Testing Biomaterials

- How to characterize the material that will be processed into a medical device/implant?
- How biomaterials can be evaluated to determine if they are biocompatible?
- How biomaterials can be evaluated to determine whether they function appropriately in the *in vivo* environment?
- How can testing criteria be defined to proper evaluate a given biomaterials application?
  - Some biomaterials complete their intended function in seconds
  - Others are implanted for lifetime (10-70 years?)

Testing Biomaterials

- **Standards**: Consensus standards are documents developed by commitees to represent consensus opinions on test methods, devices, or procedures. Following these standards when testing new materials and/or devices is an advantage, but not mandatory, for getting marketing approval.
- Committees exist at national and international levels. (remember that several metals are even named by their ASTM standards). News and updates regarding european standards can be found at the eupean society for biomaterials webpage: http://www.esbiomaterials.eu/main/index.php. The ESB is a member of the International Union of Societies for Biomaterials Sciences and Engineering (IUS-BSE)

- Technical Committee 194 of the International Organization for Standardization (ISO) meet every spring
- Set of documents 10993 (FDA's version #G95-1):
  - 10993-1: "Guidance on Selection of Tests."
  - 10993-2: "Animal Welfare Requirements."
  - 10993-3: "Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicity."
  - 10993-4: "Selection of Tests for Interactions with Blood."
  - 10993-5: "Tests for Cytotoxicity—In Vitro Methods."
  - 10993-6: "Tests for Local Effects after Implantation."
  - 10993-7: "Ethylene Oxide Sterilization Residuals."
  - 10993-9: "Degradation of Materials Related to Biological Testing."
  - 10993-10: "Tests for Irritation and Sensitization."
  - 10993-11: "Tests for Systemic Toxicity."
  - 10993-14: "Materials Evaluation."

Testing Biomaterials

- *IN VITRO* (cell cultures in glass)
  - rapid
  - inexpensive
  - poor representation of physiological conditions
  - good as the first step

- *IN VIVO* (animal experiments)
  - better approximation to human environment
  - demanding protocols (Animal Welfare Act)
  - right animal model approximate human environment
  - second step prior to clinical use

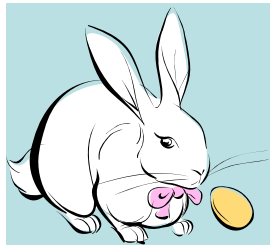
Testing Biomaterials

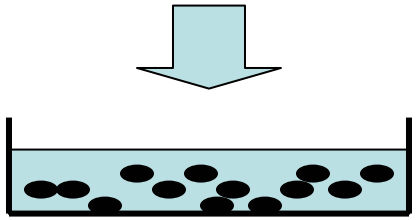
- Testing always leads to experimental variability, particulary tests in living systems.
  - The more complex the system (e.g. Humans vs. cultured cells) the larger the variability that might be expected.
- Statistics should be used at two steps in testing biomaterials.
  - Before an experiment is performed, statistical experimental design will indicate the minimum number of samples that must be evaluated to yield meaningful results.
  - After the experiment statistics will help to extract maximum useful information.
- Detailed protocols are provided by:
  - ASTM (American Society for Testing and Materials) and the
  - ISO (International Standards Organization)
  - FDA (Food and Drug Administration)
  - NIH (National Institute of Health)
  - The EU has its own directives in addition to the ISO standards. Sometimes individual EU-member states has additional, national demands.

Testing Biomaterials

- Cytotoxicity means to cause toxic effects at the cellular level:
  - death,
  - alterations in cellular membrane permeability,
  - enzymatic inhibtion,... at the cellular level.

- Evaluation by methods that use isolated, adherent cells in culture to measure cytotoxicity and biological compatibility.
  - Cells used for culture are most often
    established cell lines from cell banks
    (e.g. American Type Tissue Culture Collection)
    Cultured cell lines can be reproducibly used in many different laboratories, providing comparable results usefull for generating databases
  - Primary cells (with the exception of
    erythrocytes for hemolysis assays)
    are seldom used.
    Primary cells come directly from living tissue
    (can only be propagated a few generations in culture),
    have different genetic backgrounds, giving very high
    statistical varation in test outcome





Testing Biomaterials

- Toxicity:
  - A toxic material is defined as a material that releases a chemical in sufficient quantities to kill cells either directly of indirectly through inhibition of key metabolic pathways.
  - The number of cells that are affected is an indication of the dose and potency of the chemical.
  - If an animal is exposed to an atmosphere containing a noxious substance (**exposure dose**), only a small portion of the inhaled substance will be absorbed and delivered to the internal organs and cells (**delivered dose**).
  - Cell culture methods evaluate target cell toxicity by using delivered doses of the test substance used. –Whereas tests in whole animals relate to the exposure dose... Often resulting in different measurements of sensitivity in the two systems. To compensate for this difference *in vivo* local toxicity models are applied (direct delivery to specific organs)

Testing Biomaterials

- A highly sensitive test system is desirable for evaluating the potential hazards of biomaterials.
  - In cell culture the variables of metabolism, distribution, inflammation, and absorption are minimized and the dosage per cell is maximized to produce a highly sensitive test system.
    Testing at this high margin is considered a **safety factor** for interpolating results to whole humans
  - Typical sources of toxic materials: extractables
    - additives for manufacturability: plasticizers, antioxidants, monomers
    - Leackage from the basic material itself: cobalt, nickel from metal alloys; fluorinated polyesters from Dacron fibers; etc.

Testing Biomaterials

- Migration of chemicals from a solid phase material into liquid solvent is controlled by:
  - Diffusional resitstance within the solid
  - Chemical concentration
  - Time
  - Temperature
  - Fluid turbulence at the solid-solvent interface
  - ....
- Preparation of extractions of biomaterials have been carefully standardized to improve the reproducibility of the data.

- Complete dissolution of biomaterial is an alternative approach for in vitro testing. But:
  - May create degradation products that do not occur in the clinical application.

