

Lincoln Memorial University Continuing Education Conference April 2014

Scientific Session 304-401-14

Laboratory Diagnosis of Megaloblastic Anemia: A Case Study Approach



Presentation Objectives

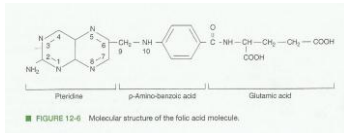
- ▶ Compare and contrast the roles of folic acid and vitamin B₁₂ in DNA synthesis.
- ▶ Describe the effects that a deficiency in folic acid and/or vitamin B₁₂ have on DNA replication and cellular division.
- ▶ Explain the concept of asynchronous development and relate this process to megaloblastic anemia.



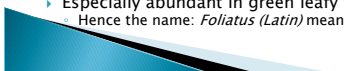
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Folic Acid

- ▶ Chemically known as pteroylglutamic acid.



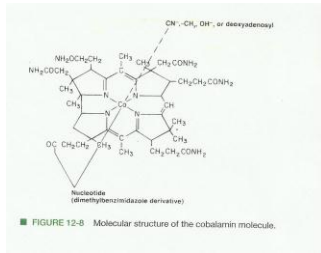
- ▶ Parent substance of a large group of compounds known as **folates**.
- ▶ Present in most foods including eggs, milk, mushrooms, and liver.
- ▶ Especially abundant in green leafy vegetables.
 - Hence the name: *Foliatius (Latin)* meaning leafy



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Vitamin B₁₂

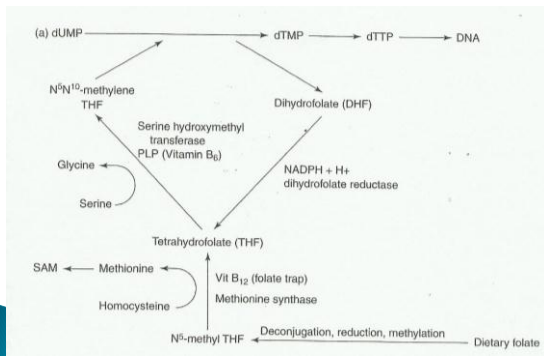
- Generic term for a family of **cobalamins** vitamins in which ligands can be chelated to cobalt.
- Present in most foods of animal origin (milk, eggs, meat, etc.)
- Intrinsic factor**, a glycoprotein secreted by the parietal cells of the gastric mucosa, is required for proper absorption.



Roles of Folic Acid and Vitamin B₁₂ in DNA Synthesis

- Folate (folic acid)**
 - Essential to the production of the thymine nucleotides used in DNA production.
- Vitamin B₁₂**
 - Essential cofactor in the production of thymine nucleotides

The Role of Folate and Vitamin B₁₂ in DNA Synthesis.



Effect of Folic Acid and/or Vitamin B₁₂ in DNA Synthesis

- ▶ When either folic acid or vitamin B₁₂ is missing, thymine nucleotide production for DNA synthesis is impaired.
- ▶ With the diminished thymine availability, DNA can unwind and replication can begin but there is an empty space in the replicated DNA sequence where the thymine nucleotide is needed.



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Effect of Folic Acid and/or Vitamin B₁₂ in DNA Synthesis

- ▶ Bottom Line:
 - Without thymine:
 - DNA repair is unsuccessful
 - DNA is not functional
 - DNA replication is incomplete
 - Cell division is halted, resulting in either cell lysis or apoptosis.
- ▶ All rapidly dividing cells, such as skin cells, epithelium of GI, and hematopoietic tissues are adversely affected.



