Hybridoma Technology and Monoclonal Antibodies

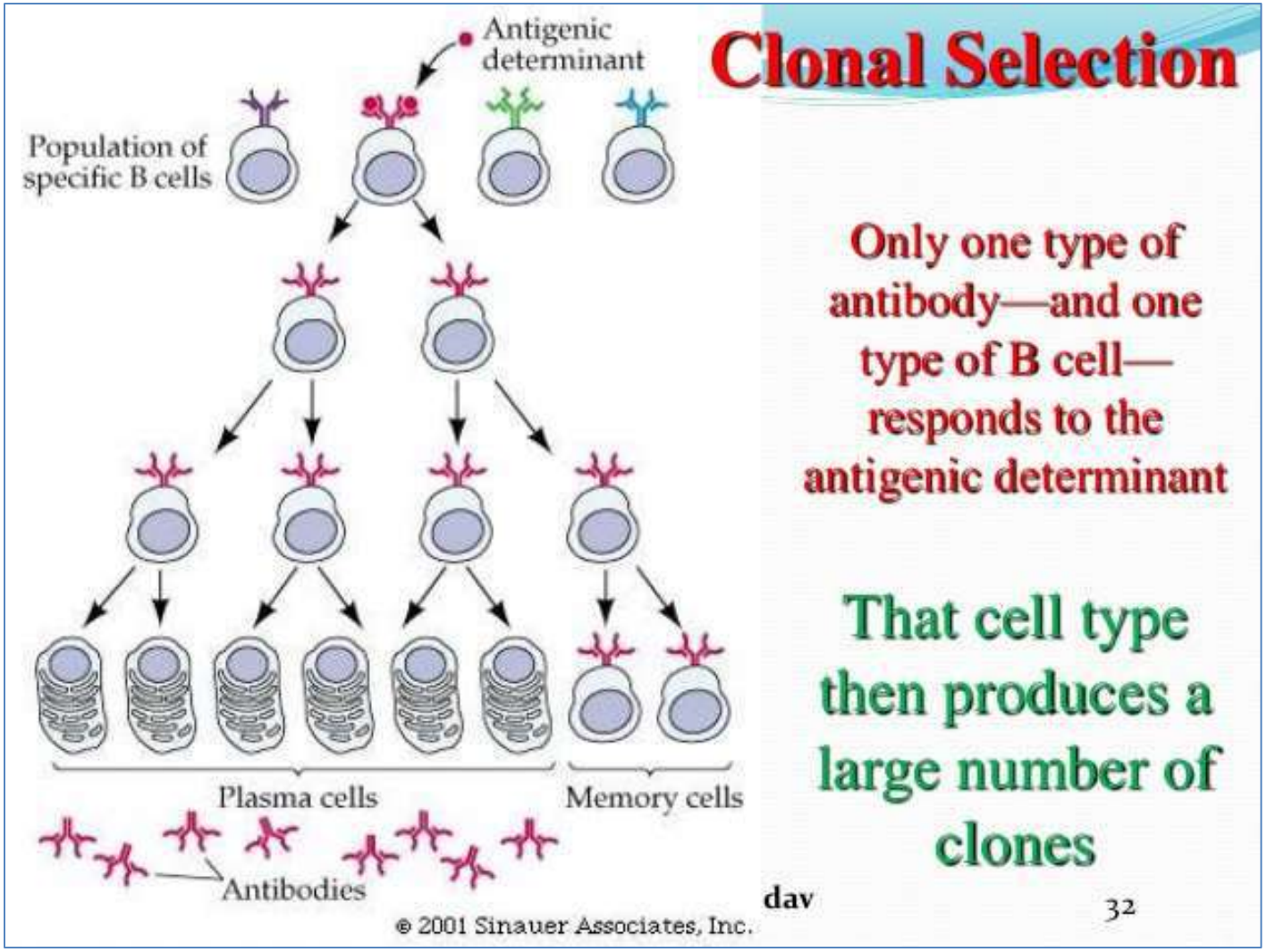
RAKESH SHARDA

Department of Veterinary Microbiology

NDVSU College of Veterinary Science & A.H.,

MHOW

Theory of Clonal Selection



What is a clone?

A population of cells derived from a single progenitor cell.

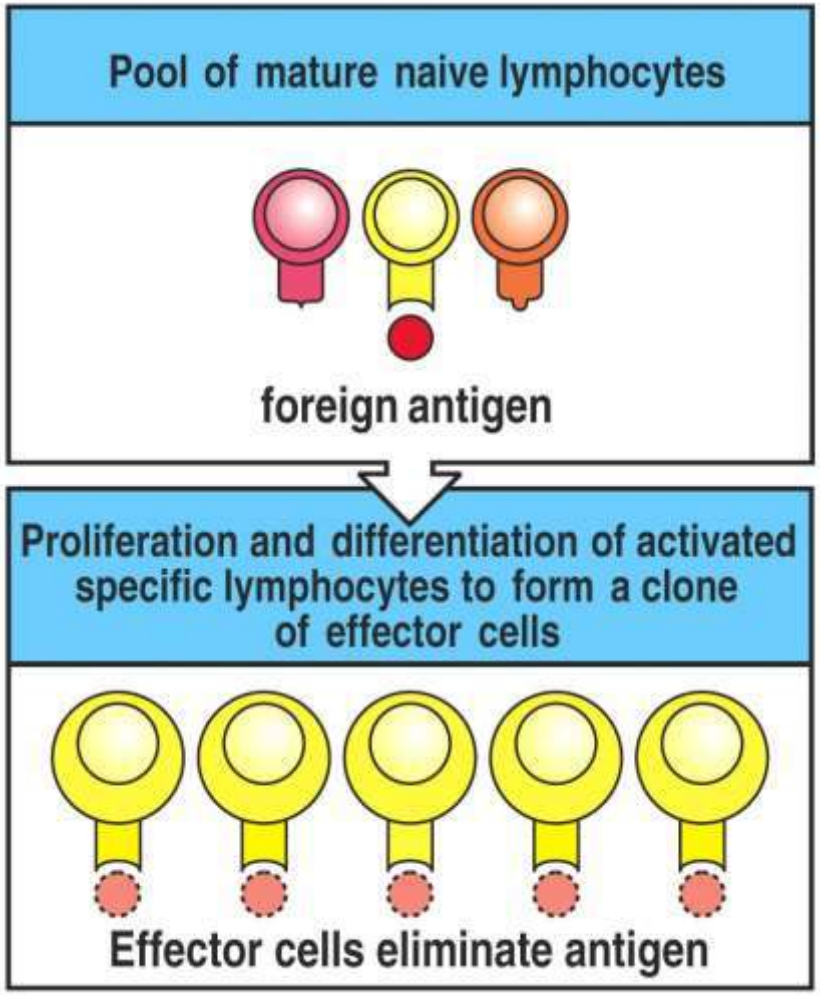


Figure 1-14 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)