Testing Biomaterials

Three morphological* cell culture assays are primarily used for evaluating biocompatibility:

- Direct contact
- Agar diffusion
- Elution

- For the results to be comparable the following parameters must be standardized:
  - Number of cells
  - Growth phase of cells
  - Cell type
  - Duration of exposure
  - Test sample size (geometry, density, shape, thickness)
  - Surface area of test sample must be carefully controlled

- Readout: Within the given quantification range:
  - Dose-response curves
  - exposure-effect relationships (Klaassen, 1986)

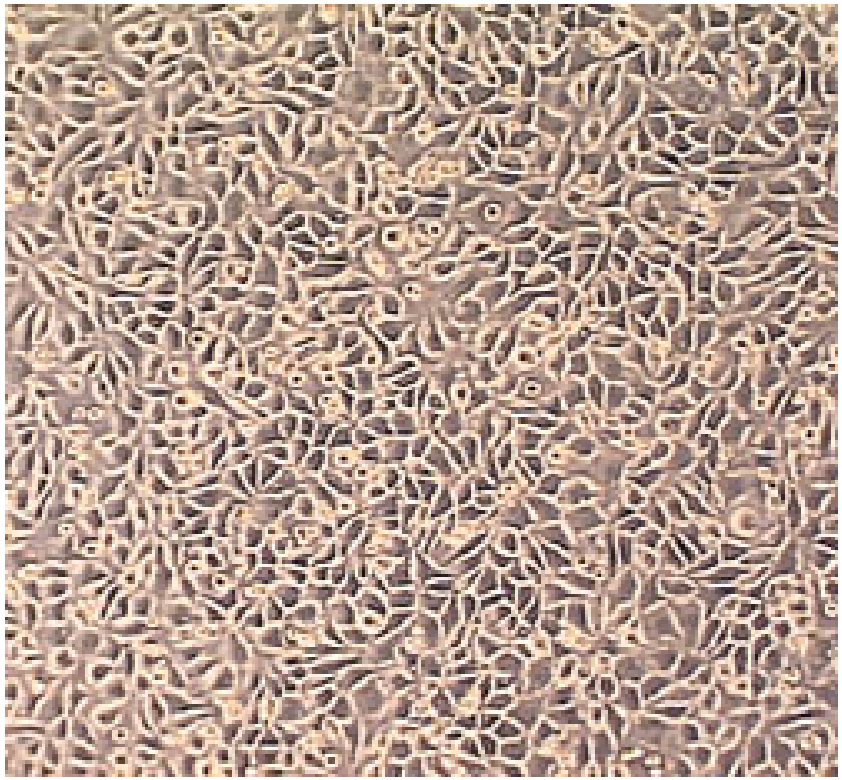
*the outcome of Morphological assays is measured by observation of changes in cell morphology (structure, appearance)

Testing Biomaterials

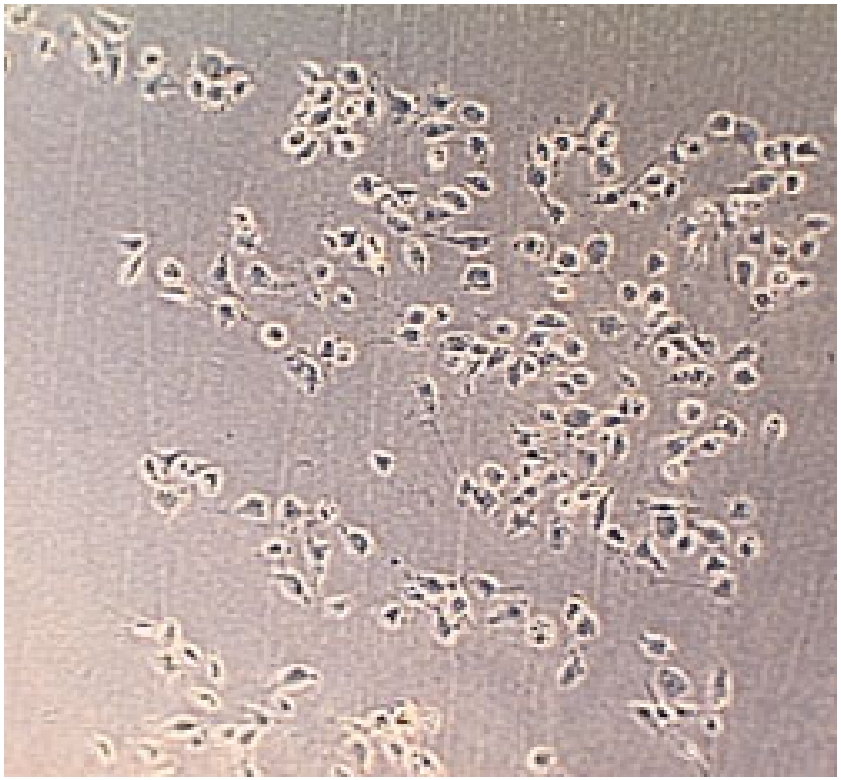
- Direct contact
  - monolayer, confluent cell culture, L-929 mouse fibroblasts
  - biomaterial in direct contact
  - 24 hours, 37±1°C
  - cells may
    - change morphology
    - die
    - lose adherence to dish

  - Cells are fixed and stained (hematoxylin blue: stains live adherent cells)
  - toxicity=dead/live !
- Why L-929?
  - easy to maintain
  - good correlation with animals tests (Northup 1986)
  - Resemble fibroblasts present in wound healing (=often the first cells to attach to implanted biomaterials *in vivo*)
  - In specific cases other, similar cell types may be used

Testing Biomaterials



A confluent monolayer (100 x magnification) of well-defined L929 mouse fibroblast cells exhibiting cell-to-cell contact. This appearance is indicative of a non-cytotoxic (negative) response

