good opsonizer

An Introduction to Antibodies and Their Applications
3rd – Edition - Millipore

Antibody Diversity

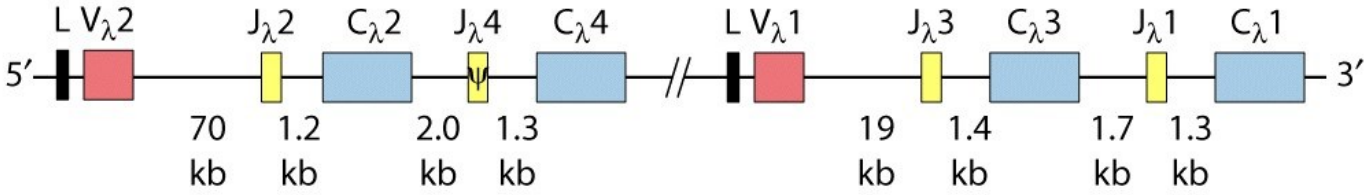


Multigene organization of Ig genes

2 types of light chains in humans:

The constant region determines the light chain's class (kappa or lambda).
The lambda class has 4 subtypes (lambda 1, lambda 2, lambda 3, and lambda 7)

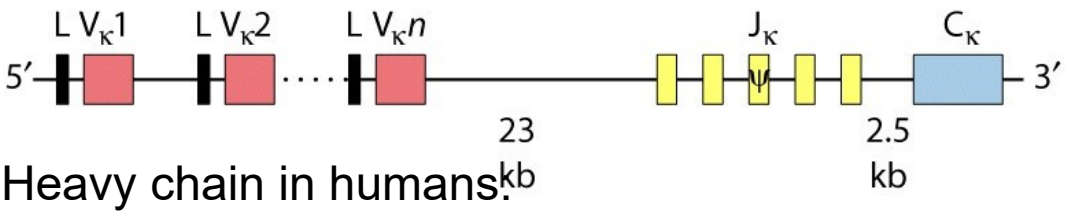
(a) λ-chain DNA



lambda (λ) chain, encoded by the immunoglobulin lambda locus (IGL@) on chromosome 22 (locus: 22q11.2)

(b) κ-chain DNA

n = ~85

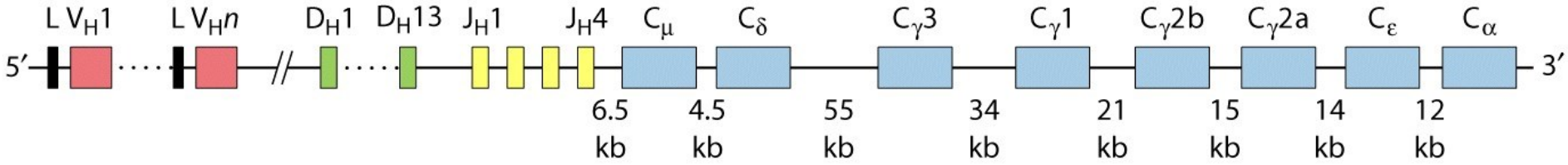


kappa (κ) chain, encoded by the immunoglobulin kappa locus (IGK@) on chromosome 2 (locus: 2p11.2)

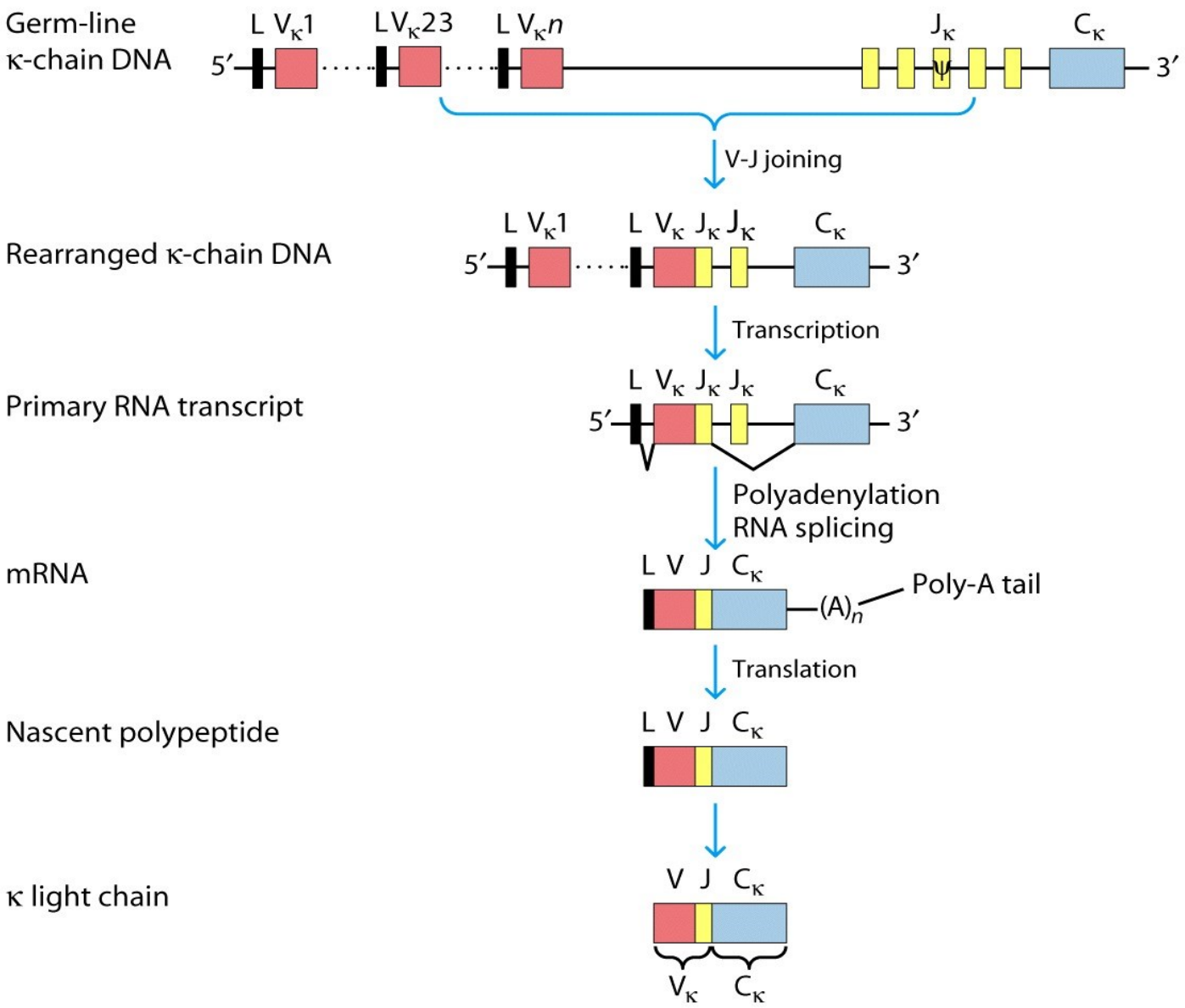
Heavy chain in humans:

(c) Heavy-chain DNA

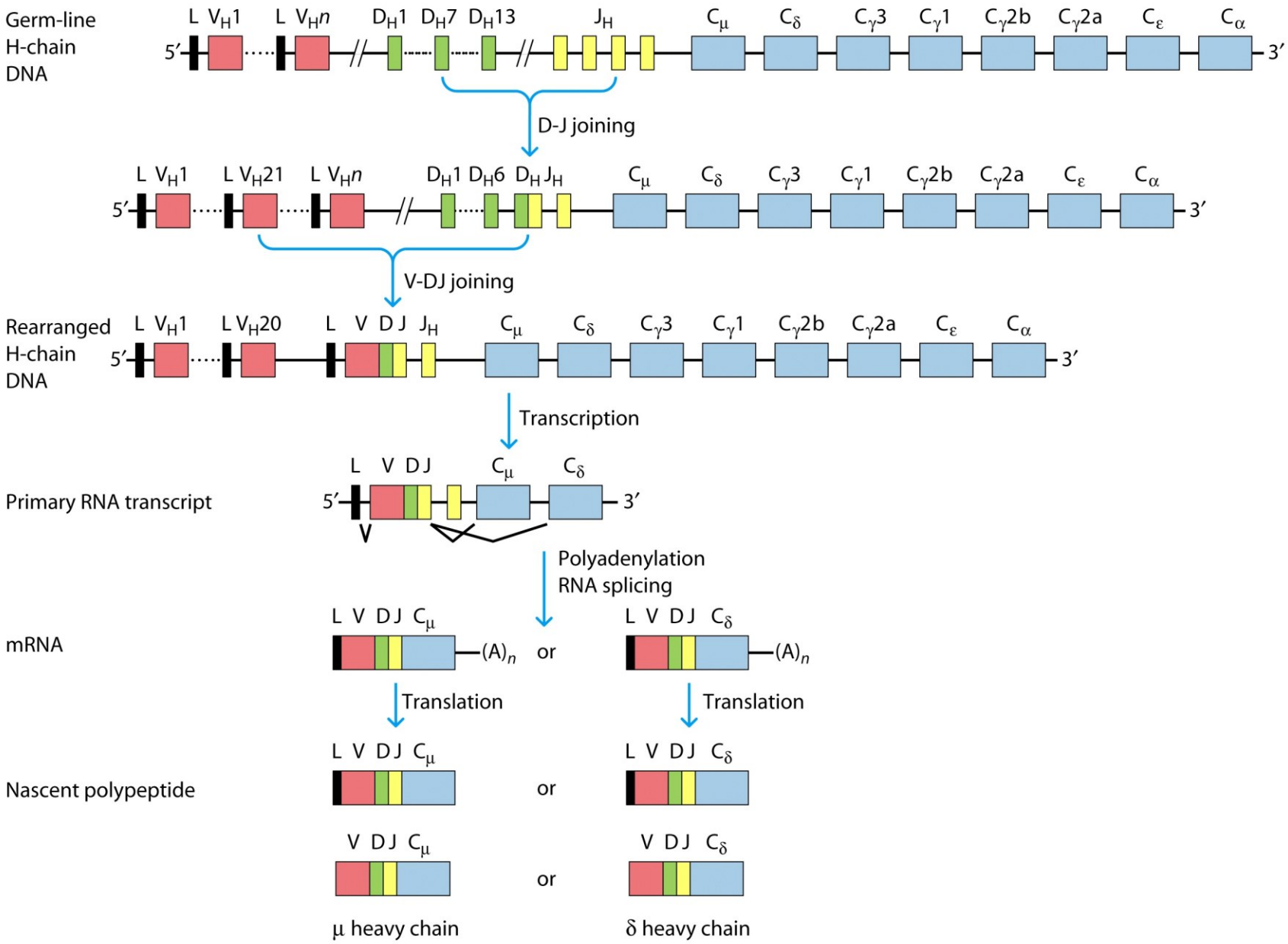
n = ~134



Kappa light chain rearrangement



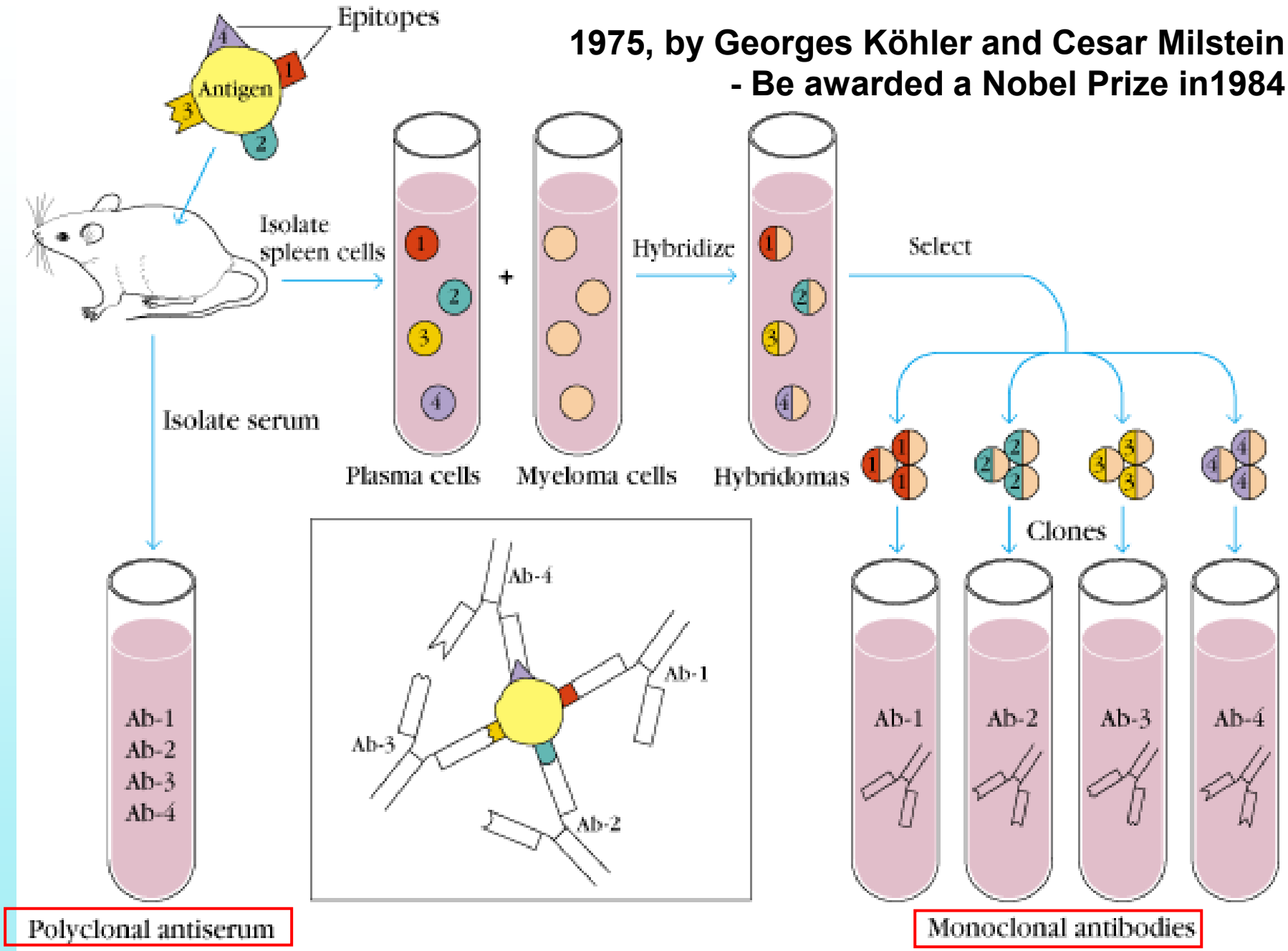
Heavy chain rearrangement



Hybridoma technology

- Fusion of an antibody-producing B lymphocyte with a myeloma
- cell generates a hybridoma cell.
- This secretes a single type of antibody and can grow indefinitely
- In tissue culture.