The adaptive immune system works by clonal selection

Antigen triggers the expansion of limited number of B cell clones that are specific for that particular antigen

Antibodies produced by a single clone of B cell are called as monoclonal antibodies

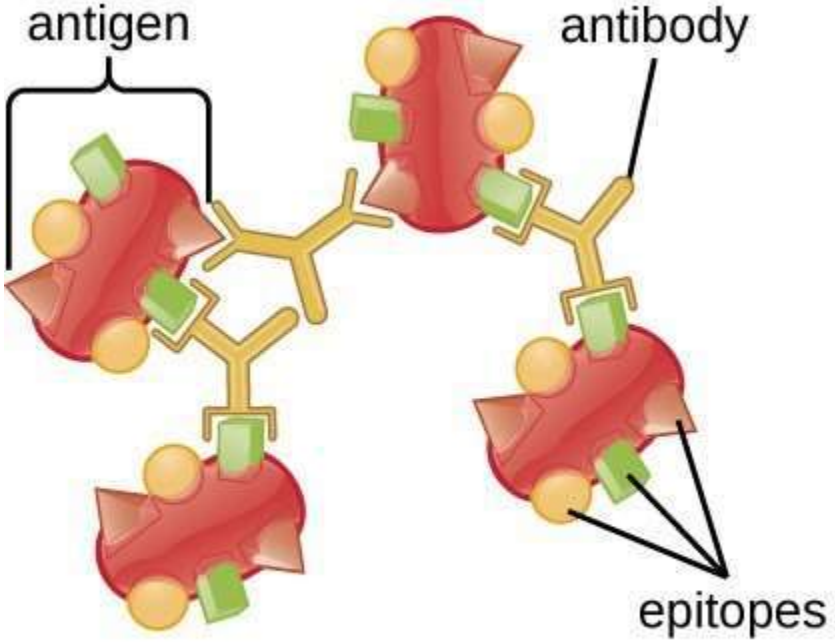
What are Monoclonal Antibodies?

- The antibodies of uniform specificity produced by a clone of a B cell against a single antigenic determinant or a hapten are called as **Monoclonal Antibodies (mAbs)**.
- Monoclonal antibodies are produced by **Hybridoma Technology** developed by Kohler and Milstein in 1975 at Oxford University
- On contrary, sum of total antibodies produced by clones of different B cells against all antigenic determinants of a multivalent antigen are called as **polyclonal antibodies**.
- Following natural infection or vaccination against a pathogen, polyclonal antibodies are produced *in vivio,* which are measured quantitatively by various serological tests.

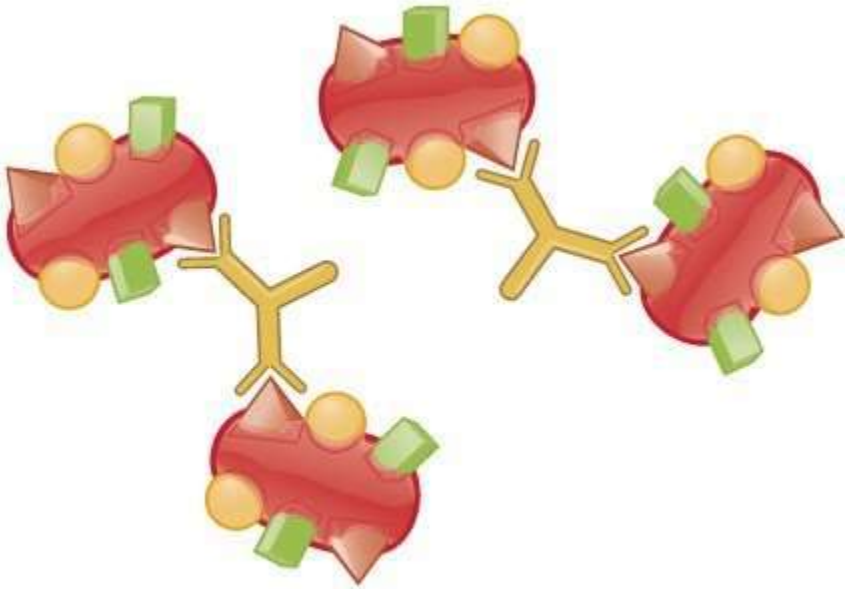
Polyclonal antibodies vs Monoclonal antibodies

Polyclonal antibodies: antibody preparations from immunized animals. Consist of complex mixtures of different antibodies produced by many different B cell clones

Monoclonal Antibodies: homogeneous antibody preparations produced in the laboratory. Consist of a single type of antigen binding site, produced by a single B cell clone



polyclonal antiserum



monoclonal antibodies

ANTIBODIES

	Polyclonal antibodies	***Monoclonal Antibodies***
Produced by:	Many B cell clones	A single B cell clone
Bind to:	Multiple epitopes of all antigens used in the immunization	A single epitope of a single antigen
Antibody class:	A mixture of different Ab classes (isotypes)	All of a single Ab class
Ag-binding sites:	A mixture of Abs with different antigen-binding sites	All Abs have the same antigen binding site
Potential for cross-reactivity:	High	Low

Polyclonals vs. monoclonals

Polyclonal antibodies	Monoclonal antibodies
Produces large amounts of non specific antibodies	Can produce large amounts of specific antibodies but may be too specific
Recognizes multiple epitopes on any one antigen	Recognizes only one epitope on an antigen
Inexpensive to produce	Expensive to produce
Technology required is low	High technology required
Skills required are low	Training is required for the technology use
Time scale is short	Time scale is long for hybridomas
Can be batch to batch variability	Once a hybridoma is made it is a constant and renewable source and all batches will be identical

How to produce mAbs?

Hyper immunize a mice against an antigen

↓

Sacrifice mice ethically and collect spleen

↓

Sieve the spleen followed by trypsinization to obtain single B-cells

↓

Seed cells *in vitro* in a suitable culture medium in a 96-well plate by limiting dilution

↓

Examine wells showing single cell

↓

Screen supernatant from such wells for production of antibody of desired specificity

↓

Expand positive well

Problem: Splenic cells are mortal and will die soon due to apoptosis

Q - How to make spleen cells to continuously produce antibodies?