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Causes of Folate Deficiency

- ▶ Dietary Deficiency
 - Inadequate diet
 - Increased need due to increased cellular replication such as pregnancy, growing infants & children
- ▶ Intestinal Malabsorption
 - Ileitis, tropical sprue, nontropical sprue, overgrowth of bacteria which preferentially utilize folate.
- ▶ Impaired Utilization
 - Antiepileptic drugs (Phenobarbital, Primidone, Phenytoin)



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Causes of B₁₂ Deficiencies

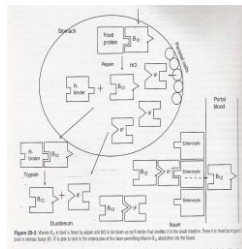
- ▶ **Malabsorption due to lack of haptocorrin and/or intrinsic factor (Pernicious Anemia)**
- ▶ **Gastrectomy**
 - Part of the stomach is removed
 - Intrinsic factor secreting cells are gone
 - Oral vitamin B₁₂ can't be absorbed
- ▶ **Crohn's Disease**
 - Intestinal enteritis, with chronic diarrhea, affects the ileum where B₁₂ is absorbed.
- ▶ **Fish Tapeworm**
 - *Diphyllobothrium latum*
 - This fish tapeworm lodges in the ileum and splits vitamin B₁₂ from intrinsic factor, rendering the vitamin unavailable for host absorption.



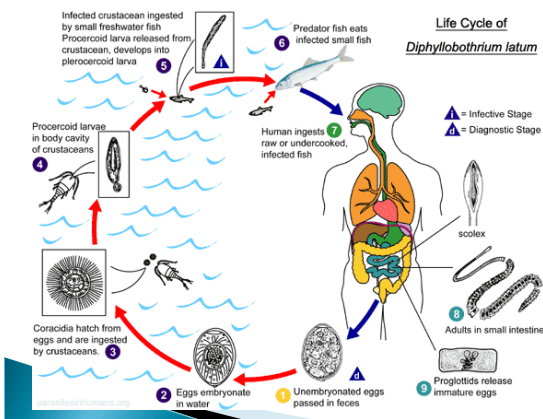
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Causes of Vitamin B₁₂ Deficiencies

- ▶ **Impaired absorption due to lack of *haptocorrin* and/or *intrinsic factor*.**
 - Vitamin B₁₂ is bound by the saliva protein haptocorrin.
 - Trypsin releases B₁₂ from haptocorrin in the stomach.
 - **Intrinsic factor binds B₁₂ and transports it across the cell membrane.**



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Vitamin B12 Deficiencies: *Diphyllobothrium latum*

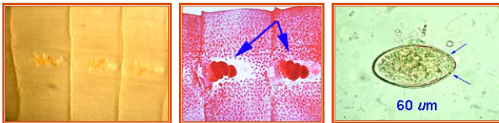


Left Frame: Close in view of the almond-shaped scolex. The genus name is derived from the lip-like folds (*diphyllus*) on either side of two central dorsoventral suckorial pits or grooves (*bothrium*).

Center Frame: Composite image showing the size of the proglottids of *Taenia* (upper) in contrast to the strobila (below) of *D. latum* with its tightly spaced proglottids that are much broader than long, from which the species name "*latum*" is derived.

Right Frame: Strobila with broad, narrow proglottids.

Diphyllobothrium latum



Left Frame: Close in view of proglottids that are characteristically much broader than long.

Center Frame: Stained section of *Diphyllobothrium latum* proglottids each with a nondescript coiled uterus resembling a compact rosette (arrows).

Right Frame: *Diphyllobothrium latum* ovum. Key identifying features are: large size (ranging from 55 - 75 µm); thin, smooth shell; inconspicuous non-shouldered slit-like operculum (arrows); and extension of inner cleavage to the inner membrane.

Diphyllobothrium latum

Infection with *Diphyllobothrium latum* is usually limited to a single worm. Most infected patients suffer few ill effects. Varying degrees of abdominal discomfort, loss of appetite, weakness, loss of weight, and anemia may be experienced.

Megaloblastic anemia is caused when the worm is located in the upper intestine and there is competition with the host for Vitamin B-12.

Laboratory diagnosis is made by the finding of operculated ova or evacuated proglottids in fecal specimens.