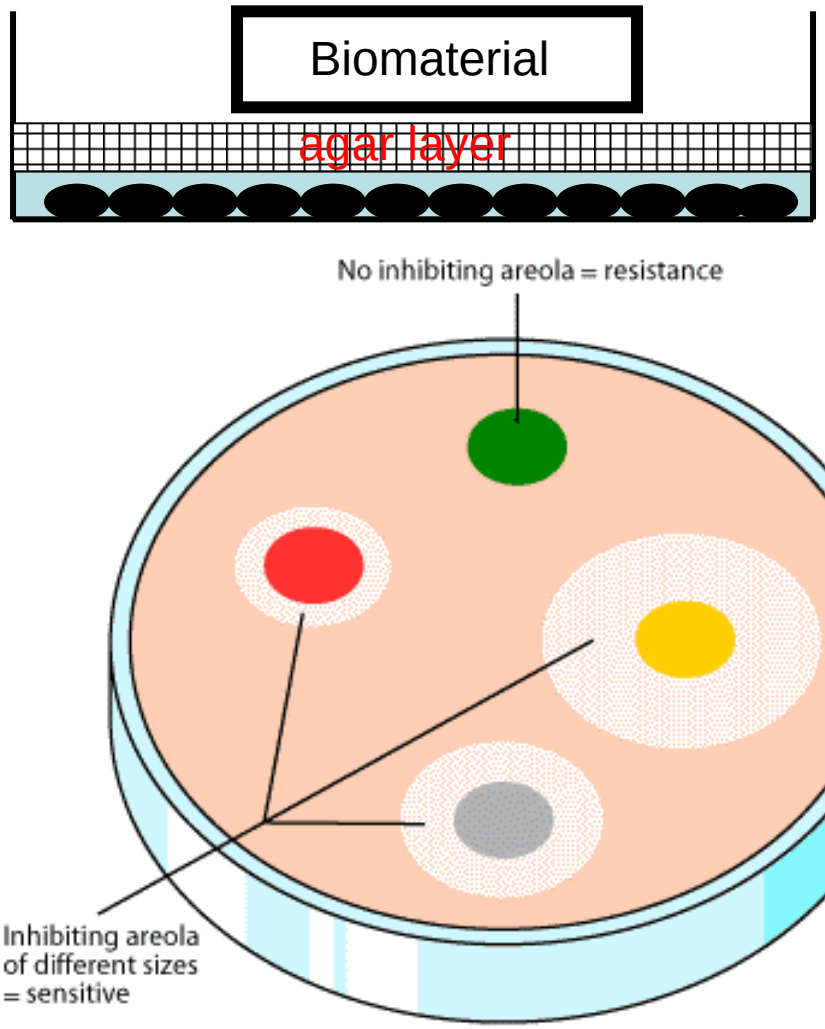


L929 mouse fibroblast cells (100 x magnification) that illustrate a positive cytotoxic reaction; the considerable open areas between cells indicate that extensive cell lysis (disintegration) has occurred.

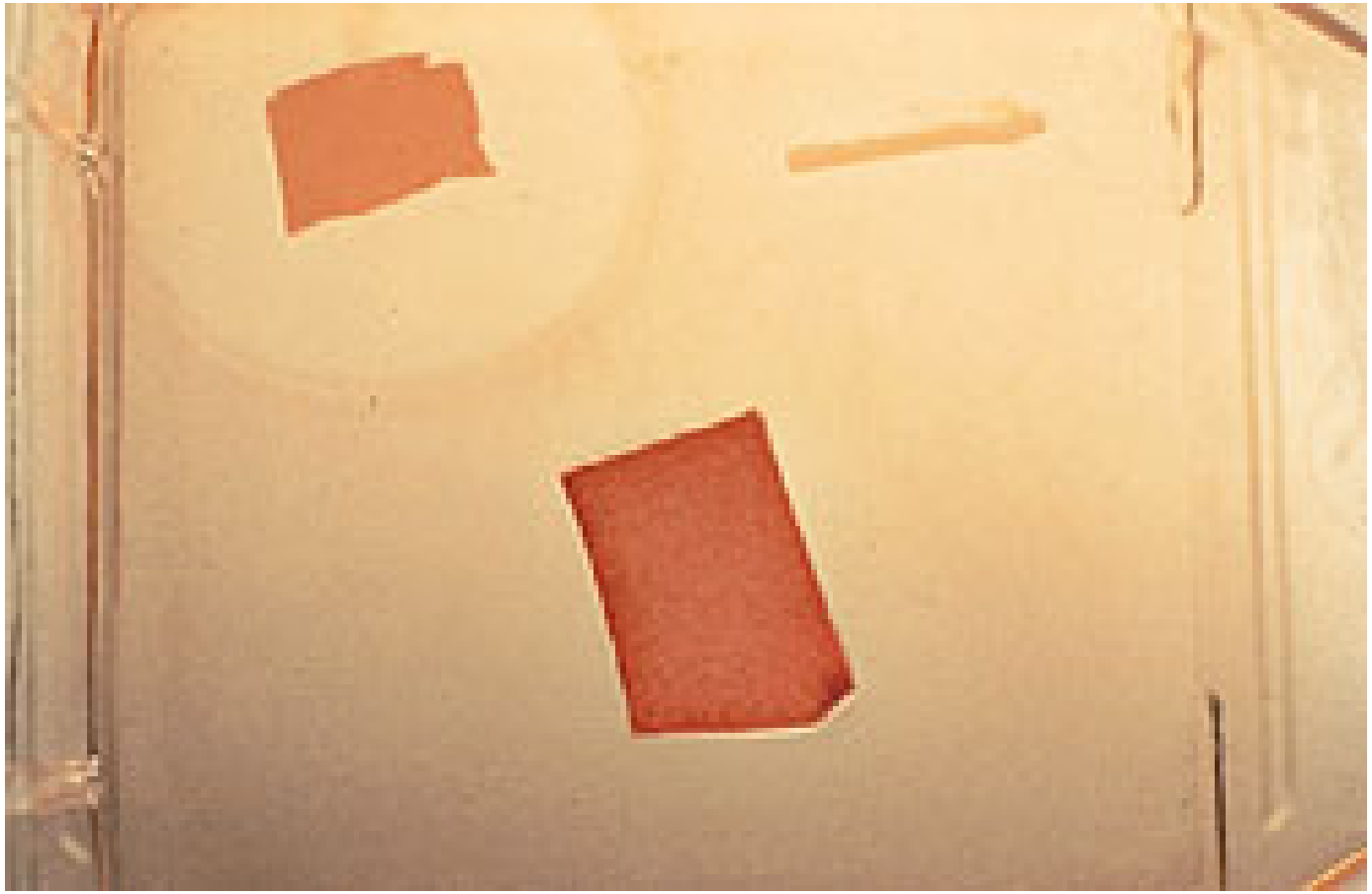
Courtesy R. Zenz

Testing Biomaterials

- Agar diffusion
  - agar layer between cells and biomaterial
  - agar: gel-like polymer derived from red alga
  - chemicals diffuse through agar
  - use special stain, embedded in the agar, to label live cells
  - Death of injured cells remain colorless (area of unstained dead cells around the biomaterial)
  - Toxicity is evaluated by the loss of vital stain



