USE OF THE GRAM STAIN FOR DIAGNOSIS OF INFECTIOUS DISEASE

Part One: Introduction and Cell Types

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INTRODUCTION

• This slide set is an introduction to the use of Gram’s stain as a rapid tool in the laboratory diagnosis of infectious disease.

• The century old Gram stain remains the mainstay of rapid diagnosis.
  – Simple and rapid, requiring only minutes as opposed to hours to even a day for sophisticated immunological techniques.
  – Inexpensive to perform.

• Requires considerable experience for the correct interpretation.
VALUE OF GRAM STAIN

When properly interpreted in the light of the clinical history, the Gram stain can provide useful, presumptive information as to the etiology of many infections.
INFLAMMATORY EXUDATE

This slide demonstrates an inflammatory exudate from the pleural fluid, obtained from a 4-year-old child with pneumonia. There is a mixture of polymorphonuclear leukocytes (PMNL) and mononuclear cells. Many of the cells contain small pleomorphic gram-negative coccobacilli. There are also a few extracellular organisms. Presumptive diagnosis of *Hemophilus* pneumonia.
An additional advantage of Gram stain is that it is immunologically non-specific. With all of the immunological tests one is restricted to the organisms for which acceptable antisera are available.
Gram Smear from a Draining Abdominal Wound of a 30 yo Man.

The specimen was submitted for a culture, but when a gram stain was done these branching filamentous gram-positive bacilli were demonstrated amidst the inflammatory cells.
Gram Smear from a Draining Abdominal Wound of a 30 yo Man.

Notice that the elongated rods stain rather irregularly. This morphologic appearance is typical of the actinomycetes. On the basis of the Gram stain, the technologist in the microbiology laboratory suggested that an anaerobic culture for *Actinomyces* sp. and a culture for *Nocardia* sp. should be considered.
ADEQUACY OF THE CULTURE TECHNIQUE

The Gram stain also allows assessment of the adequacy of the culture technique.

Bacteria may not be recovered in culture for a variety of reasons. They may have been damaged by antimicrobial therapy, so that they are no longer viable or are inhibited from growth. In addition as happened in the next specimen, the culture conditions may not have been appropriate for recovery of the organism.
INADEQUATE CULTURE CONDITIONS
This Gram smear demonstrates many inflammatory cells and several clusters of Gram positive cocci clearly grouped together in clumps. Such a Gram stain would suggest staphylococci, but no bacteria were recovered in the aerobic culture.

An anaerobic culture which was subsequently submitted yielded *Peptococcus* sp., an anaerobic organism that did not grow in the original culture and which resembles *Staphylococcus* sp. in morphology.
SENSTIVITY OF TECHNIQUE

The Gram smear is a relatively insensitive technique. It requires $10^4$-10$^5$ organisms per ml of fluid or gram of tissue in order to detect any bacteria. In some instances, this insensitivity is an advantage.

For instance, the Gram smear of the undiluted voided urine can be used with reasonable success as a screen for significant bacteriuria (greater than $10^5$ bacteria per ml). Similarly, a culture is more likely to detect confusing indigenous oropharyngeal flora that contaminate a sputum specimen than is the corresponding Gram stain.

Unfortunately, the indigenous flora is often present in such large amounts that even the Gram stain is able to detect these unwanted elements.
Oropharyngeal squamous cells are accompanied by large numbers of gram-negative bacilli in this sputum specimen from a patient in one of the special care units.
ASSESSMENT OF CELLULAR CONTENT

The Gram stain allows assessment of the cellular content of a specimen.

The first question in addressing the significance of the culture is whether the specimen came from an inflammatory process. There is no way to evaluate this question by analyzing the results of the culture. For instance, it is not uncommon to receive specimens of bile from which multiple organisms including enteric bacilli are cultured.
There is clearly a lack of any inflammatory process. The information from working up such a culture is not likely to be helpful and may be misleading. Even if the patient has fever and a post-operative wound infection, there is nothing to suggest that the isolated organisms are the ones that are involved in that infection.
Conversely, the presence of inflammatory cells establishes an inflammatory etiology and may suggest the etiologic agent. In this specimen, clumps of inflammatory cells are present and include both polymorphonuclear neutrophils (PMN) and mononuclear cells.
Although the Gram stain does not establish the etiology of the infection, it:

- does indicate that the process is not functional (e.g., caused by emotional stress) or solely related to disturbances of bowel motility
- does suggest that an organism that produces gastroenteritis by invading bowel mucosa is responsible.
- Does suggest that viral gastroenteritis or *Giardia* enteritis are less likely. In this instance, the infection was caused by *Salmonella typhimurium*. 

VALUE OF GRAM STAIN

The Gram stain can be most useful for
- assessing the adequacy of individual specimens, and
- directing attention to specimens most likely to yield the correct answer.
VALUE OF GRAM STAIN

In addition, one can provide some discrimination in those specimens that contain indigenous flora by trying to assess which morphologic bacteria are predominantly associated with the inflammatory process. Although these guides are clearly not foolproof, they do provide much needed help for interpretation of the corresponding culture.