1975, by Georges Köhler and Cesar Milstein
- Be awarded a Nobel Prize in1984



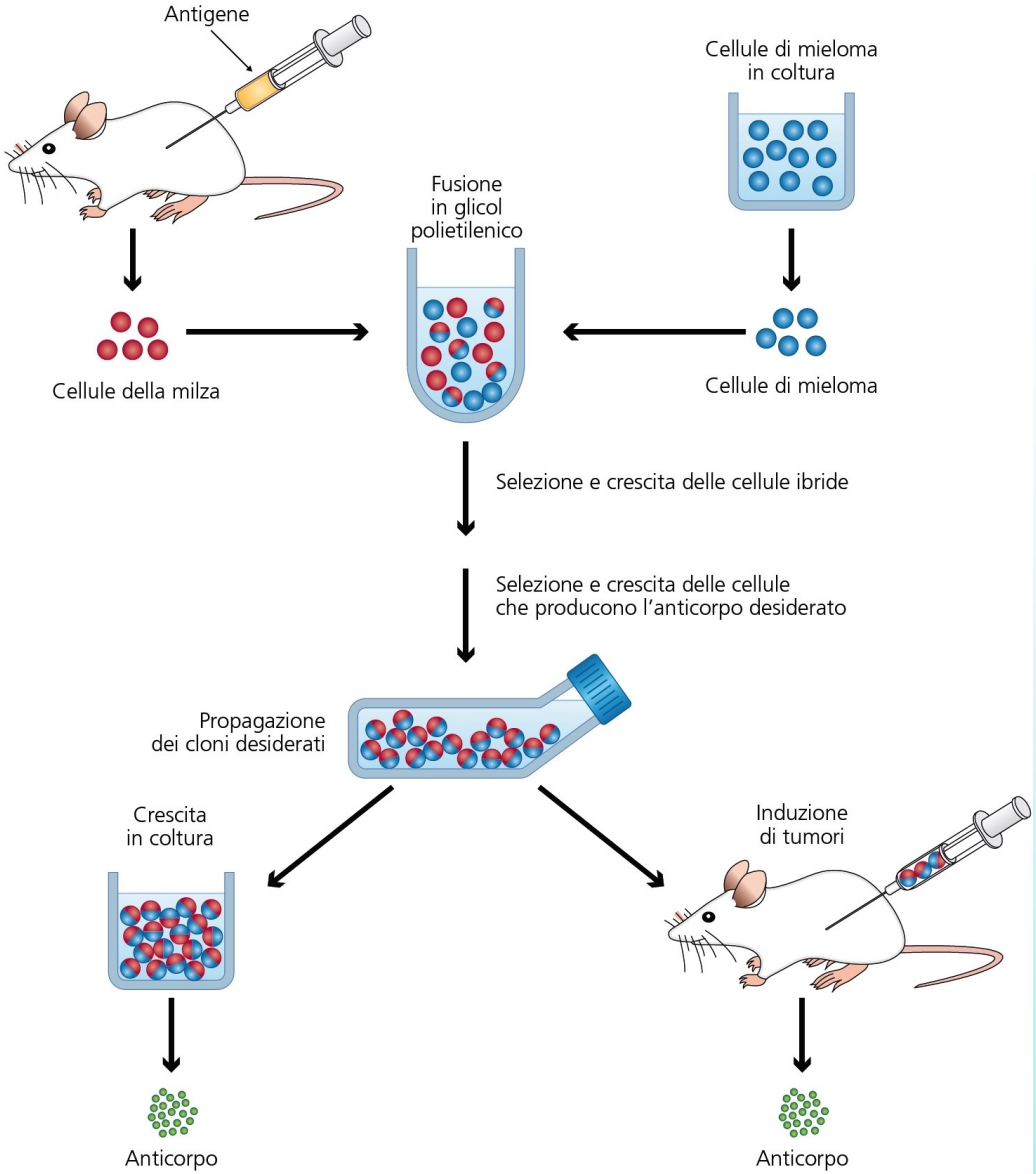


Figura 6.27
Schema della tecnica di produzione di un anticorpo monoclonale.

Raising monoclonal antibodies

- A mouse is immunised with the antigen.
- The spleen is removed and **B lymphocytes** are isolated.
- These are fused with hypoxanthine-guanine phosphoribosyl transferase negative (HPGRT -) myeloma cells (DHFR+).
- Hybridomas are selected from the mixture using HAT medium.

Selection using HAT medium

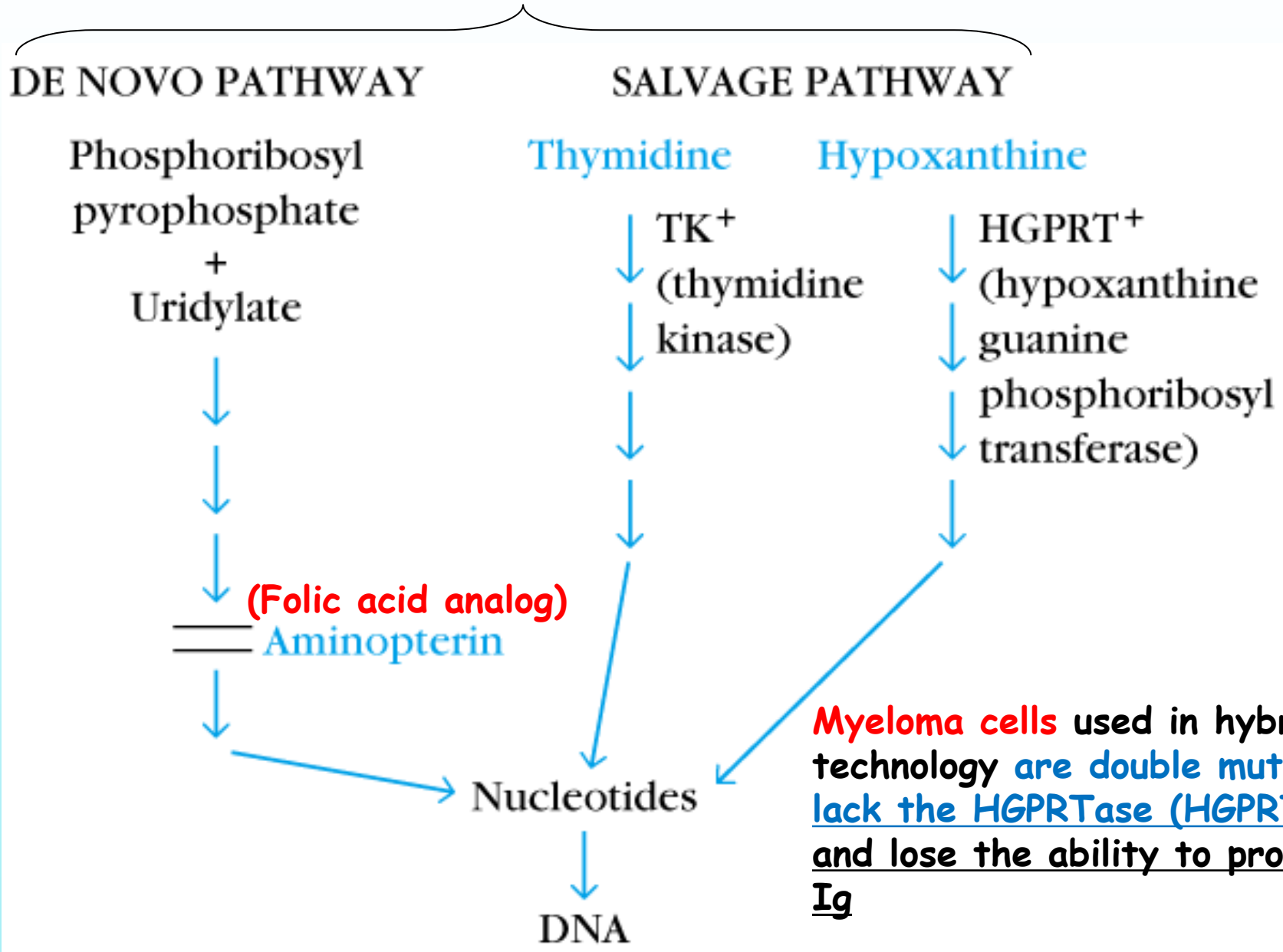
Cells make purine nucleotides

1) by *de novo synthesis*, from PRPP, Q,G,THF-For, Q, CO2, D, THF-For etc.
2) by salvage – attaching preformed purine bases to ribose.

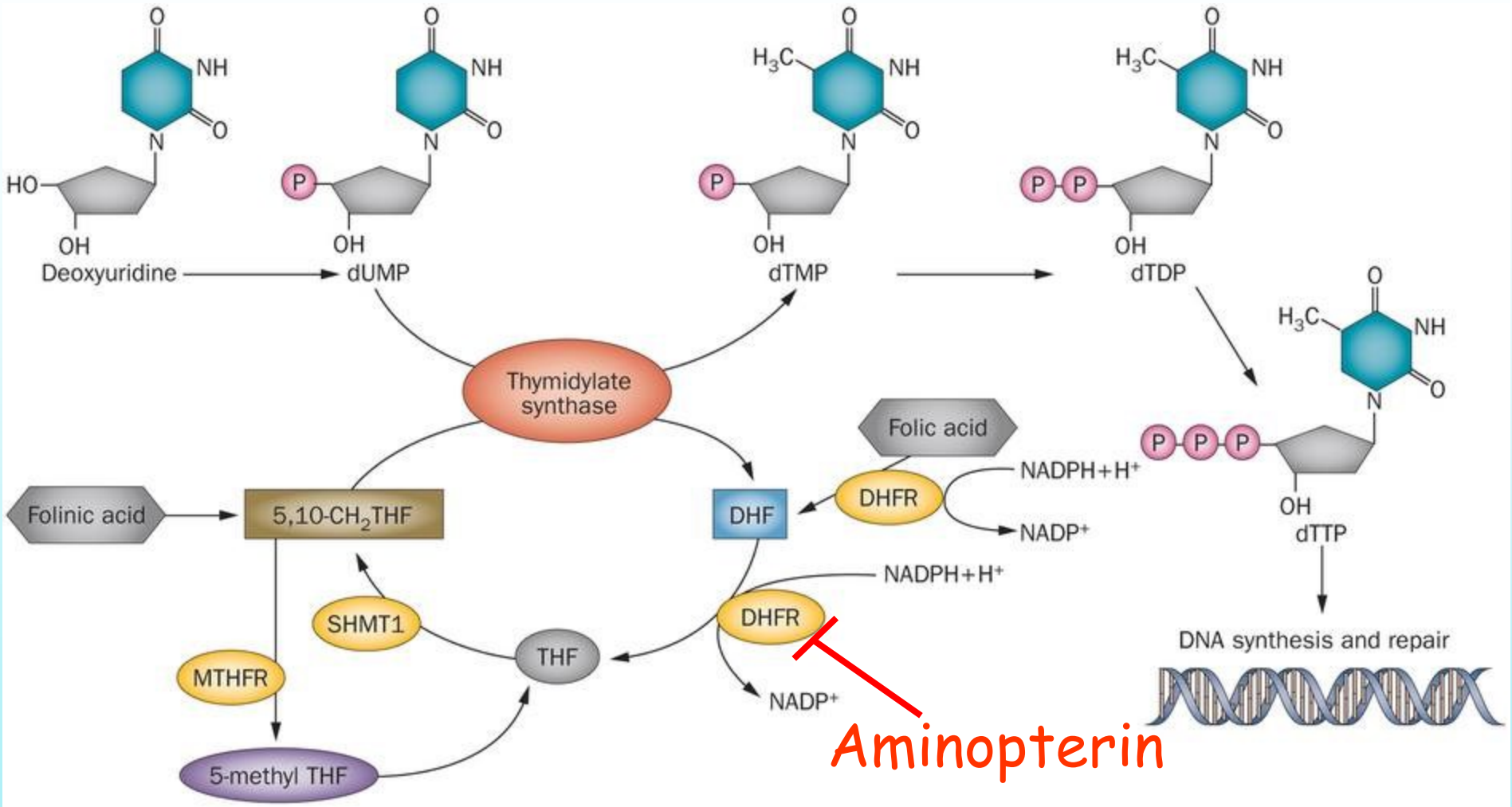
→ Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is a purine salvage enzyme

- It adds hypoxanthine or guanine to PRPP (phospho ribosyl pirophosphate) to form IMP or GMP. IMP is a precursor of AMP.

Two different pathways to synthesis nucleotide in mammalian cells



Myeloma cells used in hybridoma technology are double mutants, lack the HGPRTase (HGPRT-) and lose the ability to produce Ig

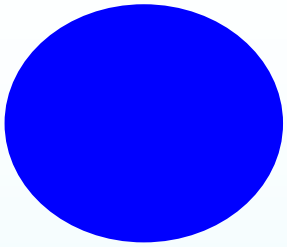

Deoxyuridine
dUMP
dTMP
dTDP
dTTP
Thymidylate synthase
Folic acid
DHFR
NADPH+H+
NADP+
DHF
Folinic acid
5,10-CH2THF
SHMT1
THF
MTHFR
5-methyl THF
DNA synthesis and repair
Aminopterin

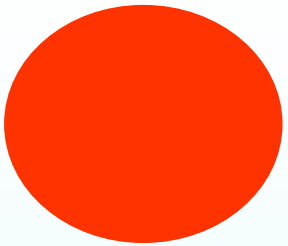
- Aminopterin
  - inhibits dihydrofolate reductase DHFR
  [and myeloma cells are (HPGRT -)]
  - *knocks out de novo purine biosynthesis* and thymidine biosynthesis
  - kills unfused myeloma cells

- Hypoxanthine
  - can be salvaged by **HGPRT+ hybridomas** enables them to make IMP, AMP and GMP and to proliferate.

- Thymidine
  - **used by hybridomas** which can't make their own thymidine when DHFR is inhibited by aminopterin.

Hybridoma





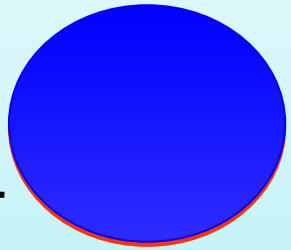
HGPRT+ B lymphocyte
Can salvage purines
Can't survive in cell culture
Produces antibody

Does not survive in culture

HGPRT- myeloma cell
Can't salvage purines
Can proliferate in cell culture

Aminopterina kills unfused myeloma cells

HGPRT+



Hybridoma
Can salvage purines
Can survive in cell culture
Produces antibody

Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is a purine salvage enzyme
- adds hypoxanthine or guanine to PRPP (phosphoribosyl pyrophosphate)
to form IMP or GMP. IMP is a precursor of AMP.

Clinical Uses for Monoclonal Antibodies

- Very useful as diagnostic, imaging, and therapeutic reagents in clinical medicine
  - Monoclonal antibodies were used primarily as in vitro diagnostic reagents
  - Radiolabeled monoclonal antibodies can also be used in vivo detecting or locating

- Immunotoxins
  - To compose of tumor-specific monoclonal antibodies coupled to lethal toxin
  - Valuable therapeutic reagent

Disappointed promises

- The early promise of the use of antibodies in the treatment of disease initially went disappointed (more than two decades) for two reasons:
  - Early antibodies displayed insufficient activation of human effector functions (i.e. the antibodies did not kill the infecting organism or cell)
  - The early antibodies were of murine (mouse) origin, and thus triggered the production of human anti-mouse antibodies (HAMA).