A - Hybridoma Technology

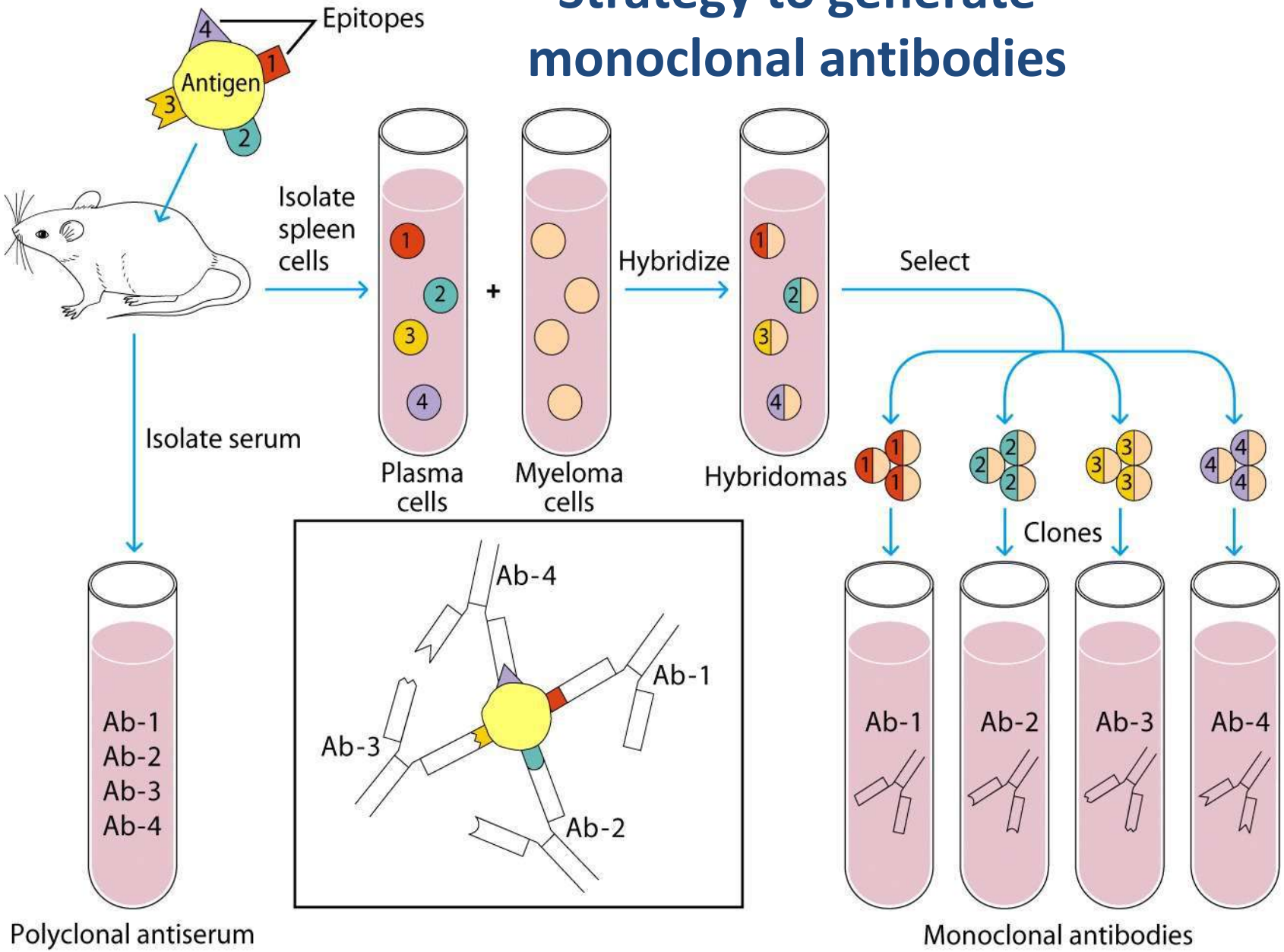
What is a hybridoma technology?

- A technique to generate continuous supply of monoclonal antibodies.

- **Hybridoma is a fusion of two cells – myeloma (plasma cell tumor) cells and splenic B cells.**

- Takes advantage of the unlimited growth capacity of myeloma cells and cellular machinery to produce antibodies of uniform antigenic specificity of B cells

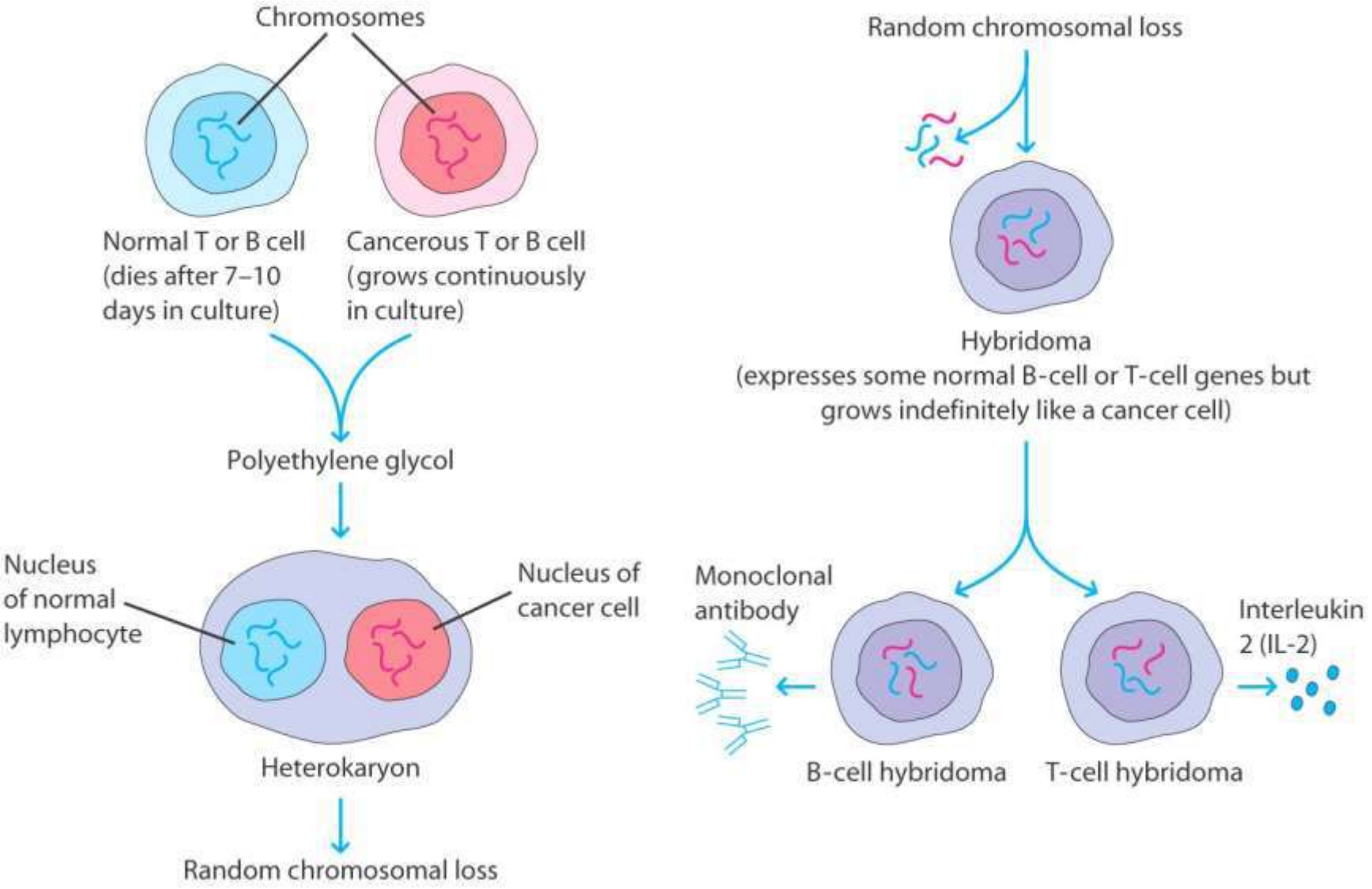
Strategy to generate monoclonal antibodies