Pernicious Anemia (PA)

- ▶ **Problem:**
 - Malabsorption of Vitamin B₁₂ due to lack of **intrinsic factor**
- ▶ Often seen in 40–60 year age group
- ▶ More common in women than men



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Facts About Pernicious Anemia (PA)

- ▶ Currently, the most common cause (85%) of vitamin B₁₂ deficiencies.
- ▶ Atrophy of gastric parietal cells is demonstrated by **achlorhydria** of gastric juice after histamine stimulation.
- ▶ While no particular genetic abnormality has been identified to date, *studies have indicated that a positive family history of PA conveys a risk of developing it by 20 times.*



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Pernicious Anemia (PA)

- ▶ **Etiology**
 - *Parietal cells* of the gastric mucosa secrete both HCL and **intrinsic factor**, which is necessary for Vitamin B₁₂ absorption across the intestinal mucosa in the ileum.
 - Without intrinsic factor, Vitamin B₁₂ can't be adequately absorbed and utilized by the body.
 - PA is an autoimmune disorder in which the immune system targets either intrinsic factor or the parietal cells that produce intrinsic factor.
 - The lack of intrinsic factor that results in an inability to absorb Vitamin B₁₂



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PA: Antibody Mediated or Cell Mediated

▶ Antibody Mediated

- 90% of PA patients have antibodies against the parietal cells.
- 75% of PA patients have anti-IF antibodies.

▶ Cell Mediated

- Recent studies suggest that the immune destruction of gastric mucosa is not antibody mediated but more likely T cell mediated.
- Patients with agammaglobulinemia have a higher than expected incidence of PA.
- T-Lymphocytes from PA patients have been shown to be hyper-responsive to gastric antigens.

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Classic Symptoms of PA

- ▶ Weakness
- ▶ Yellow skin pallor
- ▶ Pale nail beds and eyelids
- ▶ Smooth, tender tongue
- ▶ GI Symptoms
 - Abdominal pain, nausea, diarrhea
- ▶ CNS Symptoms
 - "megaloblastic madness": loss of memory, disorientation
 - Loss of balance, numbness, tingling if arms and legs
 - **May appear as if the patient has had a stroke**

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Effects of Vitamin B₁₂ Deficiencies

▶ **Megaloblastic Madness"**

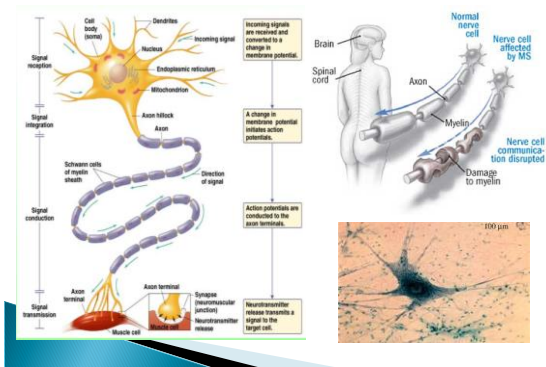
- **Root Cause:**
 - In some cases of severe B₁₂ deficiencies, there is a defective fatty acid degradation that results in odd-chain fatty acids being incorporated into membranes of neurons, causing disruption of membrane function.
- This results in the **demyelination** (destruction, removal, or loss of the lipid substance that forms the myelin sheath around the axons of nerve fibers) of the spinal cord and peripheral nerves.

▶ **Clinical Symptoms: Often Stroke-Like**

- Memory loss
- Numbness, and tingling in toes and fingers
- Loss of balance
- Impairment of walking
- Dementia, paralysis, and other serious neurological disturbances.

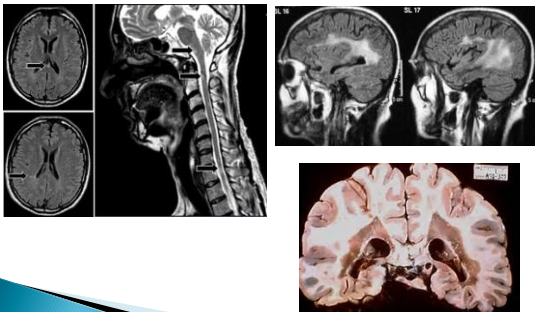
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Neurology of Megaloblastic Madness



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Neurology of Megaloblastic Madness



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Hematology Results Commonly Seen in Pernicious Anemia

- ▶ Automated CBC
 - Pancytopenia
 - Low WBC, RBC, H/H, Plt
 - Severe anemia
 - High MCV (indicates macrocytosis; usually 100–150 fL)
 - MCHC is usually normal
 - High RDW
- ▶ **Macrocytic normochromic anemia**