The types of mAb designed

- Murine source mAbs: rodent mAbs with excellent affinities and specificities, generated using conventional hybridoma technology.
- Clinical efficacy compromised by HAMA(human anti murine antibody) response, which lead to allergic or immune complex herpersensitivities.

The types of mAb designed

- Chimeric mAbs: chimers combine the human constant regions with the intact rodent variable regions. Affinity and specificity unchanged. Also cause human antichimeric antibody response (30% murine resource)
- Humanized mAbs: contained only the CDRs of the rodent variable region grafted onto human variable region framework

Antibody engineering techniques

- create chimeric or humanized antibodies by utilizing the murine variable regions or complementary determining regions (CDRs),

- in conjunction with human constant regions, in order to maintain target specificity and reduce the HAMA (human anti-mouse antibodies) response (21–23).

- Years ago, fully human antibodies are generated using hybridoma technology in transgenic mice models, such as HuMabMouse and XenoMouse, whereby the mouse immunoglobulin (Ig) gene loci have been replaced with human loci within the transgenic mouse genome (*Nat Biotechnol.* (2005) 23:1117–25. doi: 10.1038/nbt1135).

Front. Immunol., 28 August 2020 | https://doi.org/10.3389/fimmu.2020.01986

Human Monoclonal Antibodies

- Production of human monoclonal antibody
  - There are numbers of technical difficulties
    - The lack of human myeloma cells to exhibit immortal growth, be susceptible to HAT selection, to not secrete antibody, and support antibody production in the hybridoma made with them
    - Human B cell sometimes have immortality
    - That is the difficulty of readily obtaining antigen-activated B cells

- To culture human B cells in vitro to produce human monoclonal antibody
  - Transplant human cells with immune response into SCID mice (lack a functional immune system)

Humanization of mAbs

The humanization of mAbs by complementarity-determining region (CDR)-grafting

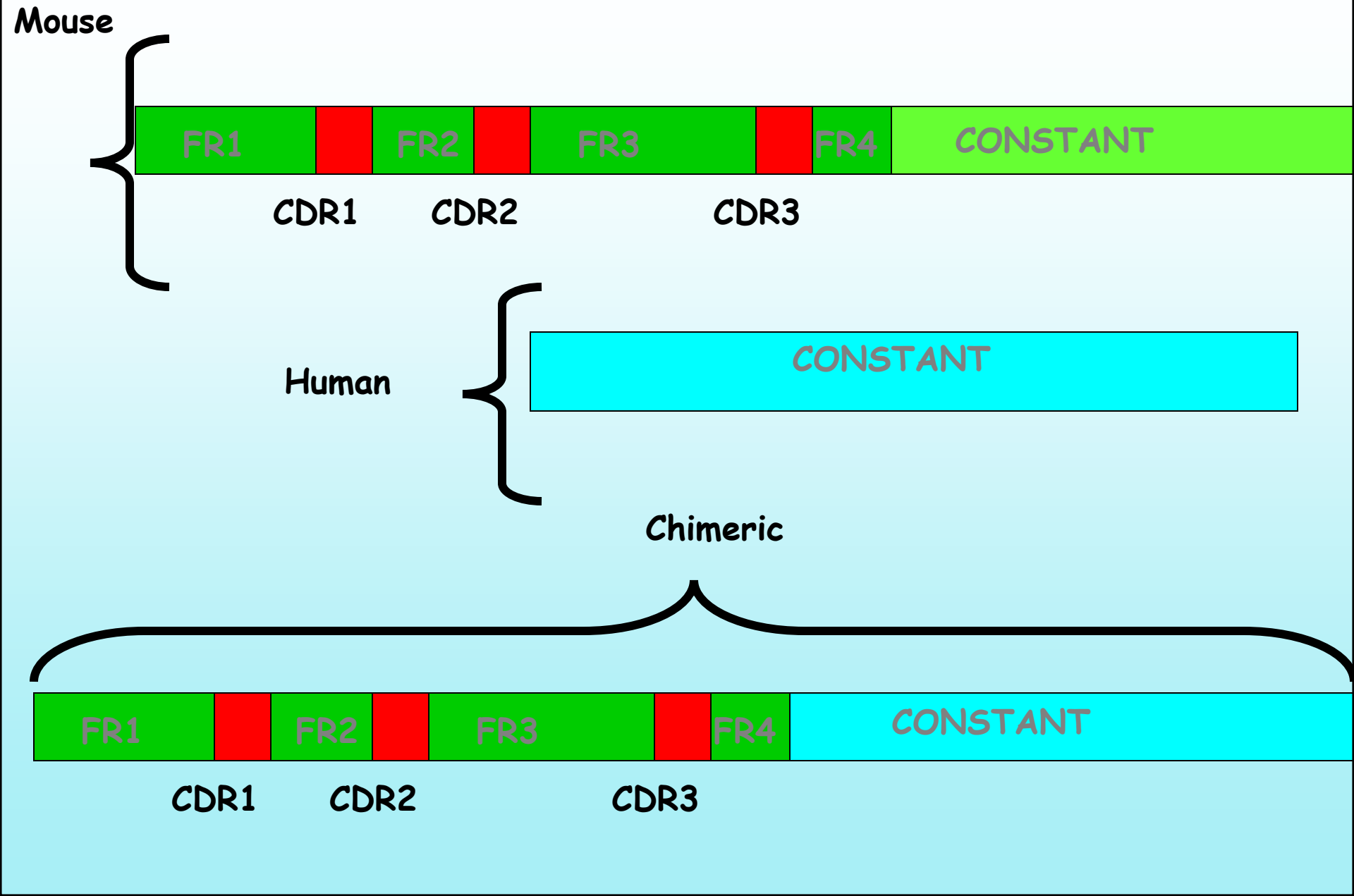
- a standard procedure to improve the clinical utility of xenogeneic Abs by reducing human anti-murine Ab (HAMA) responses elicited in patients.
- CDR-grafted humanized Abs may still evoke anti-V region responses when administered in patients.

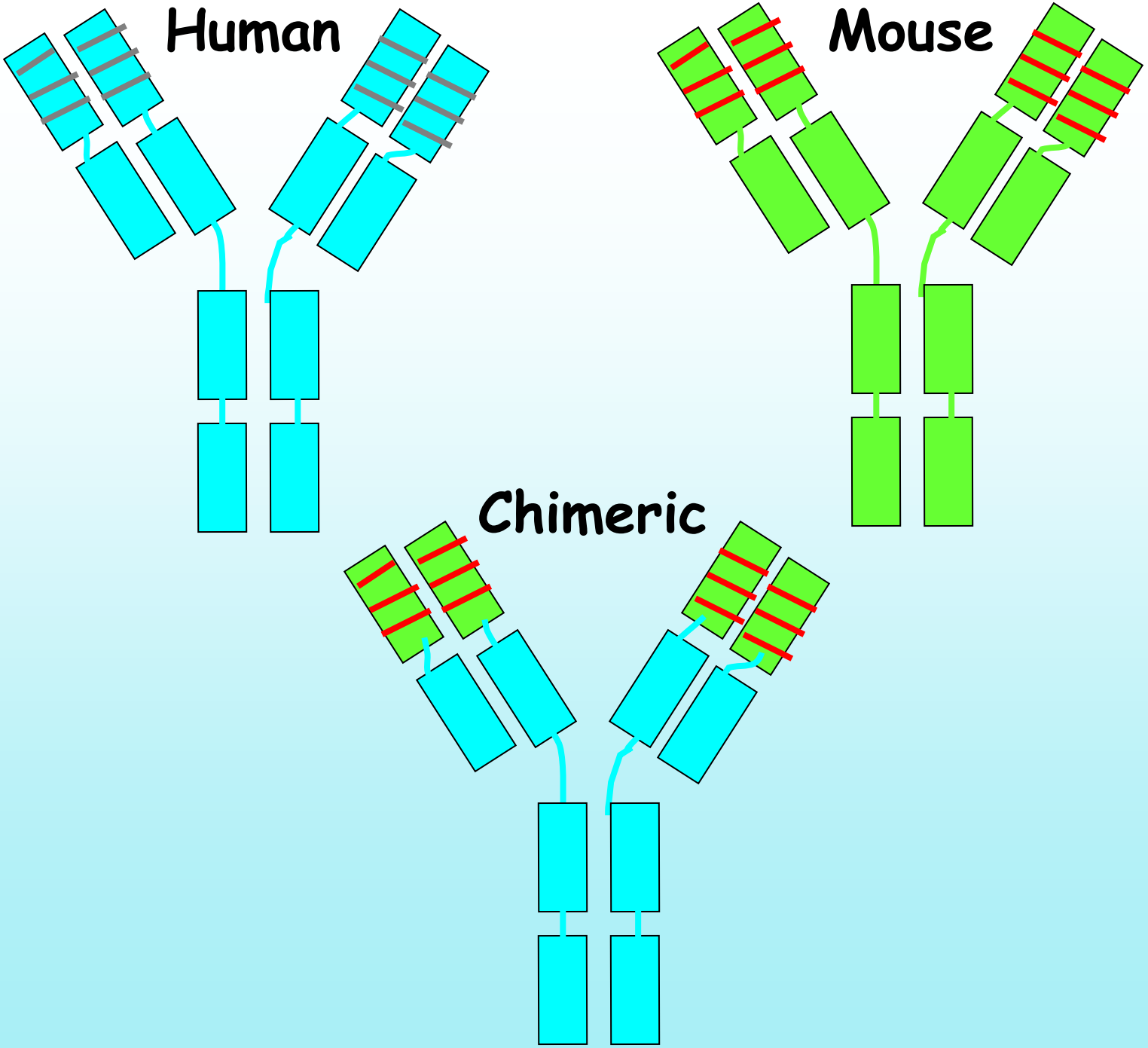
To minimize anti-V region responses:

- the Ab may be humanized by grafting onto the human templates only the specificity-determining residues (SDRs), which are essential for the surface complementarity of the Ab and its ligand.
- Typically, humanization of an Ab, whether by CDR or SDR grafting, involves the use of a single human template for the entire VL or VH domain of an Ab.

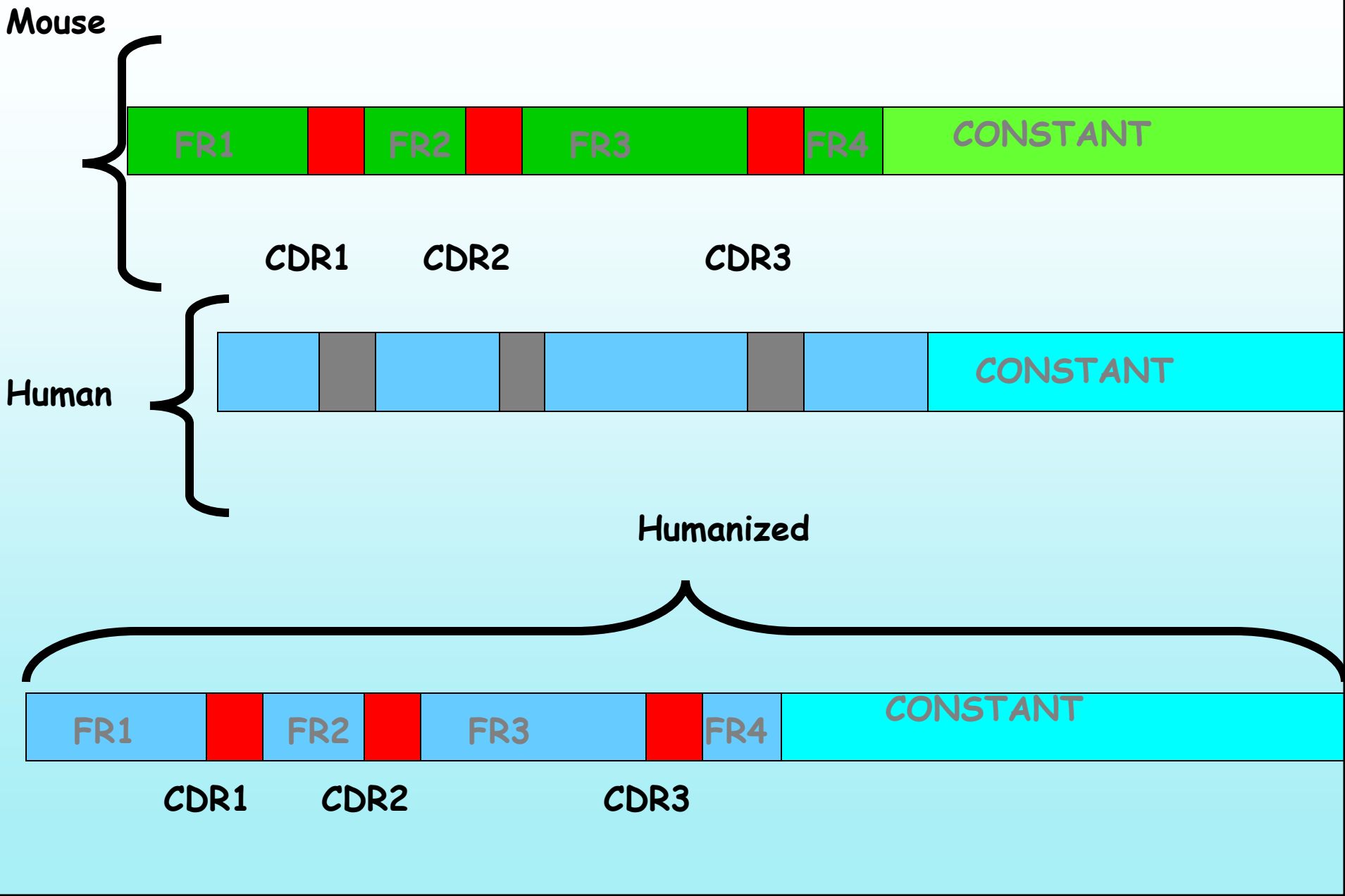
https://doi.org/10.1016/j.molimm.2004.03.041.