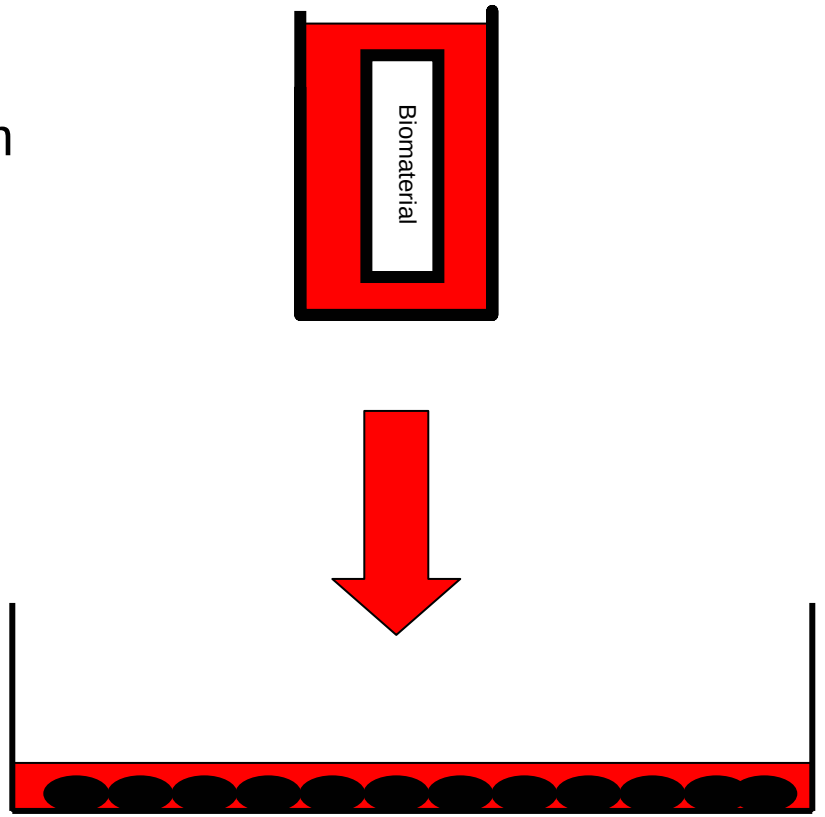
Testing Biomaterials



An agar diffusion flask containing a sample of positive control material. The discoloration that extends outward from the material indicates that the presence of the sample has caused the cells to lyse, losing the vital stain incorporated in the agar layer.

Testing Biomaterials

- Elution
  - prepare extract of a material
  - how? Standardization needed! (0.9% sodium chloride or serum free medium)
  - chemicals will leak into solution
  - apply solution to cell-culture (48h at 37°C)
  - perform stain based viability tests, microscopic evaluation.
  - experience in recognizing cell culture morphology is required.



Testing Biomaterials

- The methodologies for the three primary cell culture assays are described in the:
  - U.S: Pharmacopeia (Pharmakopöe, amtl. Arzneibuch)
- And standards published by the:
  - ASTM (American Society for Testing and Materials)
  - BSI (British Standards Institute)
  - ISO (International Standards Organization)
- Pharmacopeial* assays are legally required by the ministries of health in the US, Europe, Australia, Japan, and other countries. The ISO standards are expected to gradually replace national standards in Europe

* Pharmacopeia = a compendium containing directions for the identification of samples and the preparation of compound medicines, published by the authority of a government or a medical or pharmaceutical society. In this particular case referring to the methods listed above.

Testing Biomaterials

TABLE 1 Advantages and Disadvantages of Cell Culture Methods

	Direct contact	Agar diffusion	Elution
Advantages	Eliminate extraction preparation	Eliminate extraction preparation	Separate extraction from testing
	Zone of diffusion	Zone of diffusion	Dose response effect
	Target cell contact with material	Better concentration gradient of toxicant	Extend exposure time
	Mimic physiological conditions	Can test one side of a material	Choice of extract conditions
	Standardize amount of test material or test indeterminate shapes	Independent of material density	Choice of solvents
	Can extend exposure time by adding fresh media	Use filter paper disk to test liquids or extracts	
Disadvantages	Cellular trauma if material moves	Requires flat surface	Additional time and steps
	Cellular trauma with high density materials	Solubility of toxicant in agar	
	Decreased cell population with highly soluble toxicants	Risk of thermal shock when preparing agar overlay	
		Limited exposure time	
		Risk of absorbing water from agar	

Testing Biomaterials

- After the cytotoxicity profile more application-specific tests are performed to assess the biocompatibility of the material:
  - Products for **in vitro fertilization** procedures would be tested for adverse **effects on a very low cell population**.
  - A new **material for culturing cells** would be assayed by comparing growth rates of cells in contact with the new material with those of **currently marketed materials.**
  - Current experience: a material **non-toxic in vitro** will be **non-toxic in in vivo** assays.
  - But: the **clinical acceptability** of a material depends on **many different factors**; target cell toxicity is but one.

Testing Biomaterials

- In vivo testing: critical for development of clinical devices
  - *In vitro* tests cannot replace *in vivo* tests:
    - no inflammation
    - no immune response
    - single cell type
    - no tissue remodeling
    - No acquired toxicity through processing (eg the liver modifies many foreign compounds)
  - *In vivo* tests provide:
    - interactions of different cell types
    - effects of hormonal factors
    - interactions with extracellular matrix
    - interactions with blood-borne cells, proteins and molecules
    - Overall determination of: wether the device performs as intended and provides no significant harm to the patient or user.
  - The ISO 10993 standard, “Biological evaluation of Medical Devices” presents a systematic approach to the *in vivo* assessment of tissue compatibility of medical devices. The Standard is extended and upgraded continuosly

Testing Biomaterials

- Implant effects can be simulated *in vivo*:
  - insoluble particulate materials released by implants
  - interaction of biological factors with the implant
  - mechanical loading experienced by device
  - Time is an important variable (implant–related factors act with different time constants on the biological factors)
  - The tissue response to an implant is the cumulative physiological effect of:
    - Modulation of the acute wound healing response due to the surgical trauma of implantation and the presence of the implant.
    - The subsequent chronic inflammation reaction, and
    - Remodeling of surrounding tissue as it adopts to the implant.

