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Reducing Pyrogens in Cleanroom Wiping Materials

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In many instances, it is no longer adequate to show that a pharmaceutical or medical device is sterile, but one also must demonstrate that it is "pyrogen free." This article provides practical information on reducing pyrogens in cleanroom wiping materials prior to production, as well as eliminating residual pyrogens through sterilization.

In practice, pyrogen-free means that it has a level of pyrogens or endotoxins below a pre-defined threshold concentration which is sometimes referred to as "non-pyrogenic." Pyrogens are fever-causing materials from the cell walls of Gram-negative bacteria. Few methods of sterilization destroy pyrogens. Pyrogen control requires a "defense-in-depth" approach with cleanrooms, clean working surfaces, clean equipment as well as clean chemicals and biologicals. Fabrics and liquids used to clean surfaces in pharmaceutical and biomedical production areas need to be made with minimal bioburden to reduce the level of pyrogens after sterilization. This means starting with clean materials, keeping them clean during processing, sterilizing them effectively and auditing the level of pyrogens in the materials so produced. Such procedures have been found to enable production of cleanroom wiping materials that meet the United States Pharmacopeia 23 pyrogen standard[1] for medical devices that contact the blood or lymph in circulation, <20 endotoxin units/item (EU/item), even though wipers are not required to meet such strict standards at this time.

Introduction

Some pharmaceuticals and biomedical devices need not only to be sterile (have no living organisms present), but also must be free of pyrogens. The most common pyrogens are lipopolysaccharides (LPS) from the cell walls of Gram-negative bacteria, the kind of bacteria that often grow in ultra-pure water. "Pyrogens" and "endotoxins" are used as though almost synonymous; however, an endotoxin generically is a toxin released after destruction of the cell in which it was produced. Some forms of terminal sterilization (dry heat, gamma irradiation) can reduce the pyrogen levels on products, but may be relatively difficult and expensive and may harm the product itself. Some forms of terminal sterilization (moist heat, ethylene oxide) may not sufficiently reduce the pyrogen levels. Thus, the first line of defense is to reduce the likelihood of bacterial contamination whether or not terminal sterilization is to be used. Procedures like aseptic processing, for example, reduce both microbes and pyrogens.

Aseptic techniques are carried out in cleanrooms, typically Federal Standard 209E (US General Services Administration, 1992)[2] Class 100 or cleaner, which means the air has less than 100 particles per cubic foot (3,500 per cubic meter) that are 0.5µm in size or larger. Such rooms are regularly cleaned and disinfected, the personnel wear cleanroom garments including head and foot coverings, gloves and face masks, and the equipment is cleaned and disinfected, with validated procedures. Materials brought into the room are sterile or are sterilized (generally autoclaved) before they enter. Because there is not a terminal sterilization step, aseptic fill operations are placed under particularly intense regulatory scrutiny and require particular care.[3]

The need for sterility and minimal pyrogenicity decreases in the following order for products that contact:

- the cerebrospinal fluid
- lymph, blood in circulation
- internal organs and tissues
- the gastro-intestinal tract
- epidermal layers

Thus, the cerebrospinal fluid device limit is 2.15 endotoxin units per device (2.15EU/device) and that for lymph or blood is 20EU/device. The requirements for the environment, materials and equipment involved in making these various kinds of products will reflect the requirements on the products themselves. Similarly, the requirements for cleaning materials will be influenced by the end use of the product.

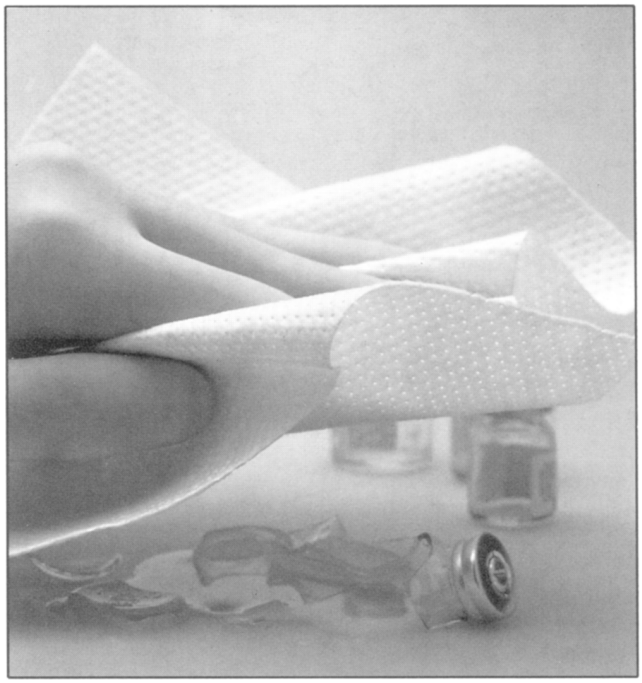

Figure 1. A sterile wiper made from a blend of polypropylene and cellulose fibers.