Hybrid Cells

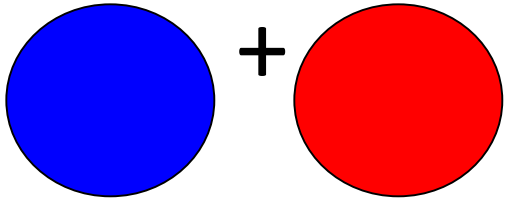
- Normal splenic cells are fused with a cancerous cell line
  - E.g. myeloma, lymphoma (NSO1)
- Fusion is accomplished with PEG (polyethylene glycol)
- The new hybrid cell exhibits properties of both cell types:
  - Unlimited growth (from myeloma cells)
  - Secretes monoclonal antibody (from splenic cells)
  - Or Secretes cytokines

Hybridomas are hybrids between a non-transformed antibody producing B cell and a transformed cell (myeloma) that can grow continuously in culture.

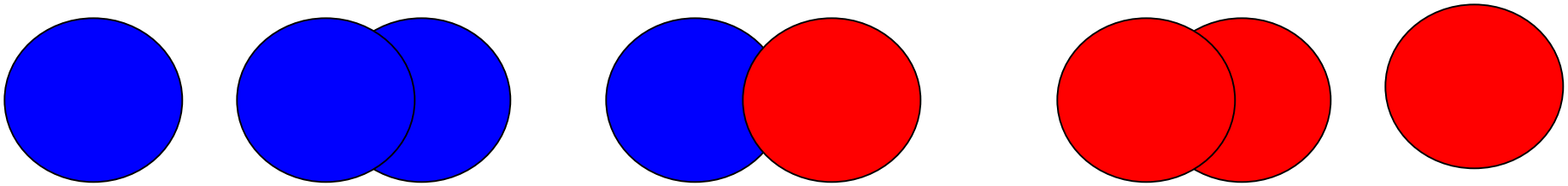


Possible Fusion Products

Plasma Cells From Immunized Animal



Myeloma cells



Unfused plasma cells

Fused plasma cells

Hybrid cells

Fused myeloma cells

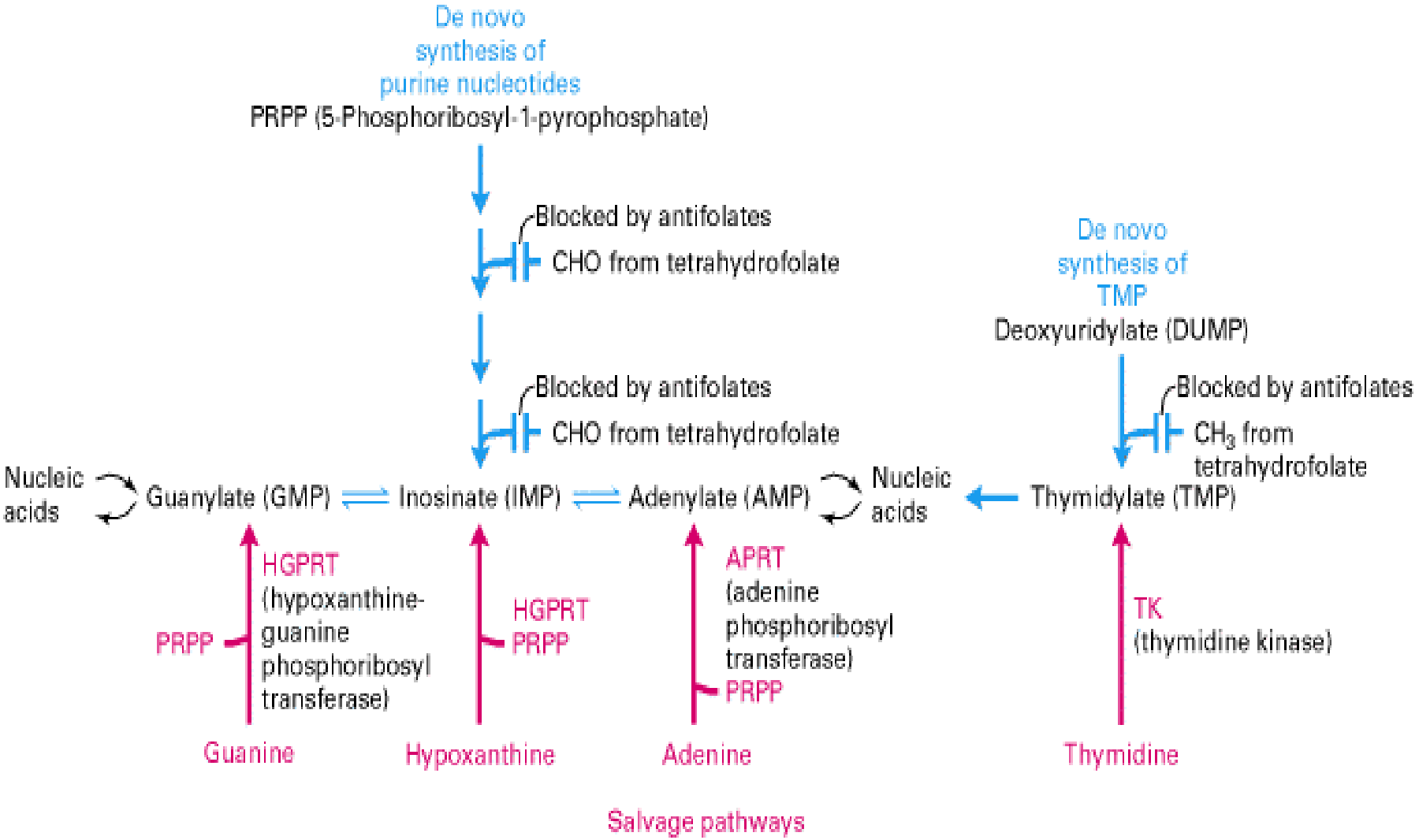
Unfused myeloma

Q- How to make only hybridoma cells to survive and secrete antibodies?