The following set of slides provide examples.
TRANSTRACHEAL ASPIRATE OF A PATIENT WITH PULMONARY INFILTRATES
Transtracheal Aspirate of a Patient with Pulmonary Infiltrates

On the right, is a group of respiratory epithelial cells. They are elongated with basal nuclei and one can clearly see the brush of cilia at the ends of the cell. There were a few scattered polymorphonuclear cells in the specimen. It was basically non-inflammatory, but obviously contained respiratory material. The culture was entirely devoid of bacteria as demonstrated by the chocolate agar plate on the left.
SPUTUM CULTURE FROM THE SAME PATIENT
Sputum Culture from the Same Patient

On the right is the Gram smear, in which one can see many squamous epithelial cells. Notice the gram negative bacilli present in this area of the smear. Once again, there were a few scattered polymorphonuclear cells. The culture, shown on the left, yielded a large number of mixed bacterial flora from this non-inflammatory process. Without the Gram smear, one would have a difficult time evaluating the significance of this culture. With it one can essentially dismiss the significance of these organisms.
Gram Smears from a Pair of Sputum Specimens Received on the Same Day from a patient with bacteremic pneumococcal pneumonia
Gram Smears from a Pair of Sputum Specimens Received on the Same Day

On the left is the first specimen, which contained strands of mucus, proteinaceous debris, a moderate number of squamous epithelial cells, a few PMNs, and a few respiratory epithelial cells. It was a minimally inflammatory specimen and it was impossible to pick an area that was devoid of oral squamous cells.

On the right is the Gram smear from the second sputum, which demonstrates clumps of inflammatory material with PMNs, macrophages, and protein exudate.
On the left side one can clearly see a large squamous cell with which bacteria of many different morphologies are associated. Several inflammatory cells are also present and there are several pairs of gram-positive cocci in the field. One might wonder about the identity of these organisms as pneumococcus, but with the mixed morphology in the presence of squamous cells, the smear is essentially uninterpretable.
Higher Power View

On the right is the second, more inflammatory specimen, in which essentially the only bacterial cells present are gram positive cocci in pairs and short chains. Many of the cocci have the pointed ends of a typical pneumococcus. In such a very inflammatory specimen, the morphology of pneumococcus often becomes distorted, as is the case here. The red staining material around the cocci probably represents the abundant polysaccharide capsule that this isolate possessed. It is treacherous, however, to try to assess the presence of a capsule with Gram-stained smear.
INTERPRETATION OF BACTERIAL ISOLATES

• In the sputum and in most other situations, the absence of inflammation makes interpretation of bacterial isolates difficult. There are a few exceptions to this rule, however.
  – One such exception is in the lower urinary tract where bacteriuria without pyuria is a concern in pregnant women, who will have increased risk of pyelonephritis if untreated.
  – Neutropenic patients may have difficulty mobilizing inflammatory cells.
  – Some bacterial infections may typically be associated with minimal inflammation. The two most important are cellulitis produced by group A beta hemolytic streptococci and *Clostridium perfringens*. These infections are characterized by a watery, edematous, spreading inflammation. An additional, more exotic example is anthrax, caused by *Bacillus anthracis*, a bacterium that produces an edema toxin.
• Refusal to culture specimens that lack inflammation is not justifiable, but blind speciation of all isolates from such specimens is not rewarding and a considerable waste of resources.
Bacterial Infection with Minimal Inflammation -- Clostridial myonecrosis.
Clostridial myonecrosis.

In this patient with gas gangrene there is a protein exudate and even at high dry power clearly visible large bacilli. The bacteria are gram-positive, but clostridia decolorize easily and may even appear gram-negative, as they do here. Inflammatory cells are characteristically sparse. The diagnosis of clostridial gangrene requires documentation of the clinical syndrome and isolation of the organism.
DISADVANTAGES OF GRAM STAIN ARE FOR THE MOST PART THE OBVERSE OF THE ADVANTAGES

- Lack of immunological specificity means that only a presumptive diagnosis can be rendered
- Identification is less definitive than that provided by immunological means
- Insensitivity of the Gram smear means that the lack of demonstration of bacteria in a clinical specimen, particularly a sterile body fluid, does not predict accurately the absence of bacteria from that specimen.
- Interpretation of the Gram-stained smear requires considerable experience. Many morphologic forms of bacteria and confusing artifacts may be present in clinical specimens.
NOTES ON SLIDE PREPARATION

In this laboratory the smears are usually prepared by laboratory personnel for your inspection. If you are in a situation where you must prepare your own smears, however, it is important to make the smear so that substantial portions are thin.

A thick specimen must be spread very thinly or diluted.

Tenacious specimens, such as sputum, may be pulled between two glass slides to give a reasonable separation of material.

Use of ringed slides facilitates the observation of relatively non-inflammatory material, where it may be hard to identify the location of the smear on the slide.