Materials for Wiping Clean

Recently, the cleaning of aseptic fill clean zones was reviewed.[4] Cleaning is performed with wipers that are clean and sterile. The cleanest are made from continuous filament polyester or other artificial material, having sealed edges, laundered and sterilized with gamma radiation by the manufacturer or sterilized in an autoclave. Figure 1 shows a sterile wiper made from a blend of polypropylene and cellulose fibers. Cleaning liquids for aseptic zones are filtered through 0.2µm-pore filters into sterilized containers or have been terminally sterilized by autoclaving or by irradiation. Wipers and cleaning liquids are sometimes combined and sterilized. Cleaning products are usually purchased double-bagged so the interior bag can be kept clean until removed and the product kept clean until used. The bagging must be able to be handled by shipping and laboratory personnel, and there is substantial literature available on testing packaging for physical and biological barrier qualities.[5] Sterilization by radiation has the advantage, however, that the packaging need not be permeable nor capable of withstanding

temperatures higher than the boiling point of water. Gamma radiation at sterilizing doses is compatible with most plastics, except tetrafluoroethylene, some Nylons and some polypropylenes.

Wipers need to be absorbent, clean, robust, sterilizable, compatible with the cleaning and disinfecting chemicals, easy to use and non-abrasive.

Liquids used in cleaning must be safe and effective. Safe means not only not hazardous to the production personnel, but also not to the users of the product being made, so its ingredients must be non-contaminating or at least innocuous. Effective means removing contamination and often it means disinfecting as well. Liquids for cleaning range from deionized water to mixtures of deionized water and isopropyl alcohol (IPA) to aqueous solutions containing various surfactants and pH-adjusters and disinfecting chemicals.

Water may not quite be “the universal solvent,” but it dissolves polar materials well, evaporates relatively slowly, and is non-flammable and non-contaminating, “environmentally friendly.” As rusting iron often attests, water can oxidize materials, changing their chemical composition. Another disadvantage of water is that it is not toxic to microorganisms, which readily contaminate it. Water is fine for dilution and, being a polar solvent, for dissolving soluble ionic compounds, but many cleaning tasks and sanitizing tasks require something stronger.