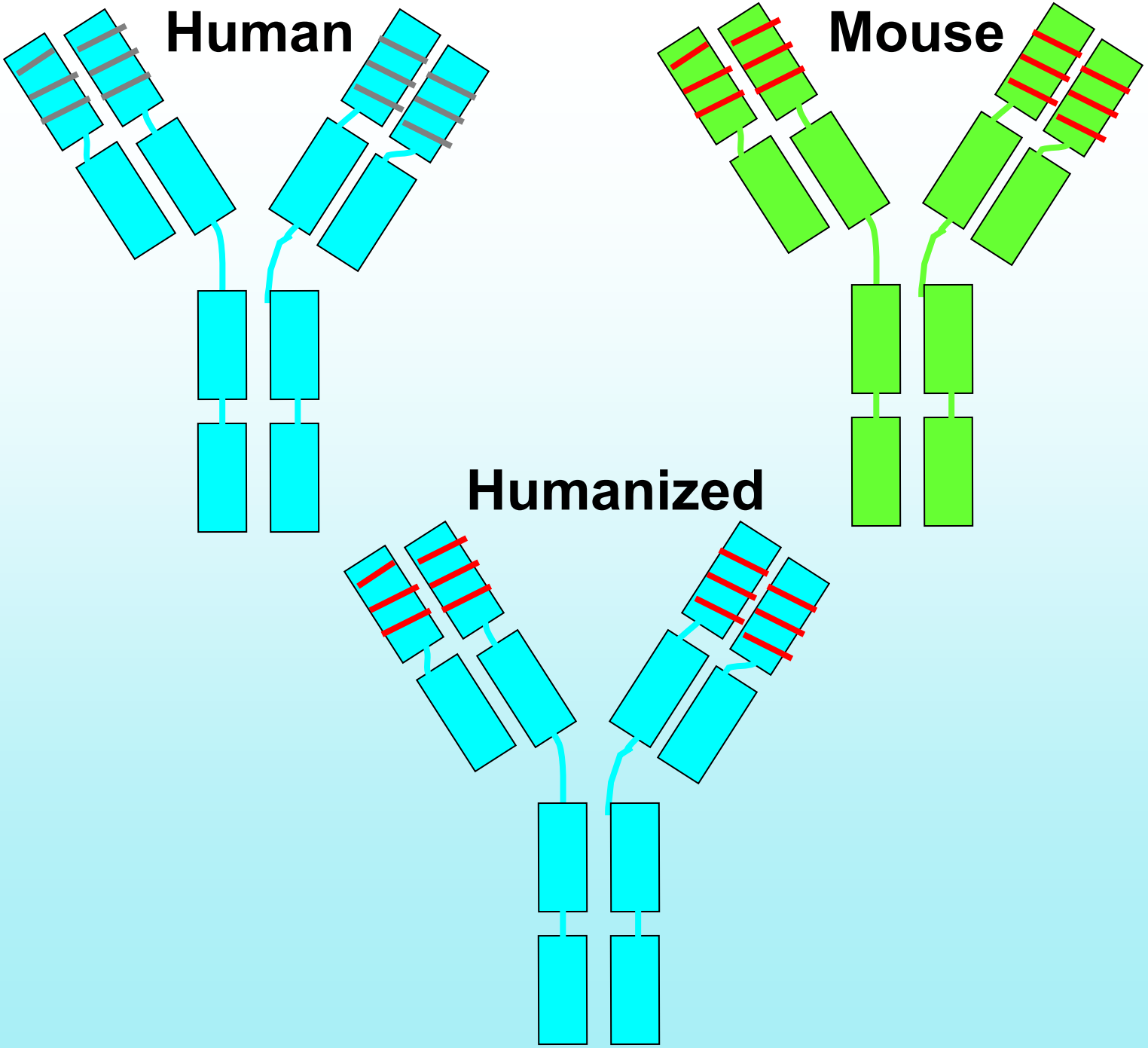
CHIMERIC HUMAN/MOUSE




Human
Mouse
Chimeric

HUMANIZED ANTIBODIES




Human
Mouse
Humanized

CHIMERIC/HUMANIZED

Reduce the HAMA response.

Maintain effector functions.

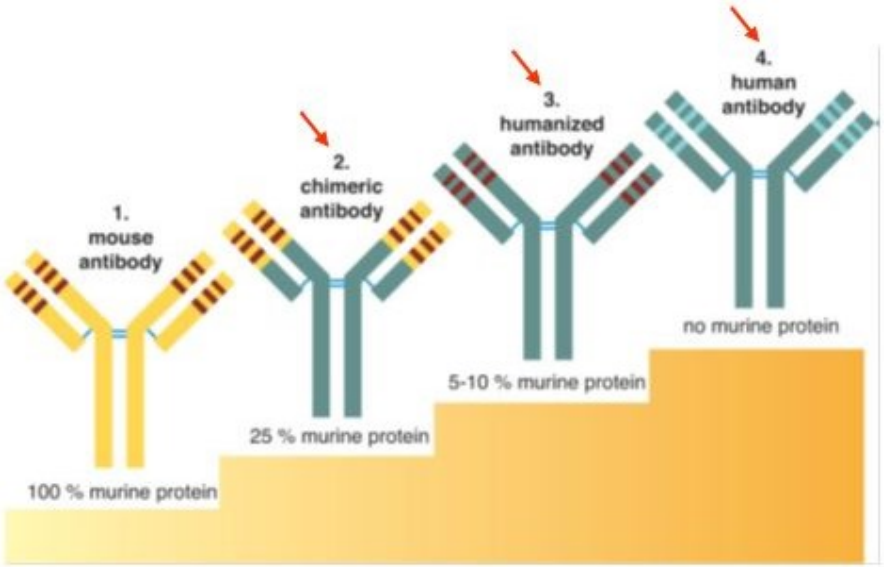
Increase the half life of the antibody.

Maintain binding affinity.

Easy to construct.

Evolution of Therapeutic Antibodies

- ximab = MAb chimerico
  (es. infliximab, rituximab, abciximab)
- zumab = MAb umanizzato
  (es. certolizumab, efalizumab, trastuzumab, gemtuzumab, omalizumab, daclizumab)
- umab = MAb umano
  (es. adalimumab)



Molecular Biotechnology: From DNA Sequence to Therapeutic Protein

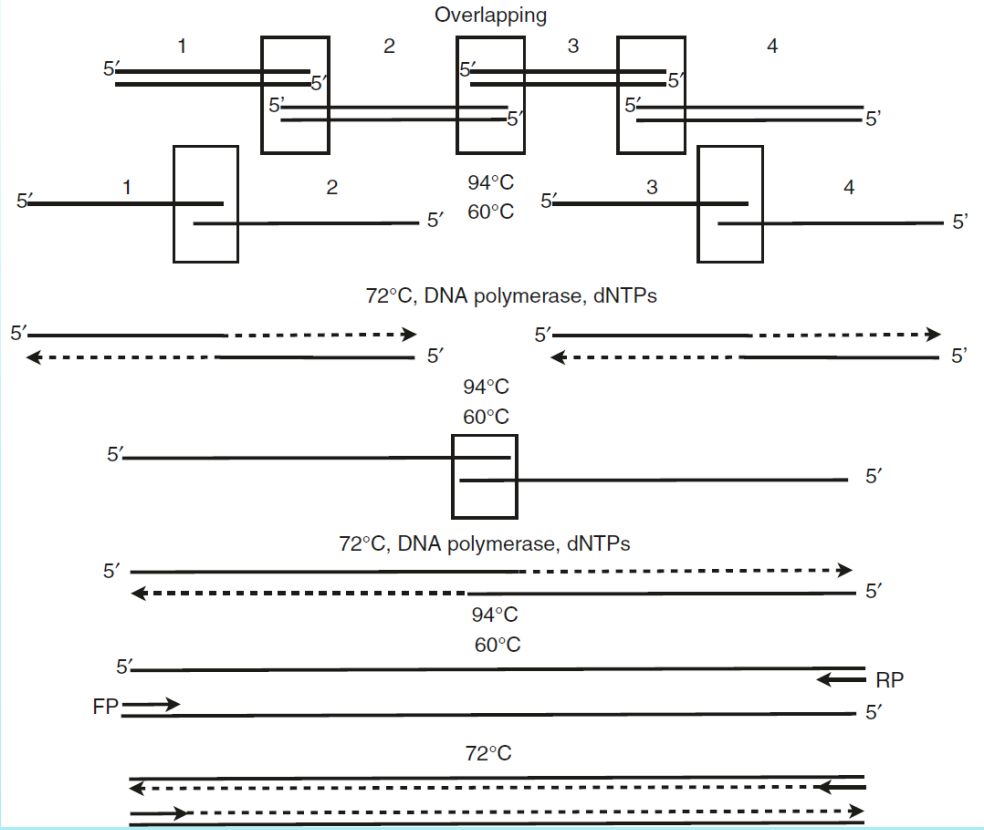
Monoclonal antibody - humanization of a mouse antibody

e.g. cell culture, 10 g/L yield of 80%. to produce 1000 kg monoclonal antibody, needs at least 100,000 L of cell culture supernatant.

- The **nucleotide sequence of each of the *V*L and *V*H regions is deduced** (contains either the murine CDRs or SDRs).
- Next, the **entire sequence is divided over four or more alternating oligonucleotides** with overlapping flanks

→ These relatively long oligonucleotides are made synthetically.

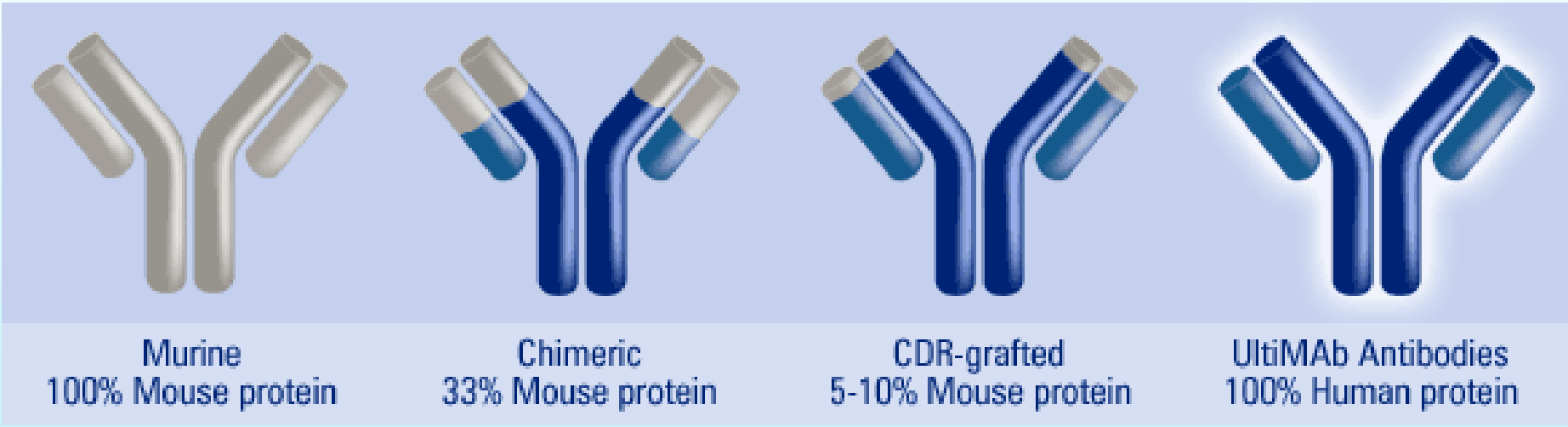
- Both the VL and VH regions contain **three highly variable loops** (known as complementarity-determining regions 1, 2, and 3).
- The **PCR product encoding the humanized *V*L and *V*H region is cloned into an expression vector** carrying the respective constant regions and a signal peptide. The signal peptide is required for glycosylation. → CHO cells



complementarity determining regions (CDRs)

ISBN 978-3-030-00709-6 - ISBN 978-3-030-00710-2 (eBook)
https://doi.org/10.1007/978-3-030-00710-2 -

Evolution of Therapeutic Antibodies



Comparison between polyclonal, monoclonal, and recombinant antibodies in terms of production method and reproducibility.

	Polyclonal	Monoclonal	Recombinant Ab
Production	A heterogeneous mix of antibodies is derived from the immune response of multiple B-cells following animal immunization with the antigen of interest.	An antibody-producing B-cell from an immunized animal is fused with a myeloma cell to produce an antibody-producing hybridoma line	The genetic sequence of the selected antibody is cloned into an expression vector, which is subsequently introduced into expression hosts to provide animal-free antibody production.
Reproducibility	Multiple antibodies to various antigen epitopes. Following numerous rounds of serum collection, the animal is dispatched and the antibody source no longer available.	A single antibody clone, providing improved batch-to-batch consistency compared to polyclonals. Potential cell-line drift and mutations can impair production and result in antibody changes that affect reproducibility.	Stable production that can be re-generated at any time through the known genetic sequence starting material.

Monoclonal Ab Production method

- Large-scale production of biopharmaceutical products, including mAbs, require **suspended cells** and also the use of a **serum-free medium (SF).**
- Adaptation to these new conditions:
  - a direct approach, where serum is completely removed in a single step
  - a gradual/sequential approach, where serum concentration in the medium is slowly reduced
- **SF adaptation can even be advantageous in terms of mAb production,**
  - in several works that indicate **significantly increased mAb production kinetics in SF medium** with respect to those done in serum-containing medium

European Journal of Pharmaceutics and Biopharmaceutics 74 (**2010**) 127–138

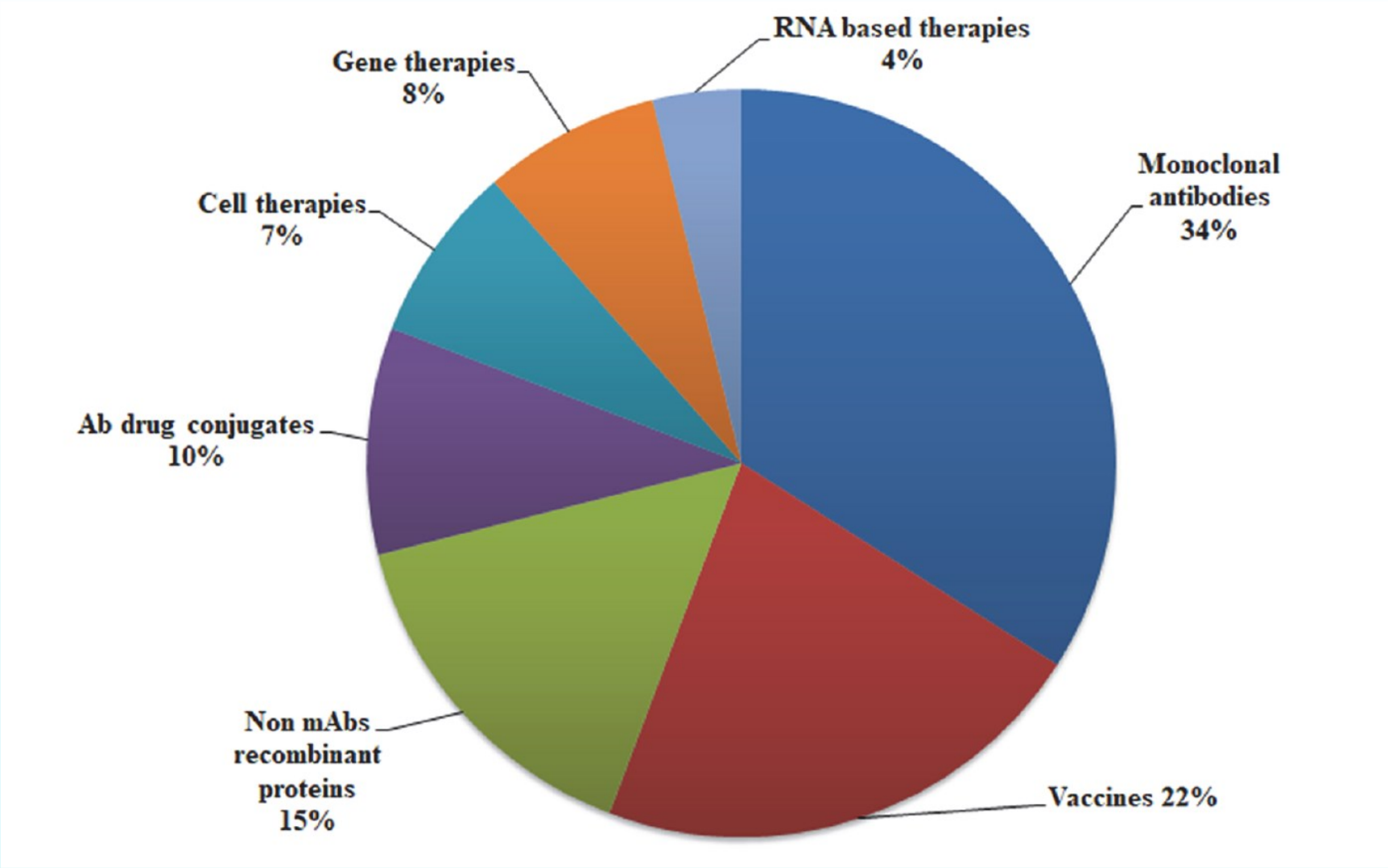
Monoclonal Ab Production method

Table 1. Recent advances in the production of full length mAbs and antibody fragments with different host organisms