A – 1. Pathways of nucleotide synthesis

2. Non-antibody secreting myeloma cells

Pathways of synthesis of Purine nucleotides



P3.653 Myeloma cells

- **This cell line can not secrete any immunoglobulins.**

- This cell line is deficient either in HGPRT (hypoxantine guanine phosphoribosyl transferase) or TK (thymidine kinase deficient), which are enzymes of “salvage pathway” .

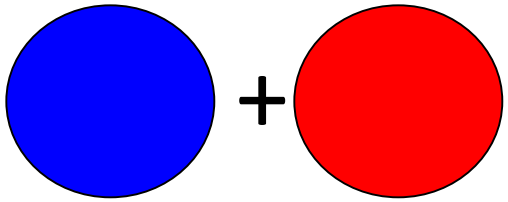
- **Hence “Salvage Pathway” is not possible.**

- Cell line cannot survive in selection medium, which contains Aminopterin
  - Aminopterin (Folic acid antagonist) inhibits “*de novo* pathway”
  - “Salvage Pathway” is not possibe due to HGPRT or TK Deficiency.

- **P3.653 cells die in the presence of aminopterin**

Possible Fusion Products

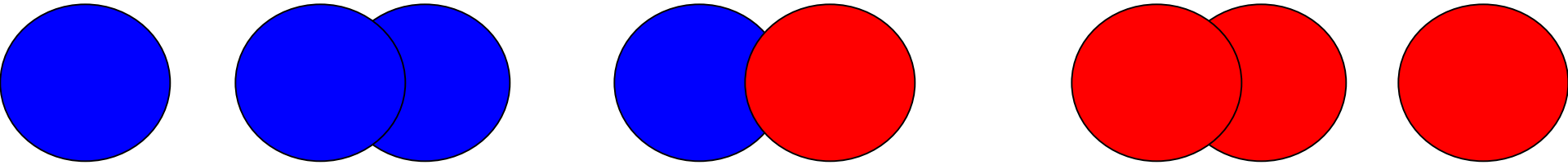
Plasma Cells From Immunized Animal



$Ig^{+}HGPRT^{+}$

Myeloma HGPRT Deficient And Ig Deficient

$Ig^{-}HGPRT^{-}$



Senescence

Senescence

Can Use Salvage Pathway

No Senescence

HAT Medium

HAT Medium

HAT selection is used to select for growth of hybrids and against the growth of the parental myeloma.

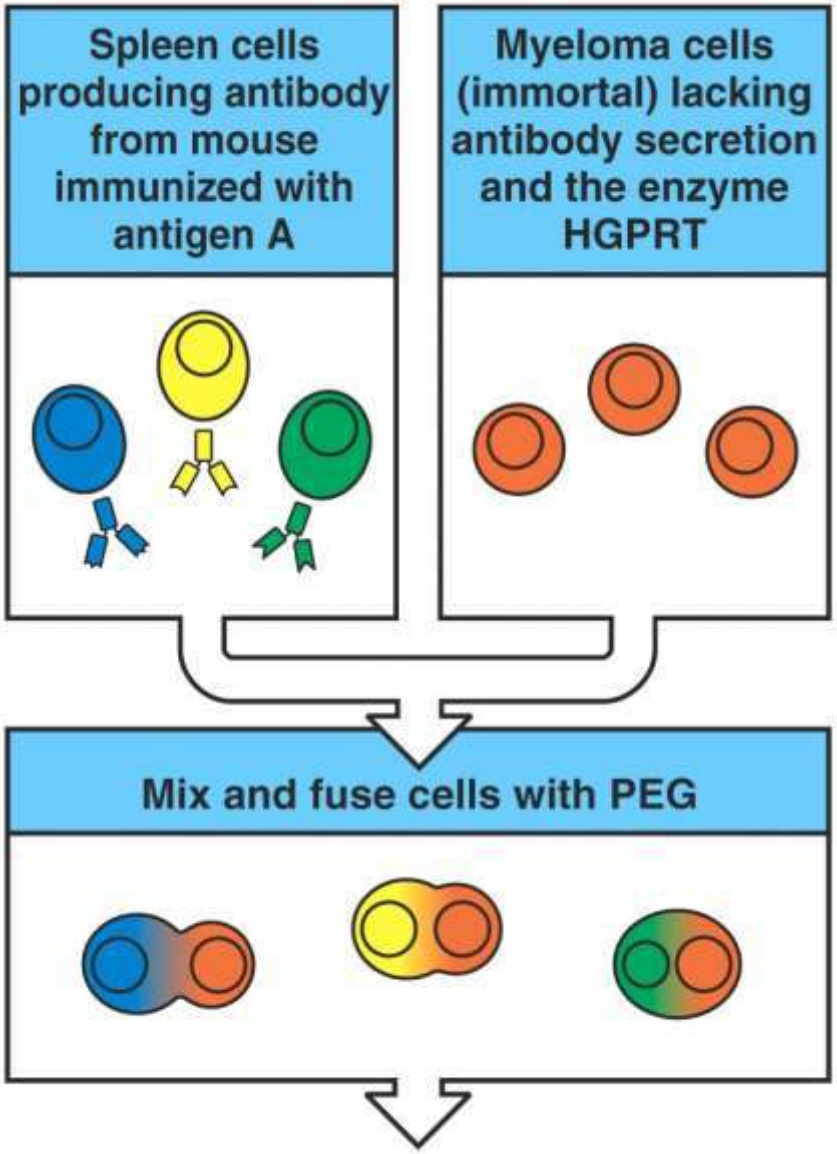


Figure A-14 part 1 of 2 Immunobiology, 6/e. (© Garland Science 2005)