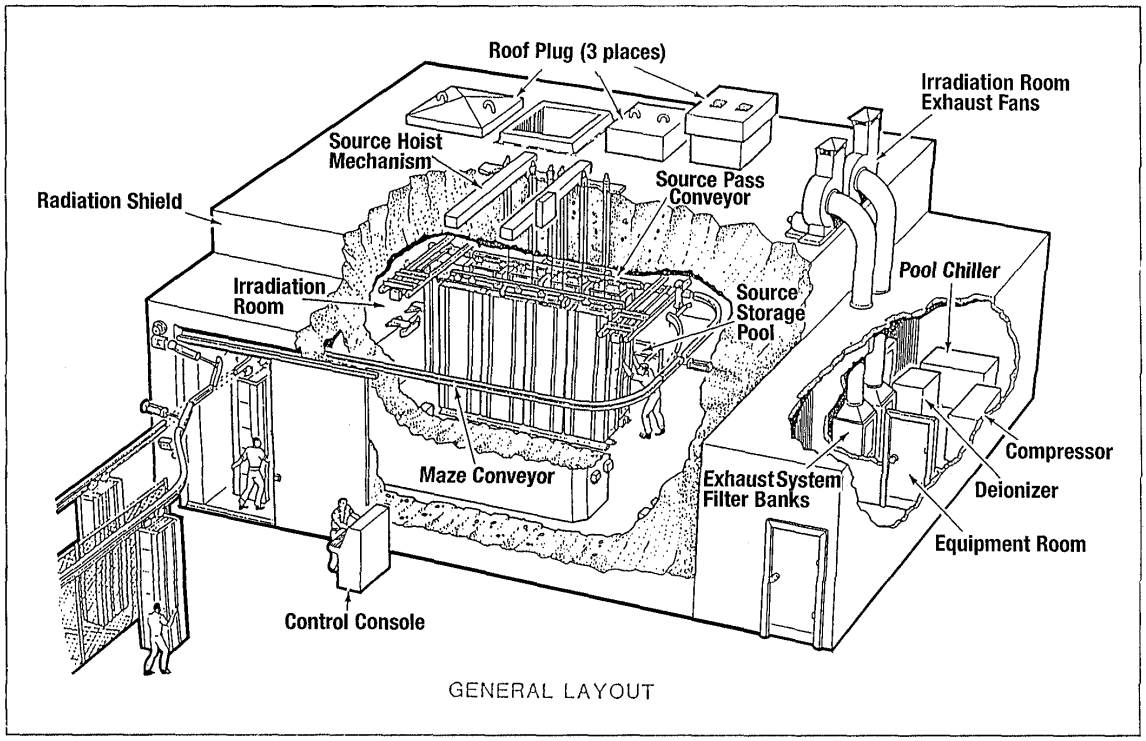

Figure 2. Gamma radiation facility (courtesy of Isomedix, Inc.).

IPA lowers the surface tension in aqueous solutions to assist soil removal, dries rapidly and can dissolve certain hydrocarbons, and 91 percent IPA/water (the azeotrope) or 70 percent IPA/water is not only good at dissolving ionics and some organics, it is a moderately good disinfectant although not a sporicide. [IPA and ethanol are hard to manufacture in sterilized form since most aseptic-filling operations do not have the explosion-prooffacilities needed and terminal sterilization is available in the US only for relatively small volumes, again for safety reasons.] There are other effective (often more effective) germicides that can be employed: glutaraldehyde, hydrogen peroxide, formaldehyde, chlorine dioxide, peracetic acid, other chlorine compounds,

phenolic compounds, iodophor compounds and quaternary ammonium compounds.[6] Some facilities like to rotate the cleaning chemicals to prevent survival of resistant strains of microorganisms. Relative safety in use, as well as issues like odor and tackiness of the residue or just the presence of any residue, also will influence the selection of the chemical agent.

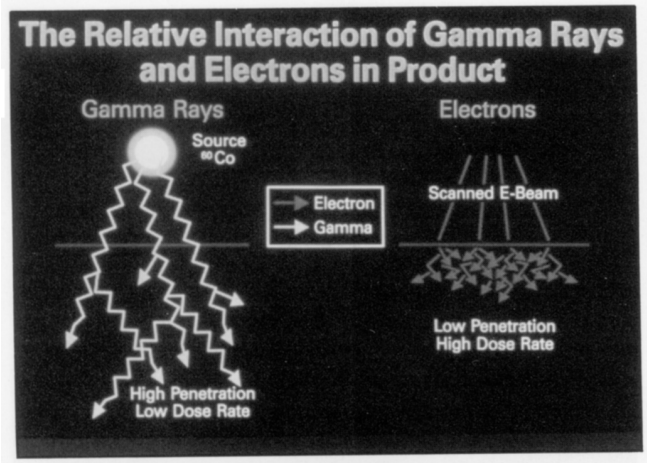

Figure 3. Comparison of gamma versus electron-beam radiation. (Courtesy of Isomedix, Inc.)

Residues left after cleaning may come from the original contamination, the wiper or the cleaning liquid. Cleaning validation is often carried out by using ultra-clean pieces of fabric or swabs to wipe the area and then be analyzed, such as by total organic carbon (TOC) through transformation to carbon dioxide and detection with infra-red absorption. Using swabs with polyester heads dipped in 1N sodium hydroxide, 59 to 111 percent recovery was realized of TOC from e. coli in fermentation residue, a "most challenging case."[7] *Validation of Aseptic Pharmaceutical Processes* is a useful reference for validation in aseptic environments.[8]

Where there are questions about the cleaning procedures, in-house validation can be carried out by repeated cleaning of the same object or area followed by analysis of the area or object or analysis of the cleaning materials. The challenge can range from colored or fluorescent dyes to naturally occurring organisms to pyrogens applied deliberately to the area. Assays can range from visual, fluorescence, to contact plate or wet culture medium recovery and incubation to LAL analysis of the cleaning liquid for pyrogens.