Testing Biomaterials

- Mechanical loading experienced by biomaterial:
  - increased local strain due to movement of device with respect to tissue:
    - hyperplasia (increased scar tissue, thicker fibrous encapsulation)
  - reduction in tissue strain due to presence of implant
    - implant takes all load: tissue undergoes atrophy (*stress shielding*)

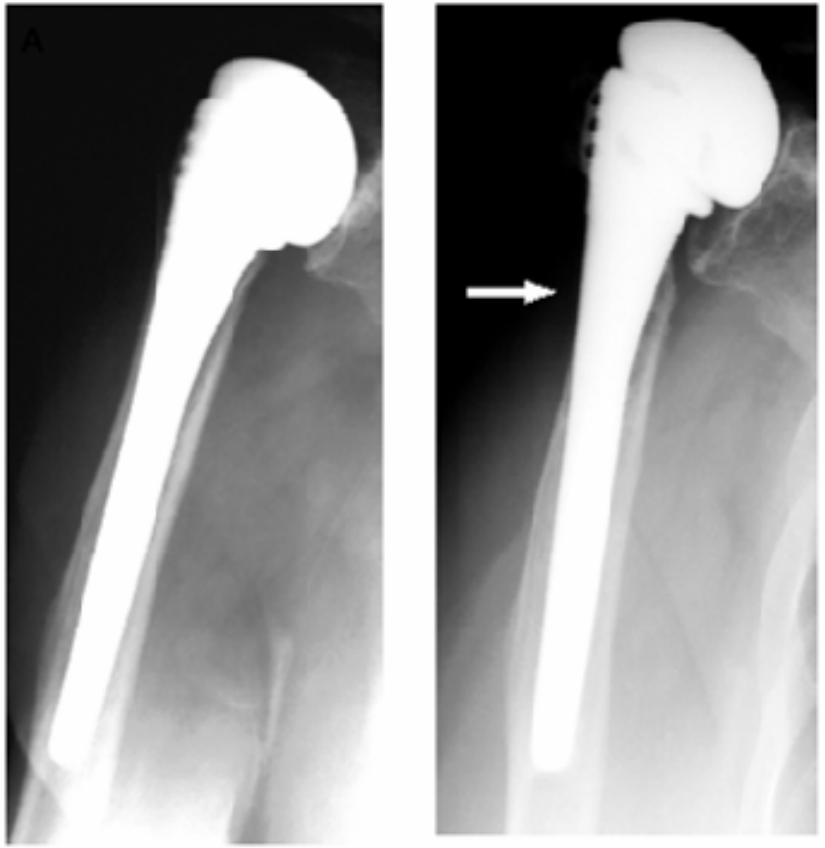


Figure 1. Example of stress shielding. (A: postoperative image. B: image of the same patient after 7 years of follow-up. Arrow indicates stress shielding)

Testing Biomaterials

- Implant sites in animal models:
  - Similarity to the site to be employed in human use of the medical device.
  - The healing and remodeling characteristics of the 4 basic types of tissue should be considered:
    - Connective tissue
    - Muscle
    - Epithelia
    - Nerve
  - In selecting an implant site consider the following:
    - Vascularity
    - Nature of the parenchymal cells (capability for mitosis and migration: determine the regenerative capability of the tissue)
    - Presence of regulatory cells (macrophages and histiocytes)
    - Effects of mechanical strain (hyperplasia, atrophy)

Testing Biomaterials

- Surgical wounds in avascular tissue (e.g. cornea, inner third of meniscus) may not heal:
  - limited potential of the proliferation and
  - migration of surrounding parenchymal cells into the wound site.
  - Gaps between an implant and surrounding avascular tissue can remain indefinitely.

- Implant sites in vascular tissue in which the parenchymal cell does not have the capability for mitosis (e.g. nerve tissue) heal by the formation of scar.

- Macrophages, along with fibroblasts of the scar often form a definable layer of cells that surround an implant: „fibrous encapsulation“.

Testing Biomaterials

- For orthopedic prostheses bone has been used as the site of implantation:
  - But the densitiy of bone formation depends on the site of implanation:
    - Cortical and cancellous bone differ in vascularization and the size of the pool of preosteoblasts that proliferate in response to surgery.

- Cutaneous or subcutaneous sites chosen to assess biocompatibility
  - readily accessible
  - thickness of fibrous capsule measure of biocompatibility
  - guinea pig

Testing Biomaterials

- Paravertebral muscle of rats, rabbits, and dogs to detect toxic leach:
  - Due to the relative motion between the implant and surrounding muscle and the
  - limited capability of the skeletal muscle for regeneration,
  - scar tissue forms around the implant.
  - Thickness of fibrous encapsulation measure of biocompatibility.

- Epithelia:
  - E.g.: Substances that might be used as temporary covering materials to facilitate re-epitheliazation of skin wounds.
  - Epidermal wounds have been produced experimentally by:
    - Heat
    - Chemical agents
    - Excision of tissue

Testing Biomaterials

- Materials for vascular prostheses have been evaluated for their blood compatibility as replacements segments in selected vesssels in various animal models:
  - Carotis-jugular and
  - Femoral arteriovenous shunts

- Nerve:
  - Nerve cells do not have the capability for division
  - The elongation of several axons allow a degree of regeneration across defect sites.
  - Certain matrices facilitate the elongation of such axons thereby accelerating the regeneration of the nerve and restoration of some function:
    - Peripheral nerves of rats

Testing Biomaterials

- Controls for in vivo investigations of tissue compatibility can include:
  - Contralateral intact tissues as anatomic controls:
    - No implant is inserted; the amount of scar formed can help to evaluate the fibrous capsule formation around the implant at the test site.
  - Sham-operated controls:
    - E.g.: A shame-operated limb can display the effects of altered load bearing on the recipient tissue.
  - Material and device controls
    - E.g.: femoral stems of total hip replacement prostheses should be of identical shape and size.