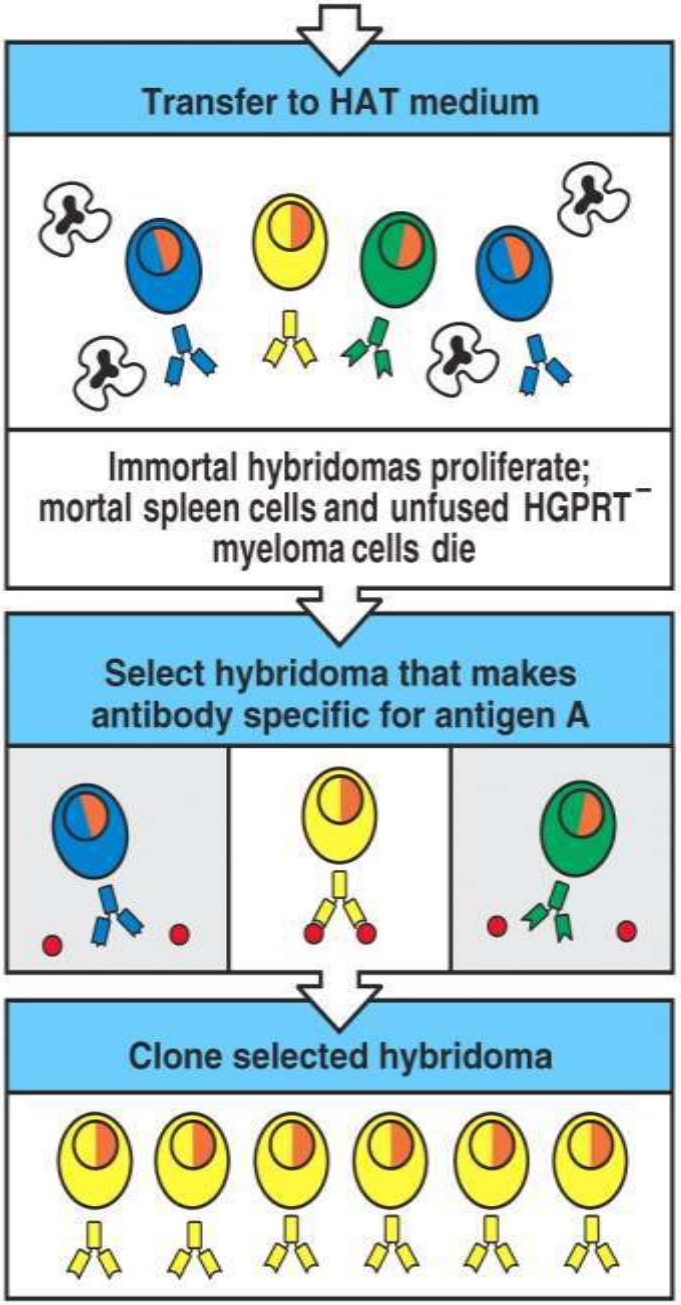
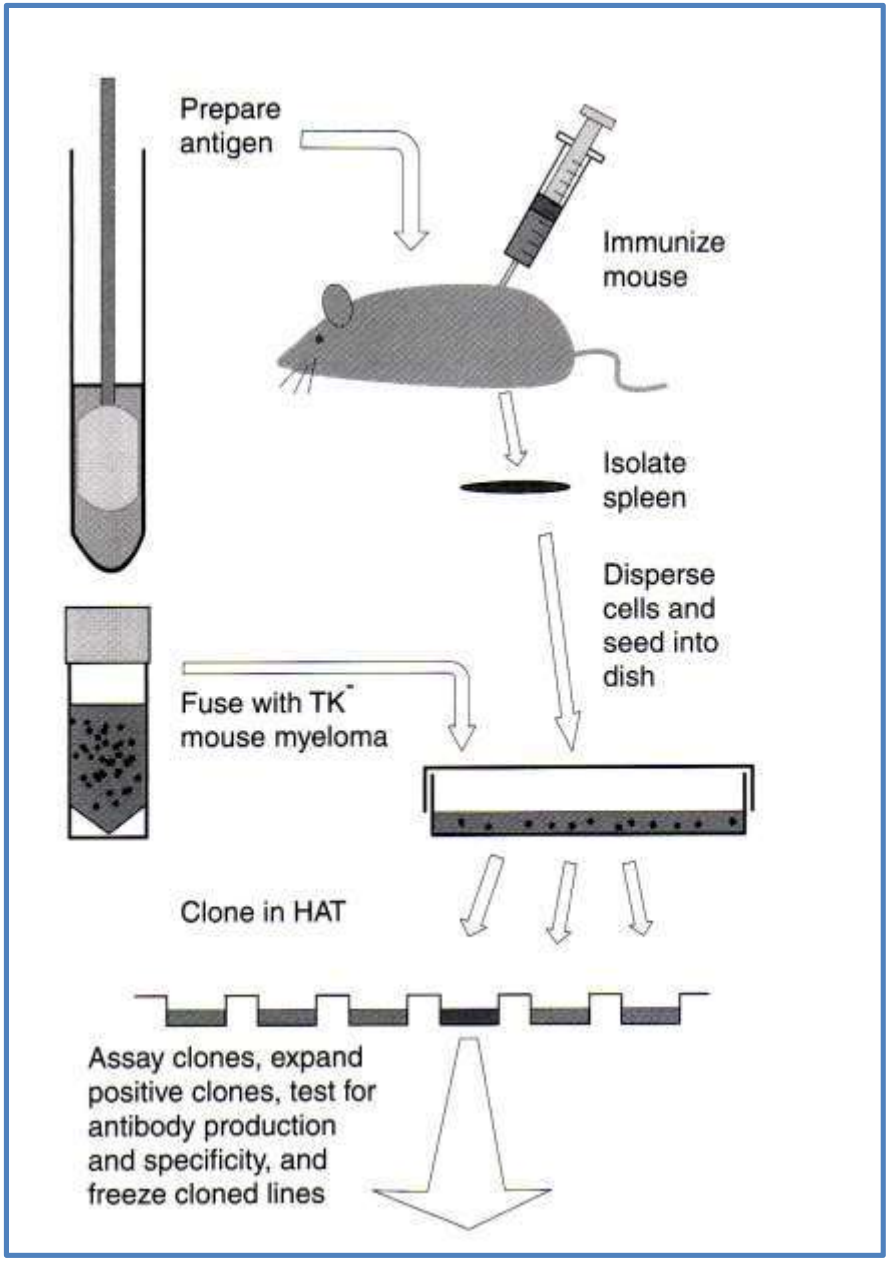


Figure A-14 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)

Making Monoclonals


Prepare antigen
Immunize mouse
Isolate spleen
Disperse cells and seed into dish
Fuse with TK⁻ mouse myeloma
Clone in HAT
Assay clones, expand positive clones, test for antibody production and specificity, and freeze cloned lines

Immunization of mice

- **For monoclonal antibody production**
  - Animal is immunized with antigen
  - Spleen cells are isolated
- **Intraperitoneal immunization**
  - Balb/c Mouse
  - Day 0: 50 μg of Ag in complete Freund's adjuvant
  - Day 14: 25 μg of Agin incomplete Freund's adjuvant
  - Day 28: 25 μg of Ag in D-PBSA
  - Bleed animal and test reactivity to antigen
    - Serum is diluted 1:30
  - Final boost of 10 μg antigen i.V or i.P 3 days before fusion
- **Aseptically isolate spleen cells**