The principles for cleaning cleanroom surfaces are:[4] fold the wiper to provide even pressure and allow creation of clean regions by unfolding, use an orderly progression from cleanest to least clean areas:

- ceiling (nearest filters) to floor
- floor: toward entrance
- clean hoods: top to bottom, wall (nearest filters) to front opening
- tables: farthest from personnel or equipment toward personnel or equipment in parallel strokes with slight overlap avoiding recontamination of cleaned areas.

The wiper is changed or at least folded to a clean area frequently after between a few and a dozen wiper lengths of wiping. [More precisely, the number of wiper lengths should be less than 1/r, where r is the fraction of the contaminants on the wiper that redeposit in one wiper length.].

An idealized wiping sequence would be:

1. dry wiping to pick up particles and droplets
2. wet wiping with a cleaning solution to remove adherent material

3. wet wiping with deionized water to remove residual cleaning solution
4. dry wiping to pick up residual water OR
5. final wiping with a disinfectant to leave a disinfecting film

Often some of these steps are combined. A wiper dampened (roughly half-saturated) with a cleaning and disinfecting solution will achieve much the same results as the idealized sequence as long as the wiper is not so wet that a large fraction of the cleaning solution is left on the surface with the contaminants and as long as the surface is not particularly dirty.

Additional information on cleaning cleanrooms is available in IES-RP-CC018.2[9] where the necessary equipment is listed along with recommendations on cleaning ceilings (filters, non-porous areas, fixtures), walls, doors, windows, floors, adhesive mats, work stations and waste receptacles. A daily cleaning checklist is provided.

Often a concern about pyrogens is accompanied by a concern about sterility and sterilization; therefore, the latter is discussed next.

Sterilization of Cleaning Materials

There are many accepted methods of sterilization:[3,6]

1. wet steam (121–132°C at 2–2.3 atmospheres pressure, 5–45 minutes)
2. dry heat (140–170°C, 60–180 minutes)
3. sterilizing gaseous chemicals (e.g., ethylene oxide, chlorine dioxide, hydrogen peroxide)
4. sterilizing liquid chemicals (e.g., glutaraldehyde, hydrogen peroxide, formaldehyde)
5. ionizing radiation (e.g., gamma rays, X-rays, 1.5–3.5 Mrad = 15–35 kGy = 15–35 kJ/kg)
6. ionizing electron beam (1.5–3.5 Mrad)
7. combination treatment of ultraviolet radiation and ozone.

For a variety of reasons, the methods of choice for sterilizing wipers are wet steam autoclaving or gamma irradiation, though occasionally ethylene oxide or electron beam processing has been used. Neither gamma nor wet steam sterilization cycles was found to make more than a five percent change in the tensile strength of polyester films, and Nylon 66 was about as resistant.[10] Polypropylene showed some yellowing and embrittlement from sterilizing gamma irradiation, limiting the subsequent shelf-life to three to five years.[10] Wipers in steam-permeable containers and liquids in “small” volumes can be sterilized (for example, autoclaved) in small batches. Autoclaving can be convenient for some users, but is not practical for large-scale producers. Autoclaving does not greatly affect the pyrogen levels and will accelerate reactions between the liquid and its container.

Sterilization of liquids is generally done through gamma irradiation, autoclaving or by filtration through 0.1µm to 0.2µm - pore filters into sterilized containers. None of these methods is universally highly effective in removing pyrogens although in some cases gamma irradiation or filtration can be effective. Sterilization is not enough, as the liquids must be dispensed in a manner that keeps contaminated air from entering the space vacated by dispensing the liquid, requiring sterile inert gas pressurization (e.g., N2) or an air filter.

To manufacture sterile wipers, gamma irradiation is often used to produce sterility. The advantages of gamma irradiation over many of the alternative methods for terminal sterilization are summarized below:[11]

1. penetrating power - even of hermetically sealed packages
2. product formulation and packaging variety - many types are compatible
3. ease of validation - dose is readily predictable and measurable
4. lack of post-treatment quarantine - no residues from treatment
5. reduction in endotoxin level - does not occur for steam or gas or electron beam[12]

The steps for successful gamma sterilization are similar to those for other methods:[11]

1. product qualification - determine how much treatment the product can withstand, how much is likely to be needed and what the minimum level will be
2. product history - determine what treatments will precede the irradiation to check for compatibility
3. bioburden determination - know what the levels are and how resistant they are likely to be
4. design for processing - make sure the product and container can withstand treatment
5. test the product - assure that no significantly detrimental effects are caused by the treatment

Figure 2 shows a schematic of a gamma irradiation facility. Material to be irradiated is moved past a bank of cobalt-60 gamma-ray sources with the dose controlled by the time the material spends in the irradiation zone(s). Dose monitors accompany the materials.