Testing Biomaterials

- Evaluation of tissue reaction:
  - Histology and histochemistry:
    - Qualitative determination of the relative numbers of various cell types.
  - Immunoshistochemistry:
    - Allows specific cell types and extracellular matrix components around an implant to be identified.
  - Transmission electron microscopy (TEM):
    - Ultrastructural examination of cells at the interface of implants.
  - Scanning electron microscopy (SEM)
  - Biochemistry:
    - Level of inflammatory mediators
    - But: Manipulation of tissues at or after explantation of a biomaterial can dramatically alter the production and release of cellular mediators.

Testing of Blood-Material Interactions

- Many devices and materials used have blood contact:
  - Heart-lung machine
  - Hollow fiber hemodialyzer for treatment of kidney failure
  - Catheters for blood access and
  - Blood vessel manipulation (angioplasty)
  - Heart assist devices
  - Stents
  - Prosthetic heart valves
  - Vascular grafts
- A device made of blood-compatible materials is not automatically blood compatible!
- No widley recognized, standard list of blood compatibility tests exists.

Testing of Blood-Material Interactions

- Many existing devices are frequently modified to improve durability and mechanical characteristics.
  - Changes may also affect blood response (is not entirely predictable): testing is required to document safety

- The performance of many existing devices is also less than optimal:
  - Prolonged heart lung machine can produce a tendency to severe bleeding.
  - Mechanical heart valves occasionally shed emboli to the brain, producing stroke.
  - Many devices are only „safe“ when anticoagulating drugs are used (e.g. Oxygenator, heart valves, hemodialyzer).

Thrombogenicity

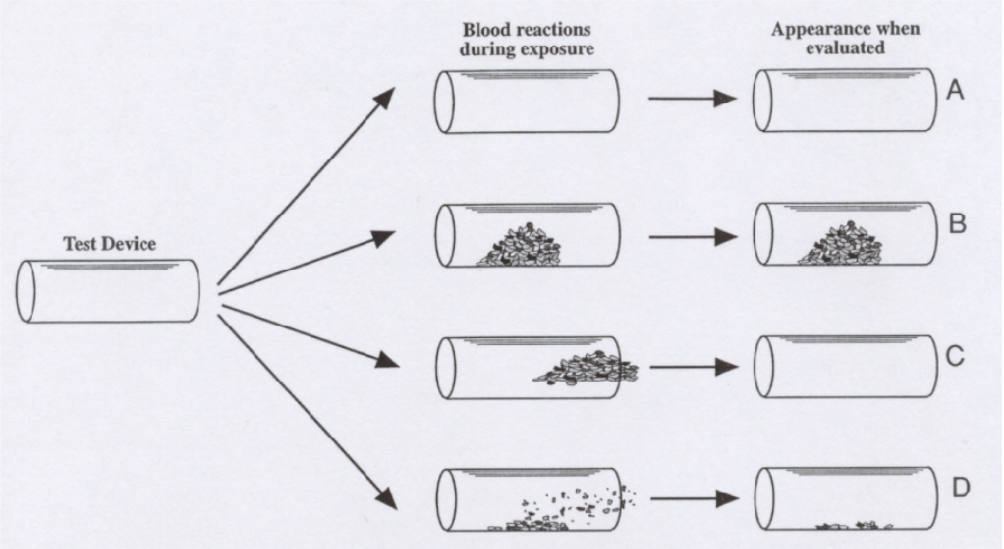
- Local effects:
  - A thrombogenic device may cause the accumulation of various blood elements (thrombus formation).
  - Cardiovascular devices may also exhibit regions of disturbed flow or stasis which lead to formation of blood clots.
  - These local effects can compromice device function:
    - Delivery of blood through artificial blood vessels
    - Mechanical motion of heart valves
    - Gas exchange through oxygenators
    - Removal of metabolic waste (hemodialyzer)

Thrombogenicity

- The local blood reaction may produce systemic effects:
  - Thrombi may detach (embolize) and impair blood flow in peripheral vessels.
  - Chronic devices may „consume“ circulating blood elements:
    - Mechanical destruction of red blood cells by heart prostheses or dialyzers.
    - Removal of platelets as a result of continuing thrombus formation.
    - Mediators of inflammatory responses and vessel tone may be produced or released from cells (platelets, white cells,..).

Thrombogenicity

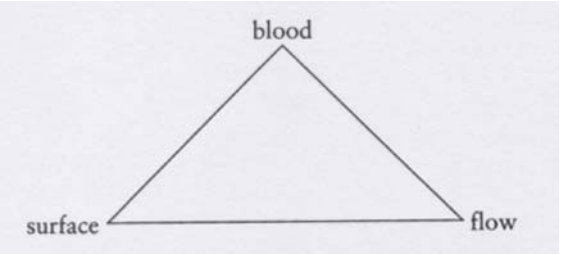
- The types of devices used are:
  - numerous, exhibit complex flow geometries, and are continuously evolving.
- The possible blood responses are:
  - numerous, complex, dynamic, and not fully understood.
- It is difficult and expensive to measure device thrombogenicity in an extensive and systematic way (experiment. animals or humans).
- Alternative interpretations can be applied to data from „blood compatibility“ tests.



Testing of Blood-Material Interactions

- We cannot generally:
  - Extrapolate results obtained under one set of test conditions to another set of conditions.
  - Use short-term testing to predict long-term results.
  - Predict in vivo performance of a device based on blood-material interactions of materials per se in idealized flow geometries.

- 3 factors contribute to the coagulation of the blood:
  - The blood chemistry
  - The blood-contacting surface
  - The flow regime



Virchow´s triade (1856)

Testing of Blood-Material Interactions

- The source and methods for handling blood can have important effects on blood material interactions.
- Initial adhesiviness of blood platelets for artificial surfaces appears to be
  - low in man and some primates and
  - high in the dog, rat and rabbit.
- Animal blood donors are relatively homogenous:
  - Age, health status, blood response
- In vitro testing generally requires anticoagulation of the blood (can have profound effects).
- In vivo testing and the use of extracorporeal circuits are also commonly performed with anticoagulats:
  - Sodium citrate (chelates Ca^{2+})
  - Heparin (used to block thrombin)