Fusing Spleen Cells With Myeloma

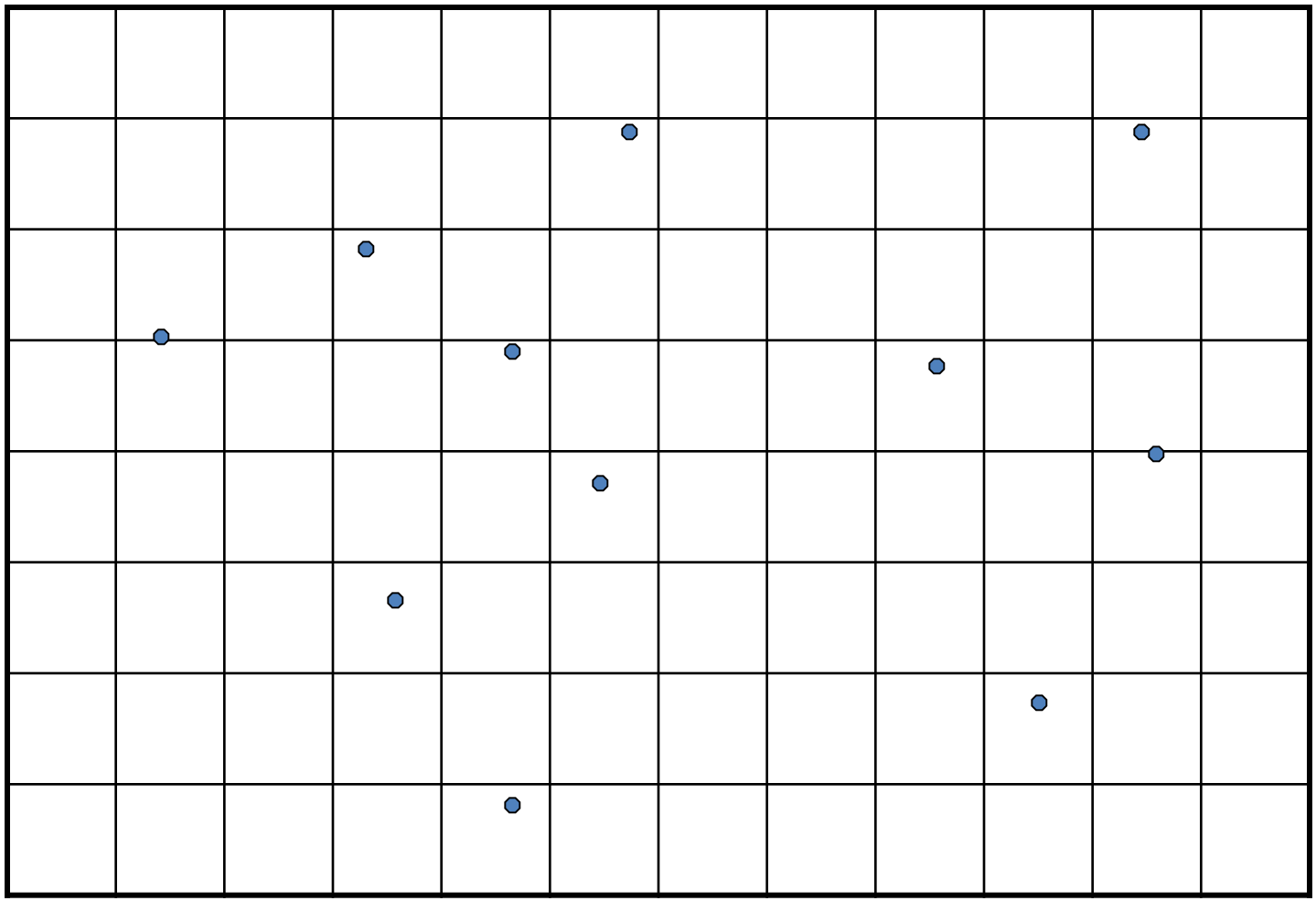
- **Purify spleen cells**
  - T-cell depletion (anti-Thy 1.2 followed by complement lysis)
  - MACS purification (CD138)
- **Fusion**
  - Mix spleen cells and myeloma in 50 mL tube (4:1)
  - Spin and resuspend pellet with 1 mL PEG over 15 s
  - Mix by swirling for 75 s
  - Add 1 mL of SFM (serum free medium) over 15s, swirl for 45s
  - Add 2 mL SFM over 30s, swirl 90s
  - Add 4 mL HAT over 30s, swirl 90s
  - Add 8 mL HAT over 30s, swirl 90s
  - Add Above volume in a new container, add HAT till cell concentration is $1x10^6$ cells/mL
  - Add 200 μL per well 96 well/plate (200,000 cells/well)

Selection of Hybridomas

- **Selecting clones**
  - Feed wells with new HAT medium (remove old, add 150 μL new)
  - Feed cells twice a week
  - After about 2 weeks test supernatants of potential clones using ELISA
  - Retest +ve clones in 48 hrs
  - Expand clones in new 96-well plate with **HT NOT HAT**
  - Retest suspensions, expand in 24-well plate, switch to normal medium, retest
  - Expand in 4-well plate
  - Cryopreserve

Cloning hybridomas from fusion

Plate at limiting dilution (<1 cell/well) in 96 well plates
Allow clones to expand
Expand positive well and test for production of antibody of desired specificity in supernatant



Cloning hybridomas from fusion

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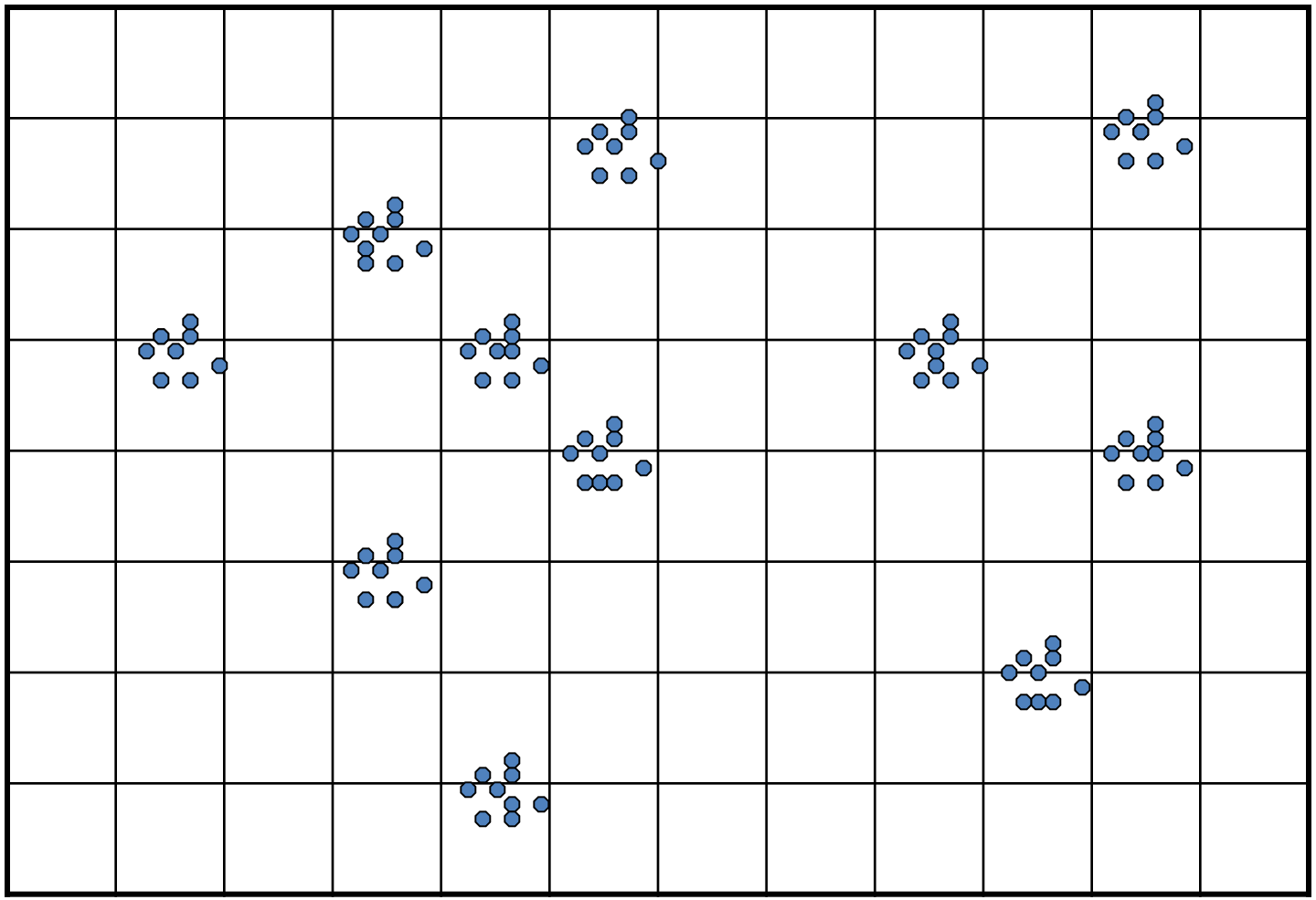