Sterile wipers and wiper/liquid combinations are often sterilized according to the guidelines of the Association for the Advancement of Medical Instrumentation (AAMI):[13] Initially, bioburden is determined from samples from 10 wipers apiece from each of three lots. A production dose is selected to give a one ppm Sterility Assurance Level (SAL), a probability of one in a million that a wiper or wiper/liquid combination will have a viable organism after sterilization at that dose. A sample of 100 wipers is treated with a dose having SAL=0.01 for the level of bioburden corresponding to which the production dose was selected. The 100 wipers are tested for sterility by being separately incubated in biological growth medium for seven days. If two or fewer of the 100 treated with the sub-process dose are non-sterile, the process dose has been confirmed. For routine auditing, the bioburden is determined from 10 wipers and the subprocess dose sterility test is run on 100 wipers. If the sterility test is passed, the production dose remains validated.

Depending on the original bioburden and on the depyrogenating effectiveness of the sterilization technique, there will be more or less pyrogenic material (endotoxins) in the cleaning materials.

Pyrogens

An excellent survey of “microbes, endotoxins and water” was presented almost a decade ago:[14] “injection fever” was found to be caused by lipopolysaccharides (LPS) from the cell walls of Gram-negative bacteria. LPS, like a typical surfactant (detergent) molecule, has a water-seeking (hydrophilic) end and water-avoiding (hydrophobic) end, sometimes forming membranes or microscopic structures in water and giving it an affinity for various surfaces when in an aqueous solution. This affinity depends on subtle factors (such as surface history) as well as on pH, ion concentrations, surfactants etc., and can make the materials hard to remove by mere rinsing with pure water. LPS pyrogens can cause fever, shock, even death, when in the blood stream (or cerebrospinal fluid), but they also can mediate an enhanced immune response to Gram-negative bacterial infections.

Control of pyrogens generally requires starting clean and working clean to minimize the presence of pyrogens, then removing or destroying residual pyrogens. Methods of pyrogen removal include:[15] adsorption on activated charcoal or onto deep-bed filters or LPS-specific filters, ultrafiltration or reverse osmosis, distillation and chromatography. Methods of destruction include:[15] dry heat, moist heat, storage at high temperatures, chemical reagent treatment, and ionizing radiation. The following section will discuss minimization. Pyrogen assays are discussed in the last section.

Pyrogen Minimization in Cleaning Materials

The first line of defense is to limit the bioburden, the colony-forming units, particularly bacteria. Natural products, such as cotton, are more likely to have microorganisms, other things being equal, than man-made products such as polyester, polypropylene, Nylon or even Rayon (reconstituted cellulosic material). Polyester wipers are made from filaments extruded from a hot melt of pure polyester so they start biologically clean. Formation of yarn from the filaments and formation of knitted cloth from the yarn is done under clean, but not sterile conditions so

chemical, particulate and biological contamination can occur. The fabrics are cut, some have special sealed edges created, and washed with clean water and surfactant in special cleanroom laundry facilities to remove contaminants, dried in heated and filtered air, then packaged in a cleanroom.

The oligotrophic bacteria that live in ultra-pure water systems are generally Gram-negative, so they produce pyrogens that contaminate this water. Changes in total oxidizable carbon, bacterial concentrations, and endotoxin concentrations were monitored and it was concluded that these were interrelated.[16] Minimizing the organic compounds which nourish the bacteria should help reduce these populations and thus reduce the pyrogens in the water. Thus, methods like activated charcoal adsorption, perhaps as part of a recirculation loop, can be expected to contribute to pyrogen reduction by removing nutrients and some of the pyrogens themselves.