Production milestone	Recent advances					
	Mammalian cells	Refs	Yeasts	Refs	*Escherichia coli*	Refs
Stable and efficient expression system	Site-specific homologous recombination Vector engineering and marker attenuation Expression of anti-apoptotic genes	[73] [74,75] [76]	Targeted gene integration Concomitant expression of several genes Co-expression of chaperones Reduction of proteolysis Over-expression of Hsf	[15] [17] [34] [21,35] [22]	Plasmid-free expression system Marker-free selection system Co-expression of chaperones	[41] [42] [44]
Clone selection	Robotics and fluorescence-activated cell sorting	[75]	Targeted gene integration Optimization of codons, gene copy number, and promoters	[15] [77]	Not an issue	
Disulfide bridges	Intrinsic feature of the ER		Intrinsic feature of the ER		Transport to the periplasm	[46]
Product secretion	Intrinsic feature		Over-expression of SNAREs Mutation studies on MFα1 System biological analysis	[20] [78] [77]	Transport to the periplasm	[46]
Chemically defined medium (CFD)	Serum-free CFD	[56]	Already applied		Already applied	
Efficient bioprocess	Concentrated fed-batch strategy	[79]	Fully aerobic strain Dynamic processes	[23] [80]	Manipulating the native acetate formation pathway Engineering the glucose uptake system	[49] [50]

Trends in Biotechnology January **2014**, Vol. 32, No. 1 -
https://doi.org/10.1016/j.tibtech.2013.10.002

Biopharma therapeutics that are currently available according to the biopharma survey in March 2019.



J. Microbiol. Biotechnol. **2021**. 31(3): 349–357
https://doi.org/10.4014/jmb.1911.11066

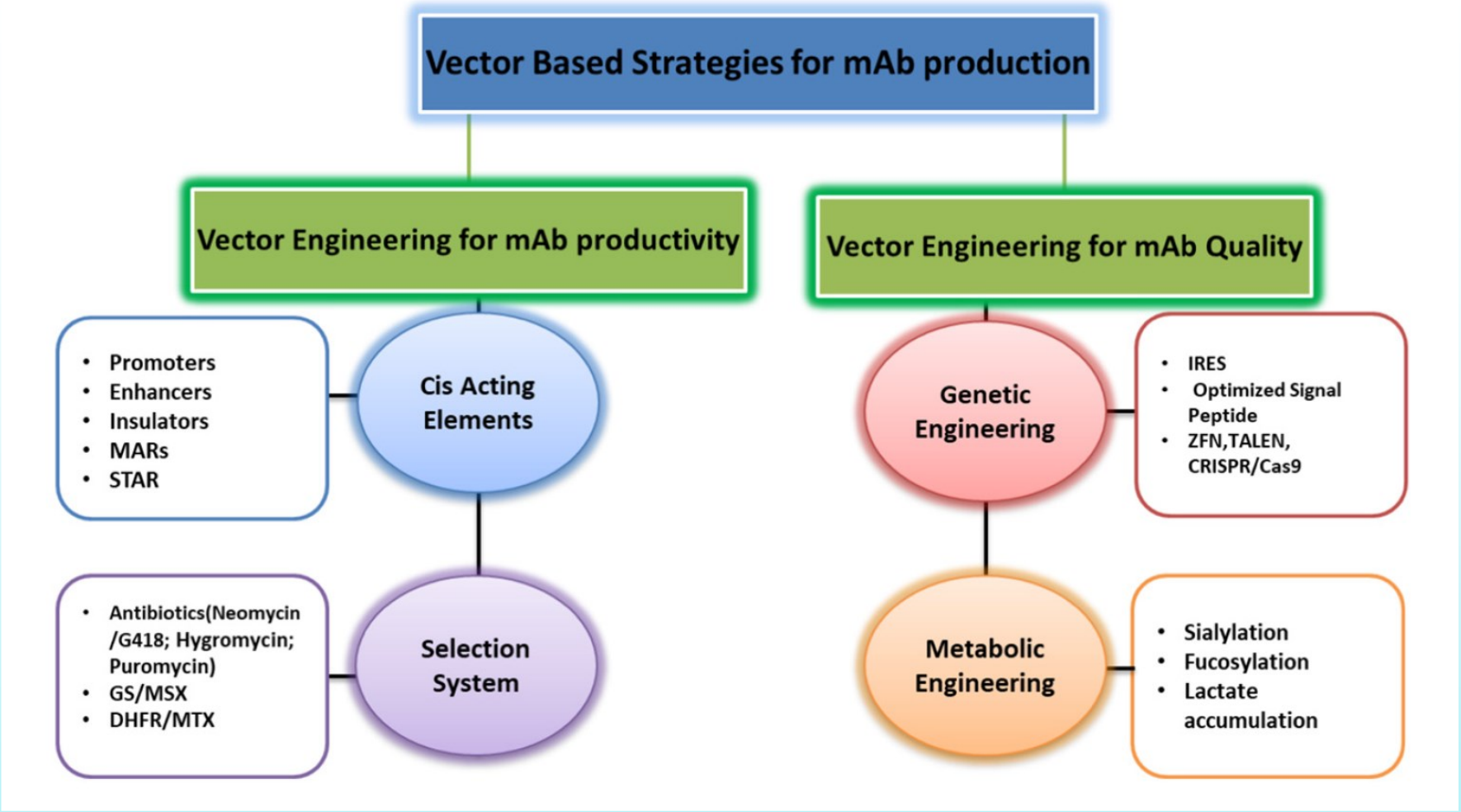
Monoclonal Ab Production method

Table 1. Monoclonal antibodies, source cell line and cultivation method.

Sl. No	Antibody	Host cell line	Culture system	Ref
1	RF-HBs-1(Anti HBs Ag mAb)	Hybridoma	Perfusion	[8]
2	CB72.3 Chimeric IgG4	GS-CHO 46	Fed Batch /Perfusion	[9]
3	Alemtuzumab(Anti CD 52 mAb)	CHO	Perfusion	[10]
4	Anti IL-8 mAb	CHO	Shake flask /wave bag	[11]
5	Anti-Salmonella Enteritidis O-Ag mAb	Hybridoma	Roller bottles/Stirred tank/ Disposable bioreactor	[12]
6	100F4 mAb (specific to HA protein of H1N1 influenza)	Drosophila Schneider 2	Perfusion	[13]
7	Anti enrofloxacin IgG1 mAb	Hybridoma	Batch/Fed Batch/Perfusion	[14]
8	CRL-1606 (Anti fibronection IgG mAb)	Hybridoma	Batch/Fed Batch	[15]
9	Anti digitoxin IgG	Hybridoma	Batch/perfusion	[16]
10	Anti CD22 IgG_2a	$SP_{2/0}$	Batch/Fed Batch/Perfusion	[17]

J. Microbiol. Biotechnol. 2021. 31(3): 349–357
https://doi.org/10.4014/jmb.1911.11066

Vector Based Strategies for enhancing mAb production



Vector engineering for mAb Productivity by:
Cis Acting elements which regulate the transgene expression,
selection system enhances cells constitutively and stably producing mAbs.

Vector engineering for mAb Product Quality by:
metabolic and genetic engineering approaches which optimize mAbs secretion, folding and glycosylation pattern of mAb.

Biotechnology Advances Volume 37, Issue 8, December 2019, 107415
https://doi.org/10.1016/j.biotechadv.2019.107415.

Phage Display Derived Monoclonal Antibodies: From Bench to Bedside

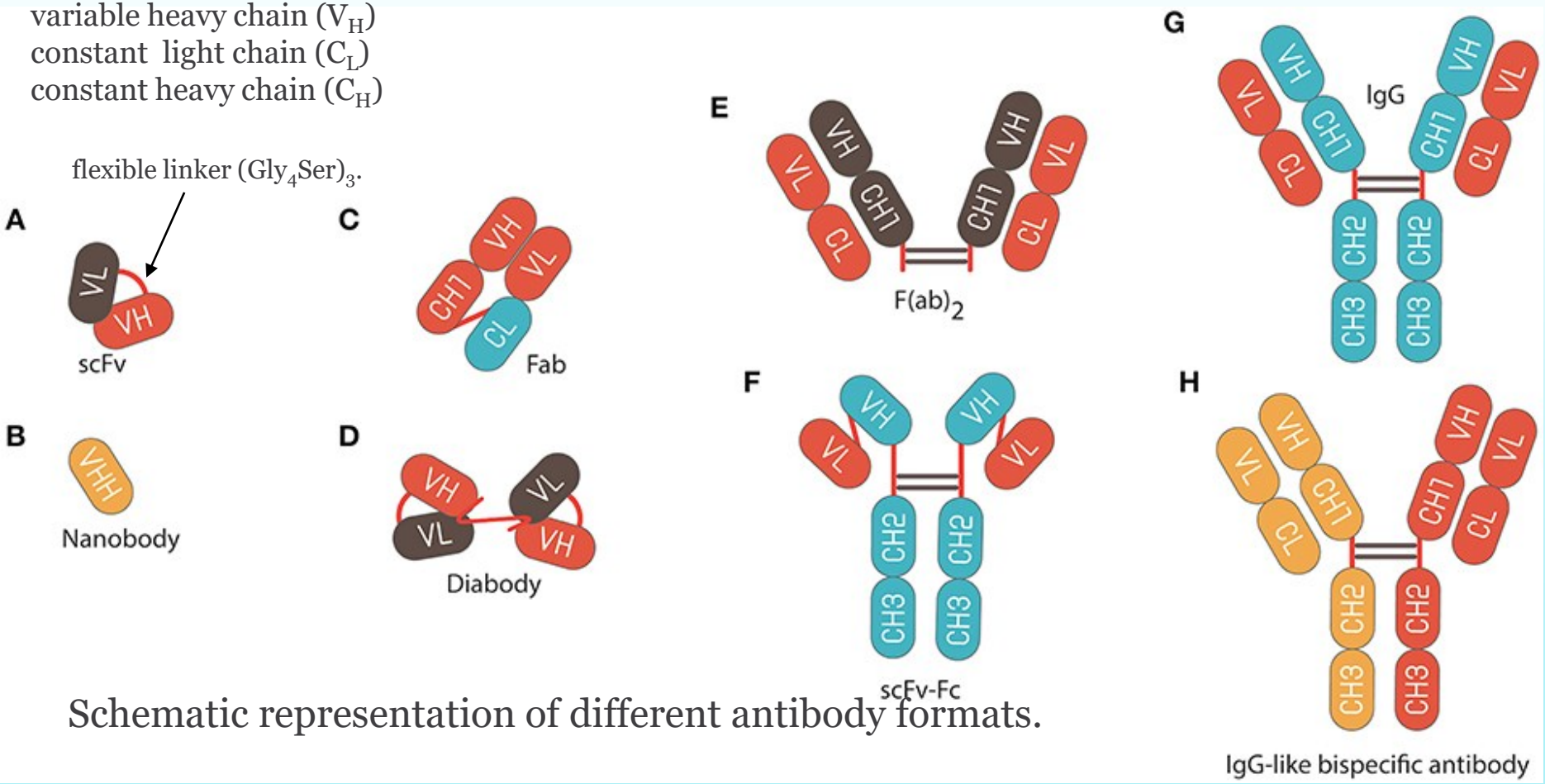
last 120 years, the research and development of antibody-related technologies.

- 1901, Emil von Behring won the first **Nobel Prize in Physiology or Medicine** for the successful therapeutic use of horse hyperimmune serum containing neutralizing polyclonal antibodies against diphtheria and tetanus toxins (12).
- 1984 Kohler and Milste received the **Nobel Prize in Physiology or Medicine** for developing the ground-breaking hybridoma technology which facilitated the isolation of mAbs and their subsequent production in laboratories (13).
- 2018, George P. Smith and Sir Gregory P. Winter were awarded with the **Nobel Prize in Chemistry** for their development of phage display of peptide and antibodies (14–16).
  2018 James P. Allison and Tasuku Honjo were honored by the **Nobel Prize in Physiology or Medicine** for their discoveries of cancer immunotherapy via the use of antibody blockade of the T-cell inhibitory receptor (CTLA-4) and programmed cell death protein 1 (PD1) to enhance anti-tumor immune responses (17, 18).