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Hematology Results Commonly Seen in Pernicious Anemia

- ▶ Differential
 - **Hypersegmented neutrophils**
 - *Be Conservative:* Even healthy people may have an occasional 5 lobed segmented neutrophil.
 - **Oval macrocytes**
 - **Other:**
 - Howell-Jolly bodies
 - Basophilic stippling
 - Cabot's rings
 - Dacryocytes



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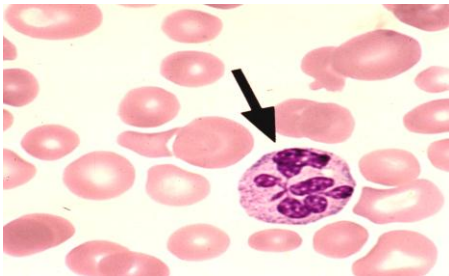
Hematology Results Commonly Seen in Pernicious Anemia

- ▶ Other Tests
 - Low Retic Count (even in the presence of anemia)
 - Impaired DNA synthesis
 - Low Vitamin B₁₂
 - High LDH (lactate dehydrogenase)
 - Caused by the breakdown of RBC precursors



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Hypersegmented Neutrophil

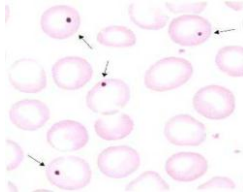


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Howell-Jolly Bodies

▶ Howell-Jolly bodies

- Appearance on Wright Stain:
 - Single, dense, round, blue granule
- Composition:
 - DNA nuclear fragment
- Associated With:
 - Megaloblastic anemia, including Pernicious Anemia
 - Hypofunction of the spleen
 - Splenectomy

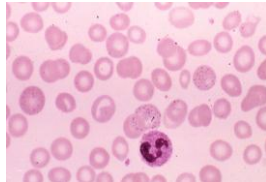


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Basophilic Stippling

▶ Basophilic Stippling

- Appearance on Wright Stain:
 - Stippled RBC
- Composition:
 - RNA (precipitation of ribosomes)
- Associated with:
 - Megaloblastic anemia
 - Lead or heavy metal intoxication
 - Thalassemia

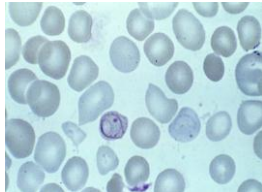


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Cabot Rings

▶ Cabot Rings

- Appearance on Wright's Stain:
 - Reddish ring or figure-8 strand
- Composition:
 - Remnant of mitotic spindle or nucleus
- Associated With:
 - Pernicious anemia
 - Lead intoxication



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Bone Marrow Findings In PA

- ▶ **Megaloblastic Erythroid Hyperplasia**
 - Characteristic of PA
 - All NRBC are larger than normal
 - Defective immature RBCs (defective DNA synthesis) die before leaving the marrow
 - Even the neutrophils are larger than normal.
 - "Giant metamyelocytes"
 - **Low M:E ratio due to erythroid hyperplasia**
 - Normal: 3:1 – 4:1
 - PA: 2:1



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Megaloblastic Anemias

- ▶ **Problem:**
 - Characterized by *defective DNA synthesis*, resulting in defects in nuclear maturation.
- ▶ **Clinical Manifestations:**
 - RNA synthesis continues, resulting in an increase in cytoplasmic mass and macrocytic anemia.
 - Thus, in megaloblastic anemia, nuclear maturation lags behind cytoplasm maturation.



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Asynchronism

- ▶ Defined as that state in which nuclear maturation and the cytoplasmic maturation takes place at different rates.
- ▶ This is an important characteristic of the megaloblastic anemias.

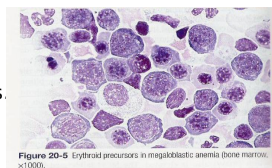
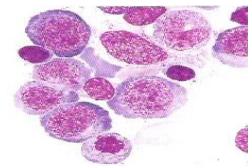


Figure 20-5 Erythroid precursors in megaloblastic anemia (bone marrow, $\times 1000$).