Testing of Blood-Material Interactions

TABLE 2 Blood–Materials Responses and Their Evaluation

System	Blood response	Assessment[a]
Device/material	Thrombosis	Direct visual and histologic evaluation; noninvasive imaging (angiography, ultrasound, radioisotope, magnetic resonance); evidence of device dysfunction.
	Thromboembolism	Emboli detection (ultrasound, laser); evidence of organ/limb ischemia, stroke.
Platelets	Consumption	Increased removal of radioisotopically labeled cells; reduced blood platelet count.
	Dysfunction[b]	Reduced platelet aggregation *in vitro;* prolonged bleeding time.
	Activation	Increased plasma levels of platelet factor 4 and β-thromboglobulin; platelet membrane alterations (e.g., by flow cytometry).
Red cells[b]	Destruction	Decreased red cell count; increased plasma hemoglobin.
White cells[b]	Consumption/activation	Decreased counts of white cell populations; increased white cell plasma enzymes (e.g., neutrophil elastase).
Coagulation factors	Consumption[b]	Reduced plasma fibrinogen, factor V, factor VIII.
	Thrombin generation	Increased plasma levels of prothrombin fragment 1.2 and thrombin : antithrombin III complex.
	Fibrin formation	Increased plasma level of fibrinopeptide A.
	Dysfunction[b]	Prolonged plasma clotting times.
Fibrinolytic proteins	Consumption[b]	Reduced plasma plasminogen level.
	Plasmin generation	Increased plasma level of plasmin : antiplasmin complex.
	Fibrinolysis	Increased plasma level of fibrin D-dimer fragment.
Complement proteins[b]	Activation	Increased plasma levels of complement proteins C5a and C3b.

[a]Radioimmunoassays (RIA) and enzyme-linked immunoassays (ELISA) may not be available for detection of nonhuman proteins.
[b]Tests which may be particularly important with long-term and/or large surface area devices.

Testing of Blood-Material Interactions

- In vitro tests:
  - Usually of short duration
  - Strongly influenced by the blood source, handling methods, the use of anticoagulants
  - Can not predict longer term BMI and in vivo outcome events
  - Useful in screening materials
- In vivo tests:
  - Insertion for short/long periods into the arteries or veins of experimental animals.
    - Arteriovenous (AV) or
    - Arterioarterial (AA) shunt

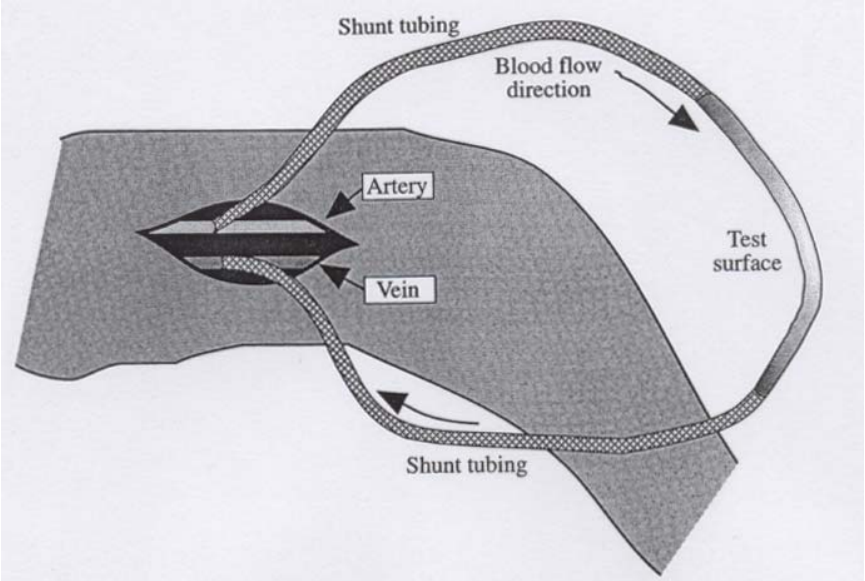
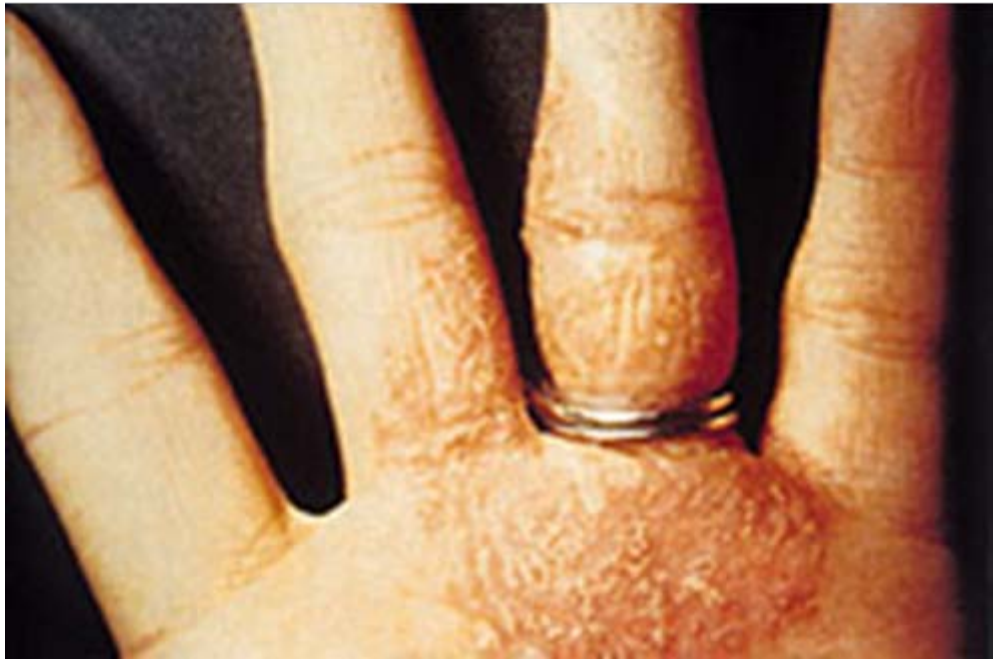


FIG. 3. Illustration of an arteriovenous (A-V) shunt placed between the femoral artery and vein (in the leg) of an experimental animal. Materials to be tested (in this case a tubular device) are interposed between inlet and outlet segments of the shunt.