Front. Immunol., 28 August 2020 | https://doi.org/10.3389/fimmu.2020.01986

Phage Display Derived Monoclonal Antibodies: From Bench to Bedside

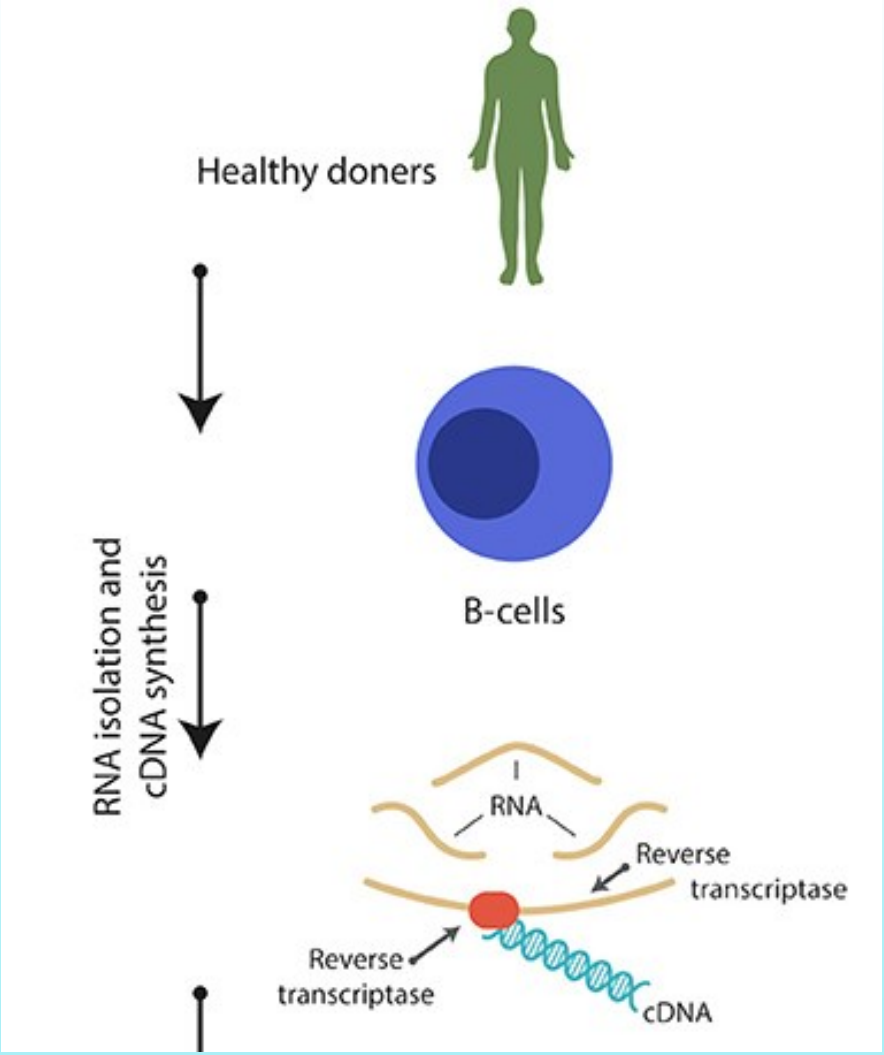
Single chain fragment variable (scFv)
variable light chain (V_L)
variable heavy chain (V_H)
constant light chain (C_L)
constant heavy chain (C_H)



Schematic representation of different antibody formats.

Front. Immunol., 28 August 2020 | https://doi.org/10.3389/fimmu.2020.01986

Phage Display Derived Monoclonal Antibodies: From Bench to Bedside



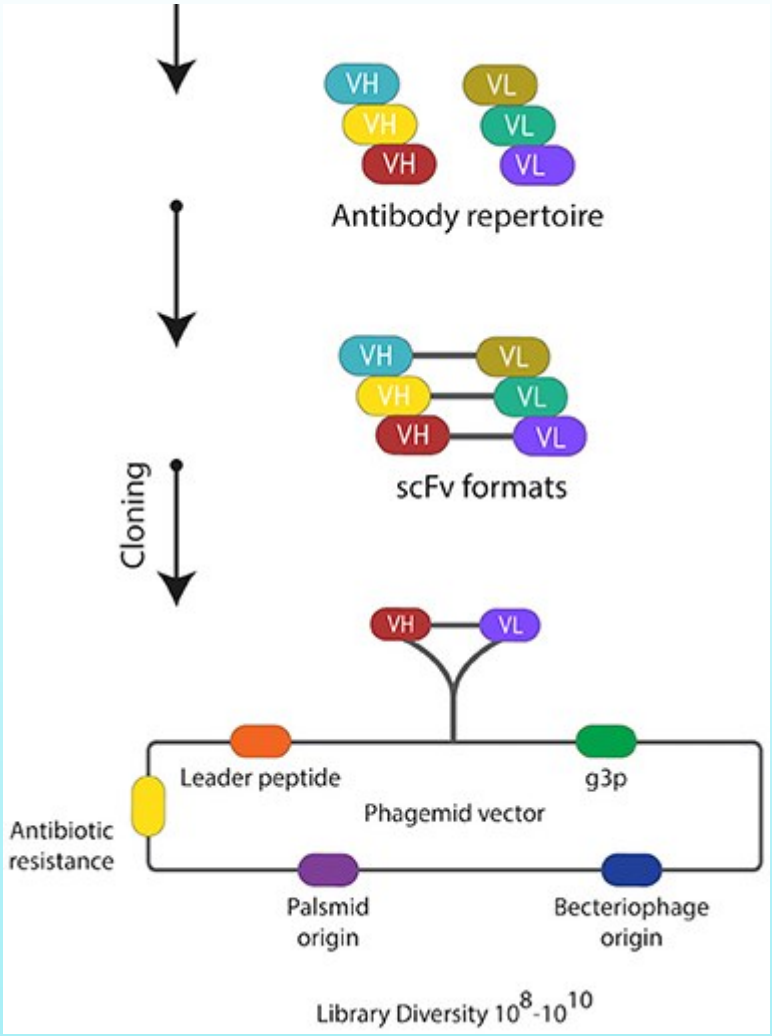
Strategy used for construction of naïve scFv-phage display libraries. (I)

Total RNA is isolated from B-lymphocytes from non-immunized healthy donors.

Then cDNA is synthesized from the isolated RNA using reverse transcriptase enzyme.

Front. Immunol., 28 August 2020 | https://doi.org/10.3389/fimmu.2020.01986

Phage Display Derived Monoclonal Antibodies: From Bench to Bedside



Strategy used for construction of naïve scFv-phage display libraries. (II)

Then the repertoire of the V_H and V_L genes is amplified from the cDNA using forward and reverse primers hybridizing to the variable domains.

scFvs are constructed and cloned into phagemid vector and a naïve phage library of 10^8-10^{10} is usually generated.

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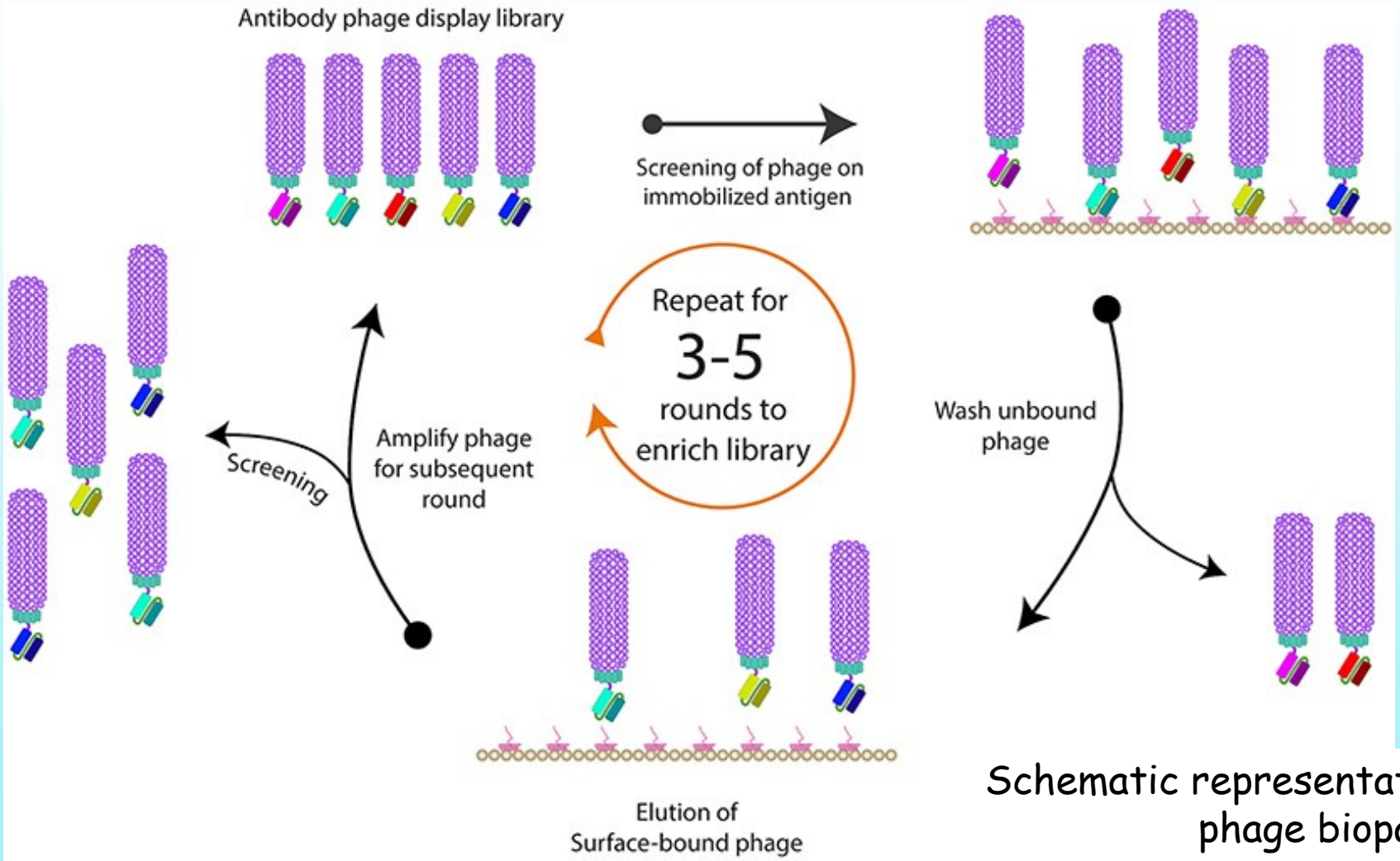
Phage Display Derived Monoclonal Antibodies: From Bench to Bedside



Schematic representation of phage biopanning.

Front. Immunol., 28 August 2020 | https://doi.org/10.3389/fimmu.2020.01986

Obstacles to the use of monoclonal antibodies in cancer treatment

- Antigen distribution of malignant cells is highly heterogeneous, so some cells may express tumor antigens, while others do not.
- Tumor blood flow is not always optimal
- High interstitial pressure within the tumor can prevent the passive monoclonal antibody from binding.

Biophysical and Biochemical Characteristics of Therapeutic Proteins

PROTEIN STRUCTURE

- **Primary Structure**
- **Secondary Structure**
  (α-Helix; β-Sheet; β-turn; Loops and turns)
- **Tertiary Structure and Quaternary Structure (Forces)**
  → Hydrogen Bonds - Electrostatic Interactions - Hydrophobic Interactions - Van der Waals Interactions
- **Hydration** (Water molecules are bound to proteins internally and externally)

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https://doi.org/10.1007/978-3-030-00710-2 -

Biophysical and Biochemical Characteristics of Therapeutic Proteins

PROTEIN STRUCTURE

- **Post-translational Modifications**
  → **Glycosylation:**
  - Mammalian cells have an advantage over yeast or plant production cells.
  - The sugar chains produced in yeast are rich in mannose; their **sialic acid content is low**.
    → increase the clearance rate of these glycoproteins upon injection.
  - Plant-derived glycoproteins are rich in fucose and xylose levels; **low sialic acid content**.
    → increase immunogenicity.
    → affects the pharmacokinetic profile, i.e., accelerated clearance from the blood.
- **PROTEIN FOLDING**
  → exposition a cluster of hydrophobic groups to those of other polypeptide chains
  → resulting intermolecular aggregation.
  High efficiency in the recovery of native protein depends to a large extent on how this **aggregation of intermediate forms is minimized.**
  The use of chaperones or polyethylene glycol has been found quite effective for this purpose

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Production and Purification of Recombinant Proteins

Expression Systems (General Considerations)

- **Expression systems include pro- and eukaryotic cells** (bacteria, yeast, fungi, plants, insect cells, mammalian cells) and **transgenic animals**.

- **The choice determined by:**
  the nature and origin of the desired protein,
  the intended use of the product,
  the amount needed, and the cost.

The regulatory agencies both in Europe (EMA: European Medicines Agency) and in the United States of America (FDA: Food and Drug Administration) play a pivotal role in providing legal requirements and guidelines

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Production and Purification of Recombinant Proteins

Protein feature	Prokaryotic Bacteria	Eukaryotic yeast	Eukaryotic mammalian cells	Eukaryotic plant cells	Transgenic animals
Concentration	High	High	High	Low	Medium-High
Molecular weight	Low	High	High	High	High
S-S bridges	Limitation	No limitation	No limitation	No limitation	No limitation
Secretion	No	Yes/no	Yes	Yes/no	Yes
Aggregation state	Inclusion body	Singular, native	Singular, native	Singular, native	Singular, native
Folding	Risk of misfolding	Correct folding	Correct folding	Correct folding	Correct folding
Glycosylation (human-like)	Limited	Limited	Possible	Limited	Possible
Contamination risk	Possible (endotoxin)	Low	Possible (virus, prion, oncogenic DNA)	Low	Very possible (virus, prion, endotoxin)
Cost to manufacture	Low	Low	High	High[a]	Medium-high

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Production and Purification of Recombinant Proteins

Expression Systems

- *Transgenic Animals*
  Foreign genes can be introduced into animals like mice, rabbits, pigs, sheep, goats, and cows through nuclear transfer and cloning techniques.
  → Using milk-specific promoters, the desired protein can be expressed in the milk of the female offspring. **During lactation the milk is collected**, the milk fats are removed, and the skimmed milk is used as the **starting material for the purification of the protein**.
  - **Advantage** of this technology is the relatively cheap method to produce the desired proteins (e.g. cow)
  - **Disadvantages** are the long lead time to generate a herd of transgenic animals and concerns about the health of the animal, food safety and ethics
    - Some proteins expressed in the mammary gland leak back into the circulation and cause serious negative health effects.

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Production and Purification of Recombinant Proteins

Expression Systems

Transgenic Animals

→ The purification strategies and purity requirements for proteins from milk can be different from those derived from bacterial or mammalian cell systems.

→ the transgenic milk also contains significant amounts of the nonrecombinant counterpart. To separate these closely related proteins poses a purification challenge.

→ The "contaminants" in proteins for oral use expressed in milk that is otherwise consumed by humans are known to be safe for consumption.

The FDA and EMA approved recombinant protein:

→ **antithrombin III (ATryn®, GTC Biotherapeutics)** - produced in the milk of transgenic goats

→ **human C1 esterase inhibitor (Ruconest®, Pharming Group N.V.)** - produced in the milk of transgenic rabbits

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Production and Purification of Recombinant Proteins

Expression Systems

Plants

Therapeutic proteins can also be expressed in plants

→ These are economically feasible has yet to be established.

But → The lack of genetic stability of plants was sometimes a drawback.

→ Stable expression of proteins in edible seeds has been obtained.

→ For Oral therapeutics or vaccines might be the ideal solution to produce large amounts of cheap therapeutics → the "contaminants" are known to be safe for consumption.

But → Challenges are the presence of high endotoxin levels, a relatively low expression level of the product, and secretion of proteases limiting the shelf life of plant extracts

Biosafety concerns (such as pollen contamination and immunogenicity of plant-specific glycans) and costly downstream extraction and purification requirements,

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Production and Purification of Recombinant Proteins

Human proteins that have been studied in **transgenic plants** is provided to show the variety of what may become a viable technology in the future

Species	Protein product	Potential indication(s)
Tobacco	IgG	Systemic therapy (rabies virus, hepatitis B virus)
Tobacco	TGF-β2	Ovarian cancer
Tobacco	Vitronectin	Protease
Tobacco	RhinoR	Fusion of human adhesion protein and human IgA for common cold
Tomato Tomato Safflower	Beta-amyloid Vaccines Insulin	Study of Alzheimer's disease Infectious disease Diabetes
Carrot Corn	DTP subunit vaccine Meripase	Infectious disease Cystic fibrosis
Cherry Duckweed	Hep B surface antigen Lacteron	Hep B vaccine production Controlled release of α-interferon for hepatitis B and C
Potato	Poultry vaccine	Avian influenza (H5N1)

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Production and Purification of Recombinant Proteins

Expression Systems

Expression systems eukaryotic cells **commonly used:**

- Chinese Hamster Ovary cells (CHO),
- Immortalized human embryonic retinal cells (PER.C6® cells),
- Baby hamster kidney cells (BHK),
- lymphoblastoid tumor cells (interferon production),
- melanoma cells (plasminogen activator),
- hybridized tumor cells (monoclonal antibodies).