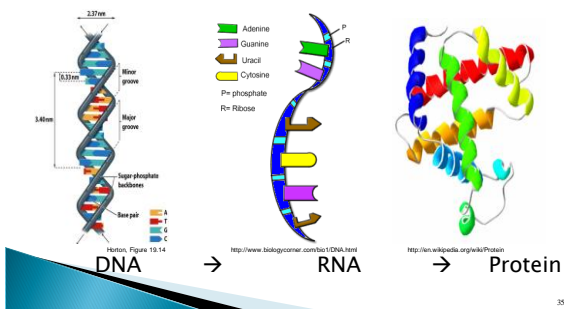


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Question...

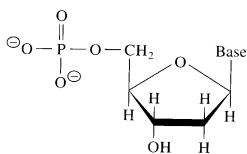
- ▶ What exactly causes Asynchronism in a Vitamin B₁₂ deficiency?
- ▶ To understand the biochemical mechanism behind Asynchronism, we must understand the basics of genetics.

Genetic Information Flow: "Central Dogma"



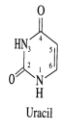
Nucleic Acid Structure

- ▶ Nucleic acids are polymers of nucleotides
- ▶ Nucleotides = phosphate + sugar + base

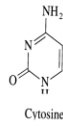
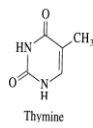


Purines & Pyrimidines = Bases

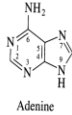
Pyrimidines



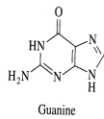
PYRIMIDINES



Purines



PURINES

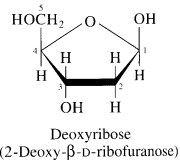
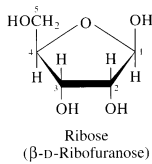


Horton, Figure 19.4

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Nucleotide: Sugar

- The carbohydrate in nucleosides comes in two forms
- Ribose** is found in RNA (ribonucleic acid)
- Deoxyribose** is found in DNA (deoxyribonucleic acid)

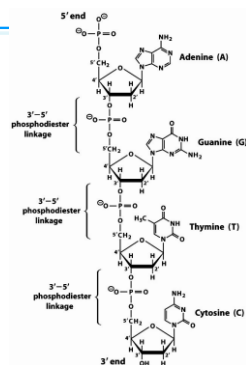


Horton, Figure 19.2

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DNA

- DNA is comprised of
 - Adenine (A)
 - Guanine (G)
 - Thymine (T)
 - Cytosine (C)
- Chargaff's Rule
 - A binds with T
 - G binds with C
 (applies to double stranded DNA only)

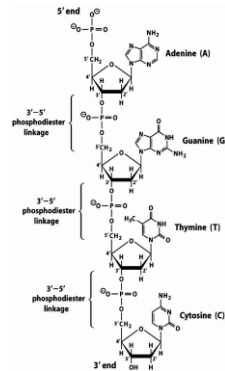


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• It is formed by nucleotides joined together by 3'-5' phosphodiesterase linkages

• One end is called the 5' end whereas the other is the 3' end

• **Replication** (duplication of DNA) and **transcription** (copying DNA into RNA) takes place in the **5' to 3' direction**



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Relationship between nucleic acid and protein sequences

- ▶ Only one of two strands of DNA is copied into RNA by the RNA polymerase
- ▶ The nucleic acid information is read in a 5' to 3' direction and the protein is made in the N terminus to C terminus direction

DNA coding strand 5' - ATGCCAGTAGGCCACTTGTCA - 3'

DNA template strand 3' - TACGGTCATCCGGTGAACAGT - 5'

mRNA 5' - AUG CCA GUA GGC CAC UUG UCA - 3'

Protein N - Met - Pro - Val - Gly - His - Leu - Ser - C

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Megaloblastic Anemia Bone Marrow

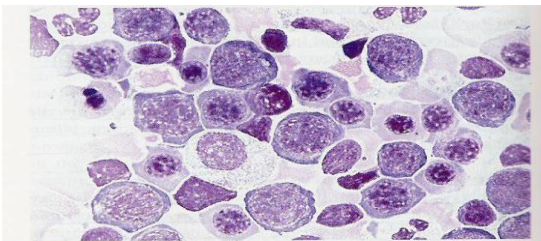


Figure 20-5 Erythroid precursors in megaloblastic anemia (bone marrow, $\times 1000$).