Testing Biomaterials

- Sensitization:
  - Prolonged contact with a chemical substance that interacts with immune system
  - Skin widely used since most reactions to biomaterials are cell-mediated type
  - Dermal sensitization marked by redness and swelling

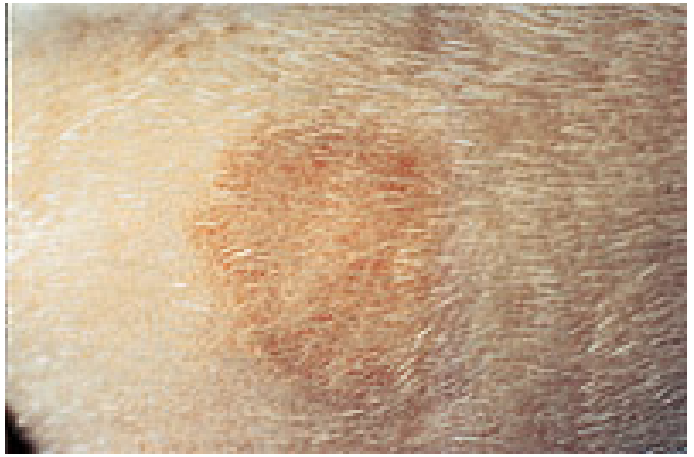


Sensitization (rash) to latex gloves

Testing Biomaterials

- Sensitization test methods (guinea pigs):
- repeated patch (Buehler):
  - Induction phase: expose shaved back directly to material under occlusive dressings. 6 hours/day, 3 days/week, 3 weeks
  - Recovery phase: 2 weeks rest to allow for development of response
  - Final exposure

- maximization (Magnuson-Kligman): used for materials that will contact areas other than the skin:
  - fluid extracts of test material prepared in saline or vegetable oil
  - inject extract with an adjuvant agent that will enhance immune response
  - two weeks rest
  - apply extract topically



Positive maximization test in guinea pig

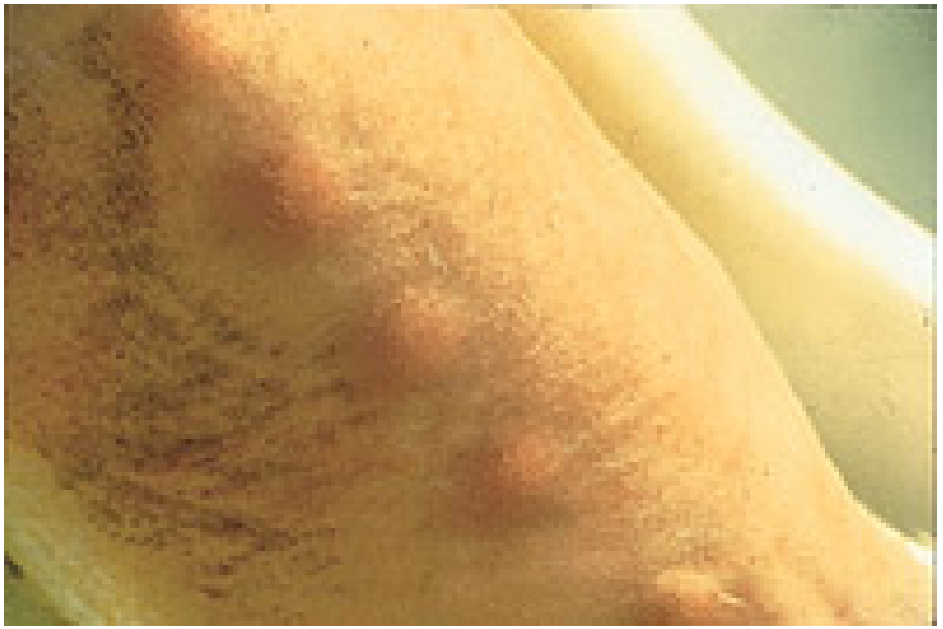
Testing Biomaterials

- Irritation: local tissue response characterized by the usual signs of inflammation:
  - redness
  - swelling
  - heat
  - pain

- In vivo tests for irritation:
  - intracutaneous
  - primary skin
  - ocular

Testing Biomaterials

- Intracutaneous test:
  - albino rabbits
  - prepare fluid extract under controlled temperature, duration, material surface/volume ratio (water and oil based solvent)
  - extract injected into multiple sites the skin (+ control injections)
  - observe for evidence of redness and swelling at 24h, 48h, 72h
  - aggressive test, extract pre-pared under exaggerated conditions
    - maximizes the chance of finding irritant chemical



Intracutaneous irritation test using albino rabbits

Testing Biomaterials

- Primary skin test
  - less aggressive than intracutaneous
  - placement of material on shaved back of albino rabbits
  - cover with occlusive dressing
  - apply between 4-24 hrs
  - observe for 72 hrs
  - score for redness and swelling
  - compare with known values for primary skin irritation
  - categorize the response: negligible, slight, moderate, severe

Testing Biomaterials

- Ocular test:
  - used for eye contact products
  - fluid extracts (occasionally solids or powders)
  - placed directly into the pocket of the lower eyelid of an albino rabbit
  - other eye untreated, control
  - observe regularly up to 72 hours
  - score based on:
    - swelling and redness of conjunctiva
    - response of iris to light
    - corneal opacity
    - presence of discharge

Testing Biomaterials

- Systemic effects:
  - Effects of released chemicals on liver, heart, kidneys, and brain
  - Mice and rats; Various routes of application
    - dermal
    - inhalation
    - intravenous
    - intraperitoneal
    - oral
  - Application:
    - fluid extracts (intraperitoneal or intravenous)
    - implantation of material (particularly biodegradable ones) (intramuscular, intraperitoneal, subcutaneous)
  - Collect
    - blood samples (hematology, serum chemistry)
    - tissue samples (pathology)

Testing Biomaterials

- A hierachy of testing, starting with in vitro systems and progressing through functionality implants in situ is implied.

Cell culture cytotoxicity (mouse L929 cell line)
Hemolysis (rabbit or human blood)
Mutagenicity (human or other mammalian cells or Ames test (bacterial)
Systemic injection acute toxicity (mouse)
Sensitization (guinea pig)
Pyrogenicity (limulus amebocyte lysate [LAL] or rabbit)
Intracutaneous irritation (rabbit)
Intramuscular implant (rat, rabbit)
Blood compatibility (rat, dog, primate, etc.)
Long-term implant (rat)