→ The cell culture has to be free from undesired microorganisms that may destroy the cell culture or present hazards to the patient by producing endotoxins.

NOTE: To prepare the **final medium, components are dissolved in purified water before sterilization**. The preferred method for sterilization is heat (≥15 min at 121 °C).
However, **most components used in the cell culture medium can not be sterilized by heat**, therefore filtration is used.
→ the **medium is filtrated** through 0.1 µm (to prevent mycoplasma and bacterial contamination) or 0.2 µm filters (to prevent bacterial contamination).

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Antibody Conjugation of Nanoparticles as Therapeutics for Breast Cancer Treatment

Challenges for Bringing ACNPs to the Clinic_

- ACNPs have failure in clinical translation.
- Lack of knowledge about the interaction between nanocarriers and biological systems,
- Poor tumor accumulation, inadequate pharmacokinetics, the safety issue of raw materials for NPs generation, and limited number of reported in vivo studies remain limitations to upgrade ACNP to the clinic.
- Not sufficient understanding about the interaction between NPs with biological macrostructures—even more so when referring to ACNPs.

Int. J. Mol. Sci. **2020**, 21, 6018; doi:10.3390/ijms21176018

Antibody–drug conjugates (ADCs) are a new emerging class

- Antibody–drug conjugates (ADCs) are a targeted therapeutics with the potential to improve the therapeutic index seen with traditional chemotherapy.

- ADC consists of three components:
  - a monoclonal antibody or antibody fragment (which targets a tumor-associated antigen);
  - a highly cytotoxic entity;
  - a chemical linker to conjugate the first two components.

https://www.genengnews.com/topics/drug-discovery/overcoming-challenges-in-the-development-of-anticancer-adcs-2/?utm_medium=newsletter&utm_source=GEN+Tech+Focus&utm_content=01&utm_campaign=GEN+Tech+Focus_20201008&oly_enc_id=8919F0564989J5C

Antibody–drug conjugates (ADCs) are a new emerging class

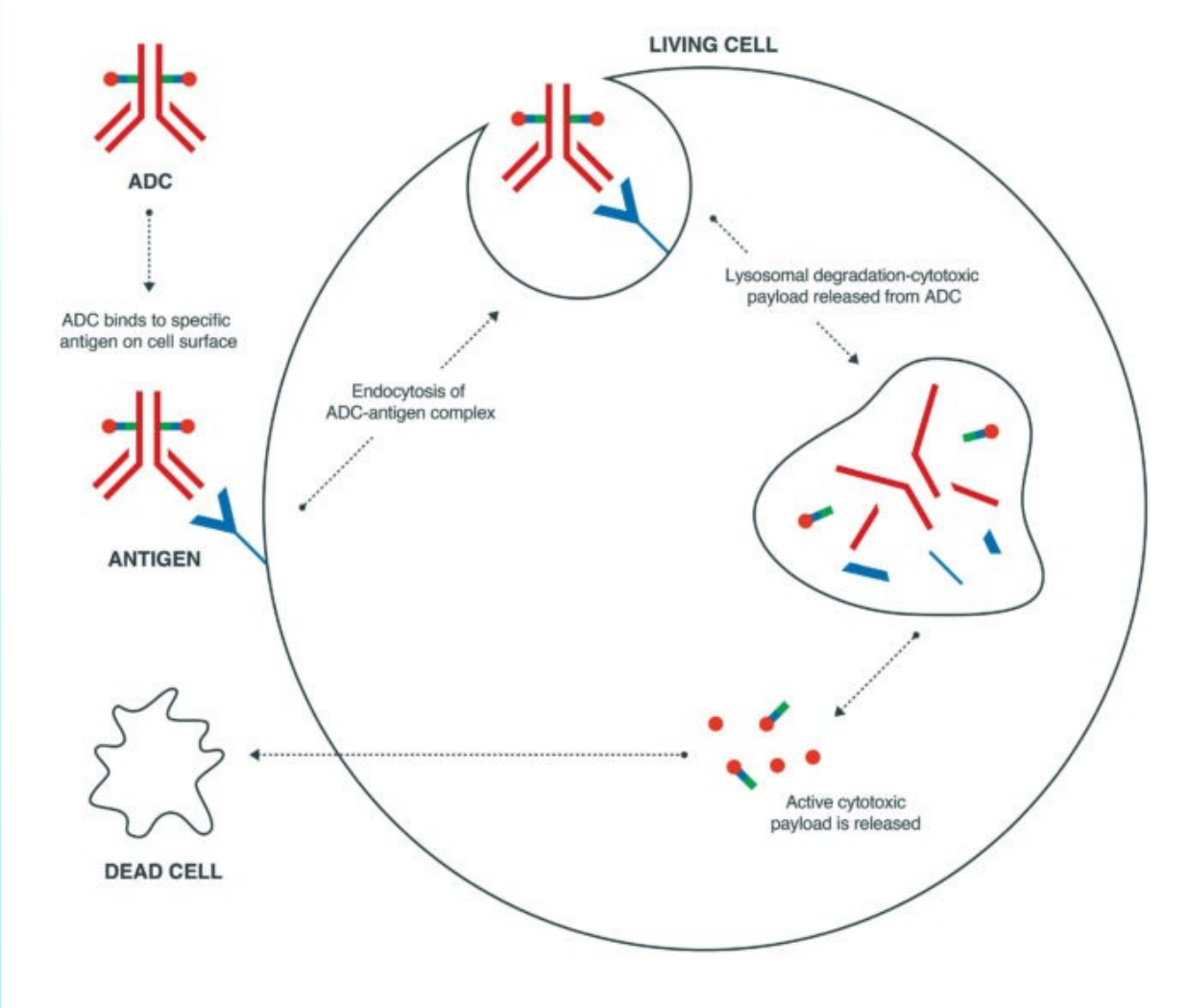
- Antibody–drug conjugates (ADCs) are a targeted therapeutics

- Binding of the antibody to the cell surface triggers internalization, and processing within endosomes or lysosomes releases the potent cell-killing molecule.

- ADC's mechanism of action combines the targeting power of an antibody with a potent cytotoxic agent, making it possible to eradicate cancer cells more effectively and selectively, while reducing the side effects which undermine patient quality of life (*Figure 1*).

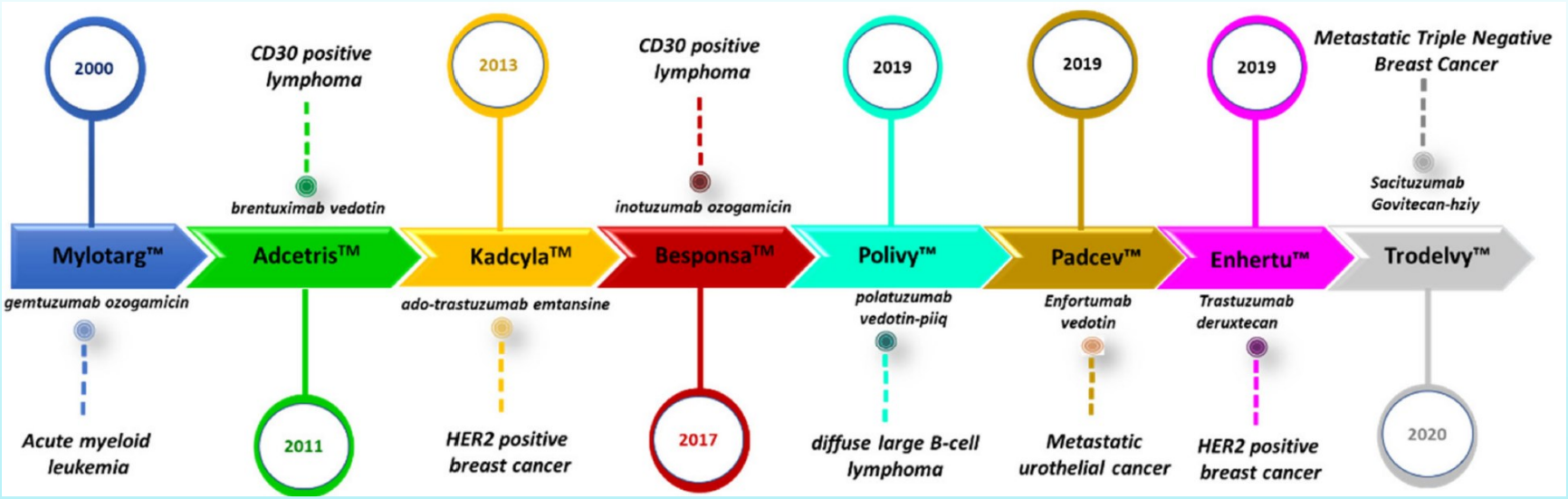
https://www.genengnews.com/topics/drug-discovery/overcoming-challenges-in-the-development-of-anticancer-adcs-2/?utm_medium=newsletter&utm_source=GEN+Tech+Focus&utm_content=01&utm_campaign=GEN+Tech+Focus_20201008&oly_enc_id=8919F0564989J5C

Antibody-drug conjugates (ADCs) are a new emerging class



https://www.genengnews.com/topics/drug-discovery/overcoming-challenges-in-the-development-of-anticancer-adcs-2/?utm_medium=newsletter&utm_source=GEN+Tech+Focus&utm_content=01&utm_campaign=GEN+Tech+Focus_20201008&oly_enc_id=8919F0564989J5C

Antibody Conjugation of Nanoparticles as Therapeutics for Breast Cancer Treatment



Antibody-drug conjugates (ADCs) approved by the Food and Drug Administration (FDA).

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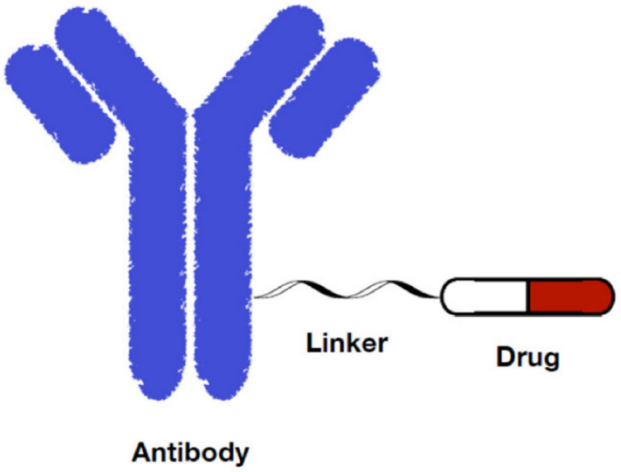
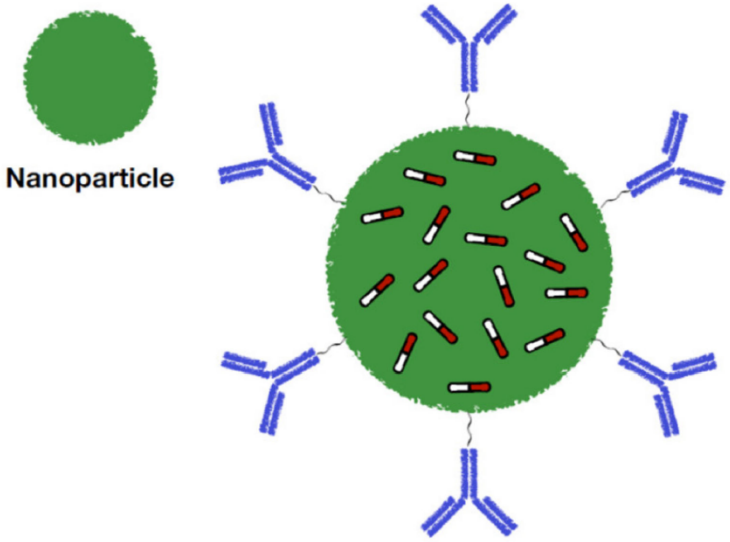
Antibody Conjugation of Nanoparticles as Therapeutics for Breast Cancer Treatment





ADC

- ✓ Linkers reduce payload potency
- ✓ Approx 4:1 for optimal balance between cytotoxicity and pharmacokinetic profiles
- ✓ Internalization of AB and linked drug at the tumor cell requires complex linker chemistry
- ✓ ADCs can only link to one drug unless using branched linkers

« *Payload modulation* *Drug to Antibody Ratio (DAR)* *Drug release profiles* *Multivalency effects* »

ACNP

- ✓ No linker required; not payload affected
- ✓ Easily modulable to ensure internalization of much higher concentrations of drug
- ✓ Simply a consequence of drug diffusion and particle degradation
- ✓ Multi-conjugation to NP surface and induction of hyperclustering of receptors

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Antibody Conjugation of Nanoparticles as Therapeutics for Breast Cancer Treatment

Conjugation Strategies for ACNPs Generation

NPs can be functionalized with antibodies or antibody fragments by adsorption or covalent binding.

- Immobilization of the desired antibodies with their correct orientation.
- The coupling method must maintain the biological activity of the antibody.
- Adsorption is a non-covalent immobilization strategy includes physical adsorption (electrostatic, hydrogen binding, hydrophobic and van derWaals attractive forces) and ionic binding.
- Covalent binding requires prior activation of the nanoparticle
  - carbodiimide chemistry (major disadvantage of lacking control over antibody orientation onto the nanoparticle surface)
  - Maleimide chemistry (binding through sulfhydryl groups of antibodies → groups are not as abundant as primary amines in the antibody structure and the incorporation of free sulfhydryl groups is required).
  - "Click chemistry" chemical reactions provide orthogonality, site-specificity, and a favorable reaction rate.
- Most covalent strategies produce low coupling efficiency and randomly oriented antibodies.

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Antibody Conjugation of Nanoparticles as Therapeutics for Breast Cancer Treatment

Conjugation Strategies for ACNPs Generation

- Non-covalent approaches using adapter biomolecules can provide orientation of the immobilized antibodies on the NP surface.

- The most relevant binding strategy with adapter molecules exploits biotin–avidin interaction as the strongest non-covalent biological interaction between a protein and a ligand.