Cytospin preparations provide excellent spreads of cells from body fluids.
PROTOCOLS FOR STAINING

• The length of time that crystal violet and Gram’s iodine are left on the smear is not critical. A minimal 10 second staining with these reagents is sufficient.

• The period of time the decolorizing agent is left on the smear depends on the chemical used. In this laboratory acetone, which is a very rapid decolorizer is employed and the exposure should be brief. In general, the decolorizing solution is rinsed across the smear until the decolorizing fluid is no longer blue.

• It is very important, no matter what the abbreviations of the earlier steps are, to leave the counterstain in place for at least 30 seconds. Many gram-negative bacilli are stained very faintly by the counterstain and may be missed if this step is abbreviated. In our laboratory 0.05% basic fuchsin is added to the safranin counterstain to enhance contrast.
FURTHER NOTES ON SLIDE PREPARATION

• The smear should be air dried. It should not be heated to speed up drying, because the heat distorts the morphology of bacteria and cells.

• It should not be placed in front of a fan or waved around the room, because such maneuvers aerosolize material on the slides, including potential pathogens such as *Mycobacterium tuberculosis*.

• These strictures will result in a small delay (as much as 5-10 minutes), but a better smear will result in the end.
IMPORTANCE OF THIN SMEAR

This is a touch preparation from a lung biopsy. Clumps of tissue and much cellular debris are present. If one looks closely, one can perceive in the background darker staining elongated gram negative bacilli, but they are not easy to recognize amidst all the other material in this rather thick area of the smear.
It is much easier to appreciate that innumerable thin, irregular gram-negative bacilli are present in the exudate. They still are not densely stained, again emphasizing the importance of the counterstain. This smear is from the lung of a patient who had Legionnaires’ disease during the 1977 epidemic in Burlington. No bacteria were grown from this specimen, because media that were adequate for recovery of *Legionella* were not available at that time. The Gram stain served as an indicator that the cultural procedures were inadequate.
When evaluating the smear one should scan the slide at low power (10x objective) and roughly quantitate the numbers of cells of different types in the smear. This overall quantitation of cell types will give an appreciation of the inflammatory character of the specimen and potential contamination from mucosal surfaces. For sputum smears our laboratory uses the following scheme for quantitation of cells:

- 1-10/low power field (LPF) = few
- 10-25/LPF = moderate
- >25/LPF = many

For other types of specimens a different scheme is used:

- 1/10 oil immersion fields (OIF) = few
- 1-10/OIF = moderate
- >10/OIF = many
QUANTITATION OF BACTERIA

- <10 organisms/smear = rare
- 1/1-10 oil immersion fields (OIF) = few
- 2-50/OIF = moderate
- >50/OIF = many
We do not quantitate cells and bacteria from fluid specimens that are centrifuged, because the number of cells and bacteria present will depend on the volume of fluid that has been processed. For evaluation of the bacterial morphotypes a thin area of the smear that contains predominantly PMNs should be selected.
There is no difficulty in selecting a field for view in this smear.
CONTAMINATING SQUAMOUS CELLS
Contaminating squamous cells are so mixed in with inflammatory cells that it is impossible to decide which bacteria are associated with the inflammatory component of the specimen. If one or two morphological types predominate it is reasonable to characterize them. If there is a greater mixture of organisms, it is probably best to lump them as mixed flora.
CELL TYPES ONE
MAY ENCOUNTER
IN CLINICAL SPECIMENS
SQUAMOUS EPITHELIAL CELL

- Distinct Border
- Cytoplasm
- Nucleus
- Folded Edge

SQUAMOUS EPITHELIAL CELL
The mature squamous cells shown here are characteristic and easy to identify. Abundant cytoplasm is present and the nucleus is small and hyperchromatic. Although squamous cells may be present in the lower respiratory tract of a patient with chronic bronchitis and squamous metaplasia, they usually signify the presence of oropharyngeal epithelium in sputum specimens. They may also be present in vaginal smears and may indicate vaginal contamination in urine specimens.
POLYMORPHONUCLEAR LEUKOCYTE (POLY)

- Lobed Nucleus
- Granular Cytoplasm
The polymorphonuclear neutrophil shown here is identified by the multilobed nucleus. Earlier precursors of the PMN in an inflammatory exudate may be more difficult to separate from other mononuclear cells.
ALVEOLAR MACROPHAGE

- Fuzzy Border
- Dust Particles
- Nucleus
- "Foamy" Cytoplasm
In respiratory specimens, the alveolar macrophage is a larger cell than the PMN. It contains abundant vaculated cytoplasm and a small nucleus.
Inclusions of various kinds may be present in these cells. They virtually fill the cytoplasm of the cells. These large pigment-containing alveolar macrophages are called dust cells.
Not all processes in the lung or other tissue are inflammatory and other cell types may be present, but the Gram stain is not a good cytological technique for identification of cells. This slide demonstrates tumor cells from a necrotic squamous carcinoma that were expectorated in sputum and detected by Gram’s stain.
End of Part One