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Megaloblastic Anemia

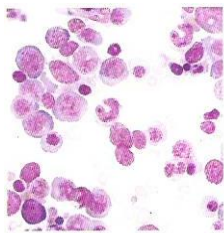


Figure 13-6C Megaloblastic (BM original $\times 500$).

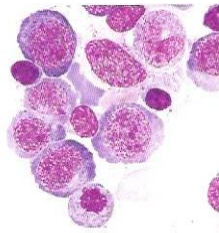


Figure 13-6D Megaloblastic (BM original $\times 1000$).

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Megaloblastic Anemia

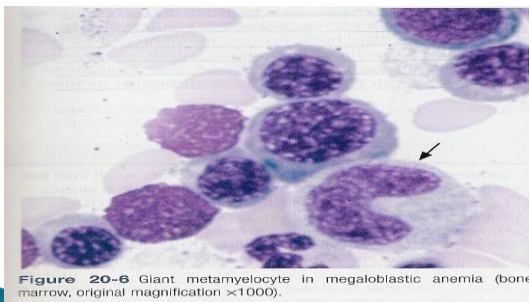
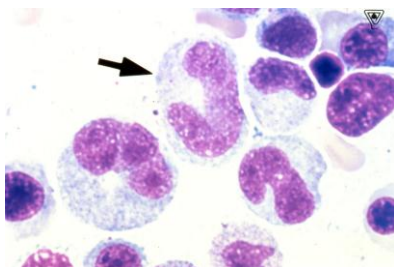


Figure 20-6 Giant metamyelocyte in megaloblastic anemia (bone marrow, original magnification $\times 1000$).

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MTCD: RBC Megaloblastic Band



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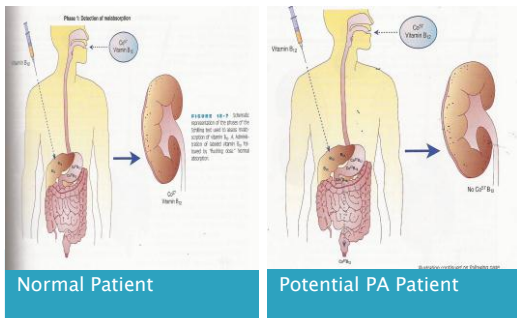
Schilling Test for PA

- ▶ Used to distinguish vitamin B12 deficiency due to malabsorption, dietary deficiency, or absence of IF.
- ▶ Involves the administration of a radioactively tagged crystalline B₁₂.
- ▶ Urine is collected for 24 hours, and its radioactivity is determined.
- ▶ If more than 7.5% of the standard oral radioactive dose is excreted in the urine, absorption is considered to be normal.



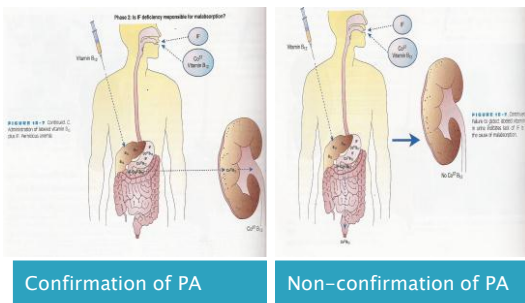
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Schilling Test: Phase I



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Schillings Test: Phase II



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Schillings Test

Final Thoughts:

- Seldom used in US due to the difficulties in using radioisotopes and the inconvenience of a 24 hour urine collection by the patient.
- Test results are not valid in the presence of renal disease.
- Methylmalonic acid (MMA) and Homocysteine are emerging as better markers for early detection of PA or vitamin B₁₂ deficiency.



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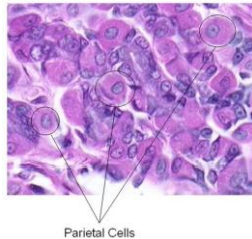
PA and Achlorhydria

▶ **Achlorhydria:** Lack of free HCL in the stomach secretions, even after histamine stimulation.

- **Reason:** Progressive loss of parietal cells with their secretory products, H⁺ and intrinsic factor.