- The most common approach using biotin–avidin interaction implies chemical of the antibody with biotin (biotinylation) and functionalization of the nanoparticle with avidin or its derivatives

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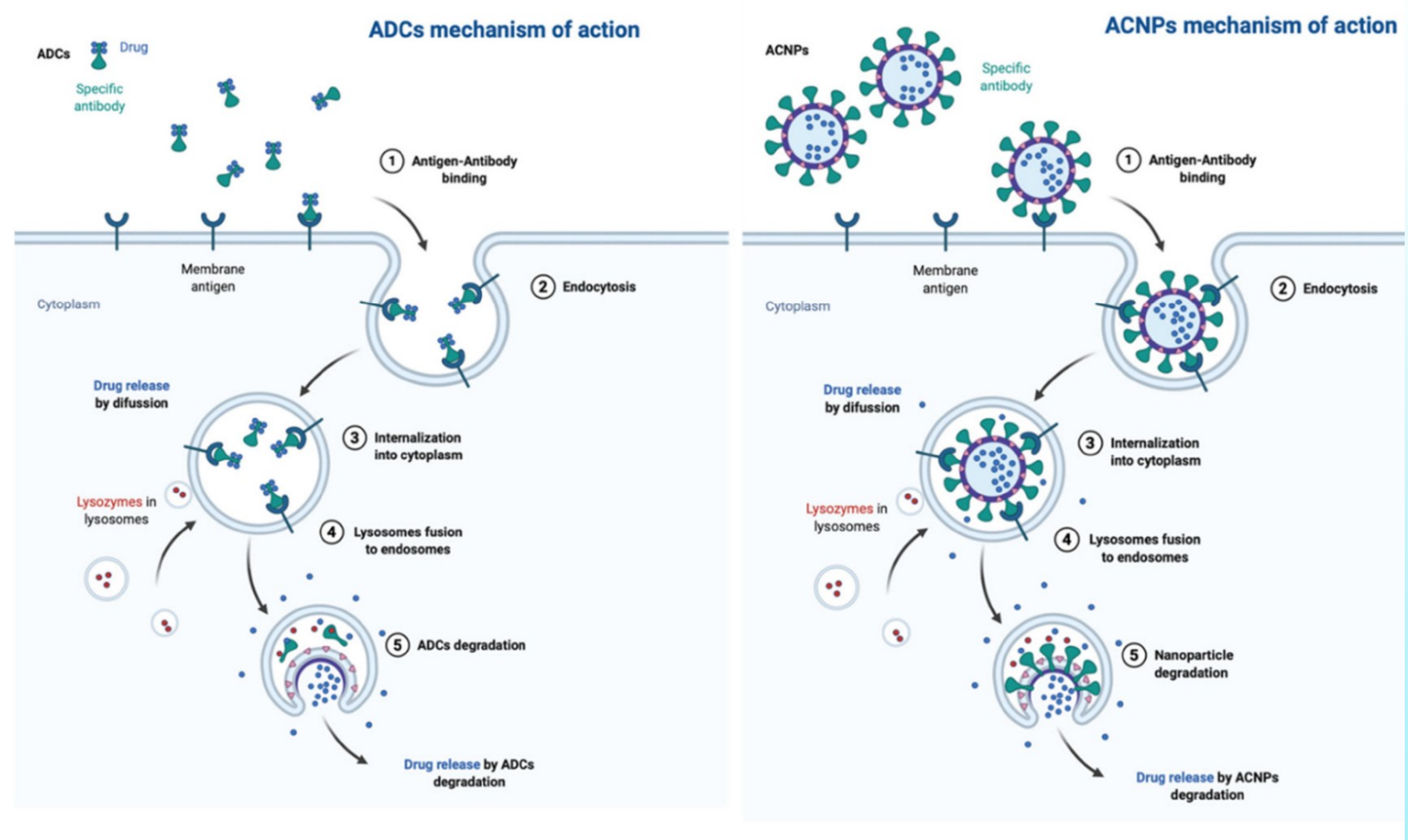
Antibody Conjugation of Nanoparticles as Therapeutics for Breast Cancer Treatment



Action mechanism of antibody-conjugated nanoparticles (ACNPs) and ADCs.

Int. J. Mol. Sci. **2020**, 21, 6018; doi:10.3390/ijms21176018

Antibody Conjugation of Nanoparticles as Therapeutics for Breast Cancer Treatment

Challenges for Bringing ACNPs to the Clinic

It is well known that the surface of the non-targeted NPs is quickly covered by serum proteins

→ implies important changes in NPs stability and metabolism

→ the influence of the size, shape, and surface charge of ACNPs are crucial to understand immune response and therefore facilitate better ACNPs design.

→ Further investigations and in vivo outcomes are required to determine the effects of NPs shape, size, and surface charge on cellular uptake.

NPs must prevent the mononuclear phagocyte system to increase circulating time.

→ the grafting of polyethylene glycol (PEG) to the nanoparticle surface as an adequate strategy for cellular internalization-

→ have been explored the use of protein and cell membrane coatings.

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Enzyme-Linked ImmunoSorbent (ELISA)

indiretto

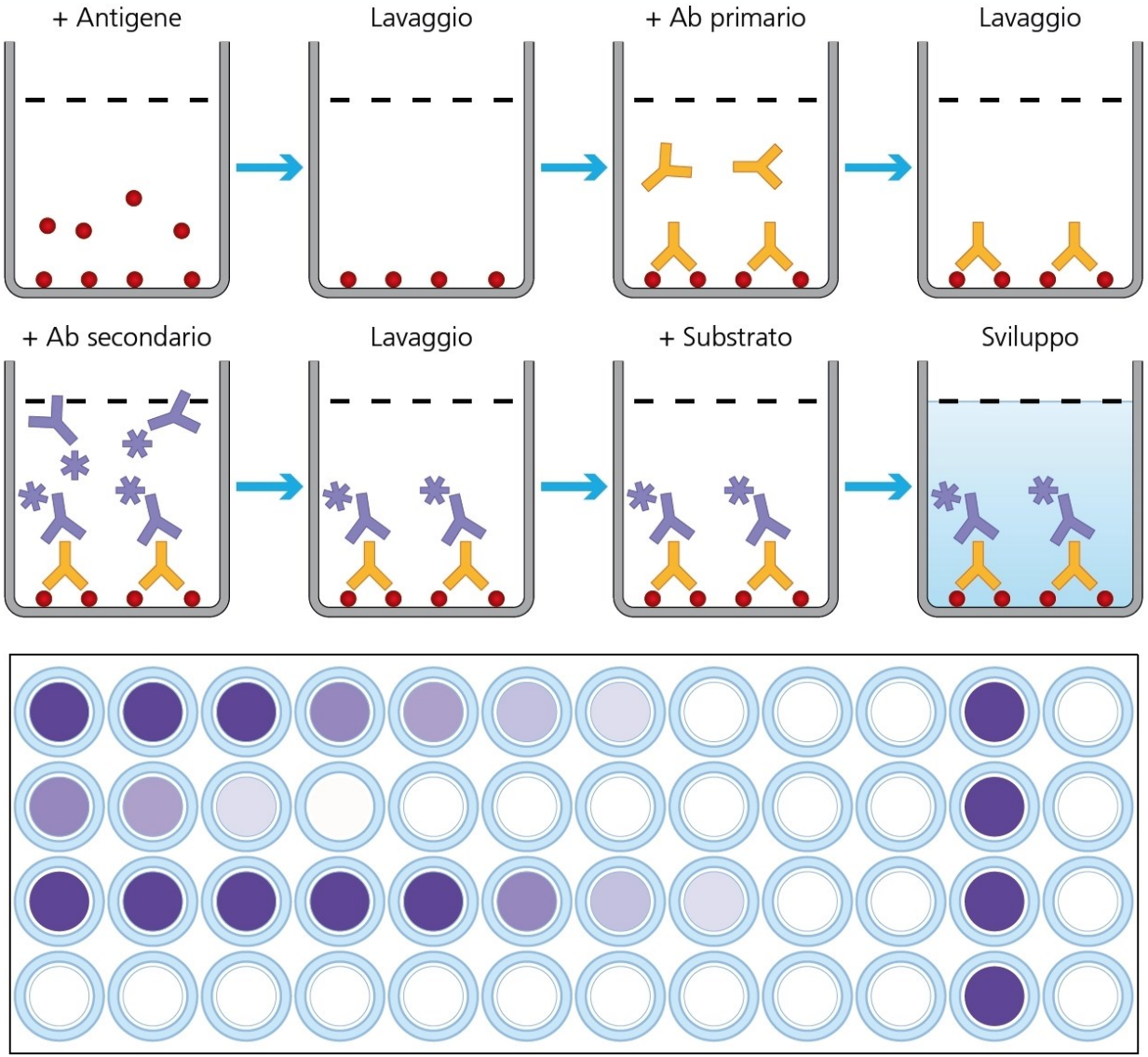
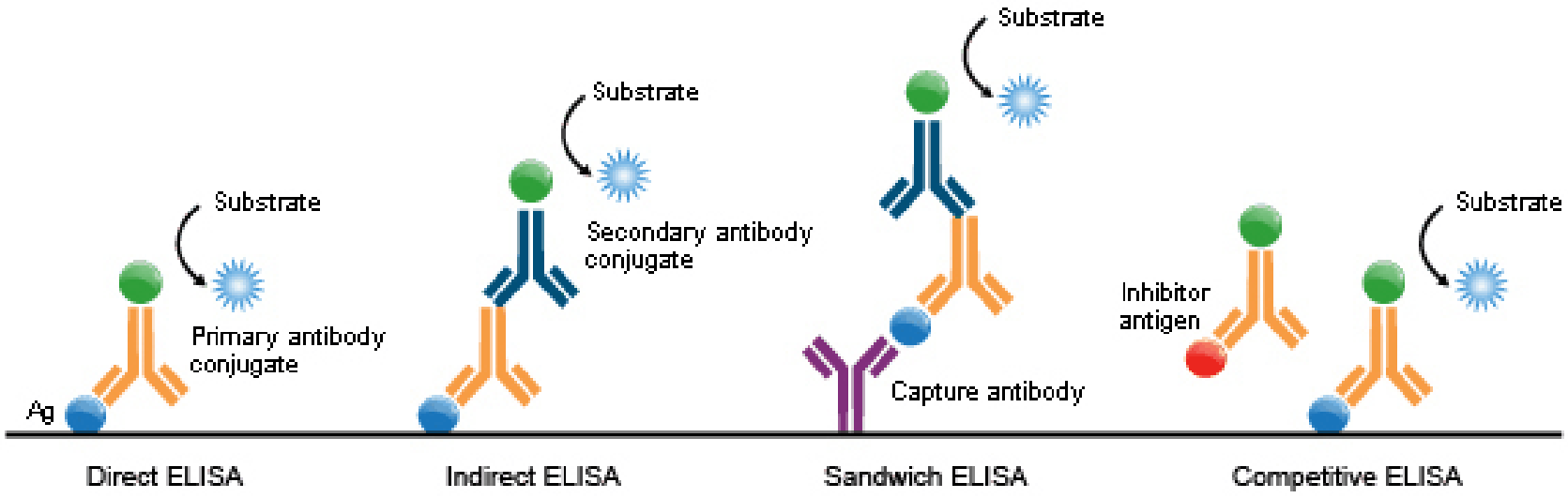


Figura 6.29
Schema di funzionamento di un saggio ELISA. La parte superiore della figura mostra i vari passaggi che sono descritti nel testo; la parte inferiore della figura mostra un esempio di risposta colorimetrica al saggio.

Enzyme-Linked ImmunoSorbent (ELISA)



http://www.abnova.com/support/

Surface plasmon resonance techniques

Based on the excitation of free electrons (called surface plasmons when excited)
by polarized light from a metal film at an interface with a medium having a different refractive index.

→ **Binding of molecules to this interfacial layer results in shifts in their reflection curves**.
→ refractive index changes are linearly proportional to the number of molecules bound,
→ technique can be used to calculate a number of binding parameters:
equilibrium association constant (KA), equilibrium dissociation constant (KD), as well as the concentration of a protein in solution.

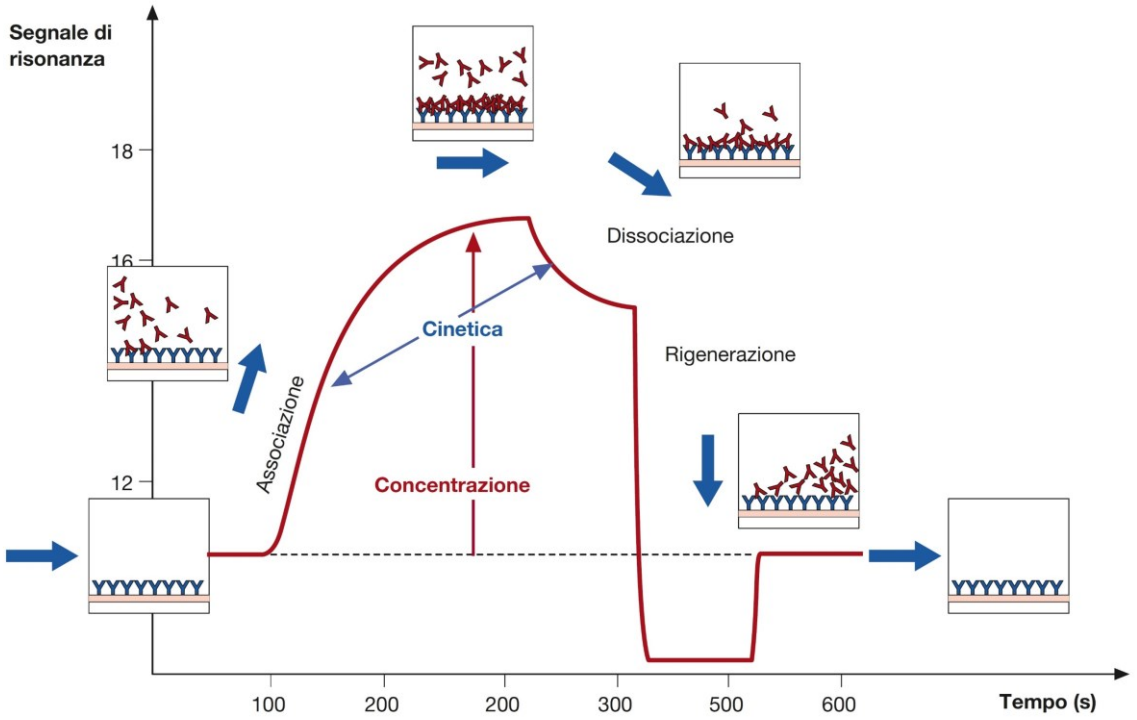


Figura 6.6 Saggio di risonanza plasmonica superficiale (SPR). Le due macromolecole in esame sono indicate dai simboli rossi e blu. La macromolecola con il simbolo blu (nell'esempio, DNA) è quella che viene legata alla superficie metallica (rosa), mentre la macromolecola con il simbolo rosso (nell'esempio, proteina) è quella che si trova in soluzione. Durante il passaggio di rigenerazione del biosensore, la molecola interagente (rossa) viene eliminata.

Protein Stability and Characterization

Other techniques:
SDS-PAGE

2-Dimensional Gel Electrophoresis and Differential Gel Electrophoresis

- Isoelectric focusing and SDS-PAGE can be combined

→ proteins are first fractionated by isoelectric focusing based upon their pI values.

→ After are then subjected to SDS-PAGE fractionated based on the molecular weights.

BIOLOGICAL ACTIVITY (POTENCY) ASSAYS/ BIOASSAYS

- **Binding Assays (ELISA),**
- **Surface Plasmon Resonance (SPR)**

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