Ensuring Monoclonality

- **To ensure monoclonality sub-clone hybridoma**
  - Serially dilute @ 1 cell per 3 wells
  - Use a spleen cells as feeder cells
  - Feeder cells concentration @ $1x10^6$ cells/mL
  - 96 well plate, $2x10^5$ cells/well
  - Colonies are observed at 5 days
  - Replenish with fresh medium @ day 7
  - Screening Is done at day 10 and 14
  - Clones are cryopreserved same way as parental clones

Antibody Production

- **Large amounts of antibody can be produced using animals**
  - Prime Balb/c animal with IFA or pristane
  - Inject clone i.p
  - Collect peritoneal ascites
- **Alternatively bioreactors are used**
  - Cells are cultured in hollow fibers
  - Fresh media and waste are recirculated
  - High concentrations of Ab produced in cell compartment
  - Collected at different time points
  - Others: Roller bottle, Microcarrier, Membrane bound cell culture processes

Advantages and disadvantages of monoclonals

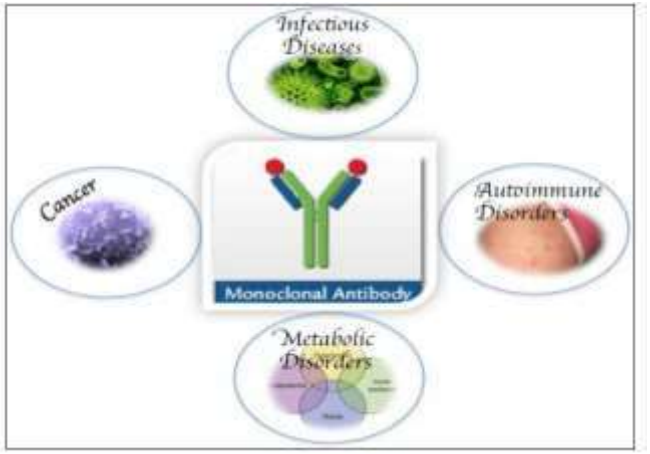
Advantages of Monoclonal Abs

- Homogeneity – binds to same epitope with uniform affinity
- High specificity
- Target oriented
- Small quantity of antigen is required
- Consistent
- Limitless supply of specific reagent
- More easily tested for cross-reactivity

Disadvantages of Monoclonal Abs

- High specificity affects use as diagnostic when there is minor differences between strains
- Limited sensitivity
- Average affinity is lower than pAb
- Immune rejection
- Expensive
- Time consuming
- Skills

Uses of mAbs


Infectious Diseases
Cancer
Monoclonal Antibody
Autoimmune Disorders
Metabolic Disorders

MONOCLONAL ANTIBODIES:A Review

- Anti-Cancer
- Transplant Rejection
- Autoimmune Diseases
- Cardiovascular and Respiratory
- Eye and Miscellaneous

- Anti-Cytokines MAb - IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, iL-23, TNF-α
- Anti-CD MAb- CD-3, CD-11, CD-19, CD-20, CD-30, CD-33 CD-38, CD-52, CD-152, CD-319
- Anti-Growth factor MAb - HER-2, PDGFR, EGFR, VEGFR
- MAb targeting PD-1, PCSK-9, Integrins, Complement C5 IgE, GPIIb/IIIa

Adverse Drug Reactions

Immune Reactions, Reactivation of T.B, Progressive Multifocal Leukoencephalopathy, Thrombocytopenia, Autoimmune disorders like Lupus like syndrome, vasculitis, Demyelinatingsyndrome, Nephritits, Cancer, Dermatitis,Cardiotoxicity and Cytokine storm

Nomenclature of Therapeutic mAbs

- Use suffix
  - –*ximab* for chimeric antibodies and
  - –*umab* for humanized antibodies.

Use of mAbs

- The **first approved mAbs was OKT-3**, which is a murine IgGa2 protein to deplete T cells in patients with acute rejection of renal allotransplant Infectious diseases
- **Auto-immune diseases**
  - **Infliximab® and Adalimumab®** are effective against rheumatoid arthritis, Crohn's disease and ulcerative colitis (block TNF-α and IL-2 on activated T-cells)
  - **Omalizumab®** inhibition of human IgE; treatment of various types of allergic asthma
- **Cancer therapy**
  - **Rituximab** is a chimeric mAb that targets the CD20 B-cell antigen – B cell lymphomas
  - **Trastuzumab (Herceptin)** – anti breast cancer (against HER2/neu (erbB2) receptor)
  - **Gemtuzumab ozogamicin (Mylotarg)** – calicheamycin conjugated mAb against acute myelogenous leukemia (AML)

Use of mAbs

- **Metabolic disorders**
  - mAbs targeting secreted fatty acid–binding protein aP2 - new treatment for type 2 diabetes
  - Evolocumab and Alirocumab substantially reduced the LDL-C level by over 50%
- **Infectious diseases**
  - Raxibazumab® - for the treatment of infectious inhalational anthrax
  - Tocilizumab ® - anti IL-6 mAb used for treating COVID-19