Procedure:

- N/G (nasogastric) tube is inserted into the stomach to remove and titrate stomach HCL.
- Patient is given an injection of histamine, which should stimulate gastric HCL production.
- PA patient: No significant increase in HCL secretion



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CASE STUDY: Background

- ▶ Linda, a 46-year old female, has recently experienced a loss of appetite due, in her opinion, to the fact that she has reoccurring bouts of an upset stomach.
- ▶ Over the past 6 months, she has experienced a 35 pound weight loss.
- ▶ She is complaining of chronic fatigue and shortness of breath upon exertion.
- ▶ She has also been experiencing increased numbness and a tingling sensation of her fingers and toes.
- ▶ Her family has noted that she is more forgetful than usual and sometimes has appeared disorientated.



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CASE STUDY: Medical History

- ▶ Has been treated for hypertension and chronic obstructive pulmonary disease for the past several years.
- ▶ Her only medication is Verapamil for hypertension.
- ▶ She quit smoking 10 years ago and denies any alcohol intake.
- ▶ When asked if she was a vegetarian or a vegan, she claims that she tries to the best that she can given her history of reoccurring bouts of indigestion, to eat a normal diet consisting of meat, eggs, and vegetables



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CASE STUDY: Physical Exam

- ▶ Pale, yellow skin tone indicating mild jaundice.
- ▶ Her tongue is red, smooth, and tender.



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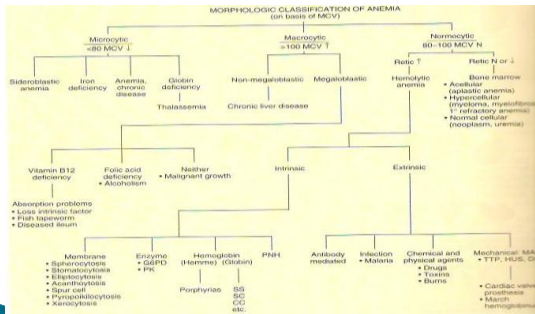
CBC Results

- ▶ WBC: 3,200/ μ L
- ▶ RBC: 2.50 M/ μ L
- ▶ HgB: 10.0 g/dL
- ▶ HCT: 31%
- ▶ MCV: 124.1 fL
- ▶ MCH: 40.5 pg/dL
- ▶ RDW: 21.2
- ▶ PLT: 109,000/ μ L
- ▶ Differential:
 - Lymphs: 36
 - Monos: 4
 - Neutrophils: 58
 - Eosinophils: 2
 - 2+ macrocytes
 - 3+ anisocytosis
 - 1+ ovalocytes
 - Basophilic stippling, hypersegmented PMNs, and Howell-Jolly bodies noted.



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Anemia Classification



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Further Testing

▶ B ₁₂ :	50 pg/mL	(low)
▶ Folate:	10.3 ng/mL	(normal)
▶ Total Bilirubin:	2.5 mg/dL	(high)
▶ Direct Bilirubin:	0.8 mg/dL	(normal)
▶ AST:	35 U/mL	(normal)
▶ ALT:	30 U/mL	(normal)

Questions:

1. What is the morphologic classification of the patient's anemia?
2. Based upon all available information at this point, what is the most likely defect?
3. What is the significance of the AST & ALT results?
4. What further testing can be done to obtain a definitive diagnosis?

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Further Testing

Schilling Test:

- Phase I (without IF): 1%
- Phase II (with IF): 10%

Intrinsic-Factor-Blocking Antibodies:

- Positive titer of 1:6400

- ▶ Question: What is this patient's definitive diagnosis?

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Therapy & Response

- ▶ **Therapy:**
 - Lifelong monthly parenteral doses of hydroxycobalamin
- ▶ **Physiological Response:**
 - Within 6 hours:
 - Rapid response by bone marrow as indicated by normal appearing pronormoblasts.
 - Within 2 – 4 days:
 - Erythroid abnormalities in the bone marrow disappear.
 - With 7 days:
 - Significant rise in retic counts.
 - Within 2 weeks and beyond:
 - HgB rises about 2 – 3 g/dL every 2 weeks until normal levels of reached.



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Questions...



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References

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