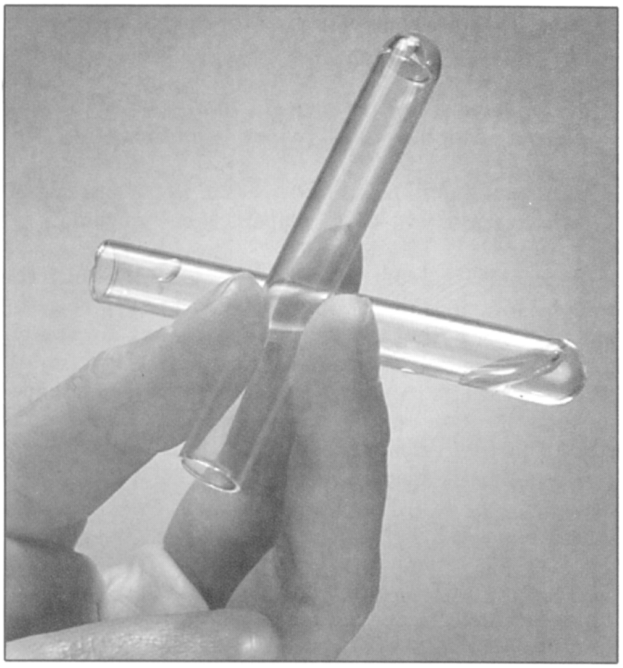

Figure 4. Gel-clot test: LAL reagent is added to samples, mixed, incubated, tested for gel formation. (Courtesy of Associates of Cape Cod, Inc.)

Pyrogen Destruction by Dry Heat

A common way to destroy pyrogens is with dry heat, such as at 250°C “for a sufficient time” as recommended in USP XXII.[17] Three-log (thousand-fold) reduction of pyrogens (LAL assay and rabbit assay) was demonstrated[18] with 250°C for 30 minutes (see also USP XXII, 1990) though not with 200°C for 60 minutes. The required time and temperature are interrelated. “Two extreme examples are glass ampoules sterilized and depyrogenated at approximately 427°C for one minute by direct exposure to a gas flame and a Viking Interplanetary Spacecraft sterilized at 104 to 113°C for 72 hours.”[19] Unfortunately, it is not practical to destroy pyrogens in wipers or cleaning liquids by dry heat.

Pyrogen Destruction by Moist Heat

Autoclaving, such as using steam at two atm pressure and 121°C for 20 minutes, has not generally been considered adequate for depyrogenation. An extensive series of tests showed this produced one- to four-log reductions (c.1/10 to 1/10,000) in pyrogenic activity for a variety of bacteria, determined by turbidometric analysis of LAL, and found that the log reductions tended to be larger as the initial challenge of endotoxin was made smaller.[20] Diminishing log reductions

were found as exposure was lengthened, however, so the exponential model cannot be relied on for extrapolation here.

Pyrogen Removal from Liquids by Filtration

The following are various methods for removing pyrogens from solutions: adsorption, ultrafiltration, chromatography, enzymatic breakdown and positively charged depth filters.[21] The use of 0.2µm-pore positively charged membrane filters was studied, as such filters are sometimes used in aseptic fill filtration already and bacterial endotoxin is characteristically negatively charged, thus attracted electrostatically to the filter. In water, such filtration produced a 10,000-fold reduction in pyrogen levels. In Ringer's solution, the reduction was 20-fold. The electrostatic interaction can be expected to be affected by pH and by the presence of various other ions that would affect the charge levels on the filter or the endotoxins or both. Further investigation and successful application of filtration for depyrogenation was reported.[22] Filtration through 0.2µm-pore positively charged filters appears appropriate for pyrogen removal from water, at least, and is likely to have broader applicability.

Pyrogen Destruction by Irradiation

Some investigators have found reductions in pyrogens and endotoxins due to gamma irradiation from cobalt - 60 sources, and some have not. An explanation for this lack of reproducibility may lie in the results reported after working with highly purified LPS that found "In contrast to endotoxin in aqueous medium, endotoxin irradiated in its dry state showed no decrease in LAL reactivity and rabbit pyrogenicity."[23] Ten-fold and 100-fold decreases were found in endotoxins at 5 and 25kGy (0.5 and 2.5Mrad) doses, respectively, for aqueous solutions; also, it required 31 to 35kGy to get a 10-fold decrease in endotoxins dried onto a substrate.[12] How these results translate to gamma irradiation of partially dry liquid-filled Gram-negative bacterial cells in air is not clear. Some destruction is expected, but how much is in doubt.

The two common methods of irradiation are by gamma rays or by electrons. Figure 3 shows schematically the differences between the two: electron beams are less penetrating, but provide a higher dose rate where they do reach, giving less time for any ancillary chemical reactions, beneficial or detrimental.

For the same total dose, gamma irradiation was found more effective at destroying pyrogens than was electron beam irradiation.[12] Electron beam irradiation has a much faster dose rate than gamma irradiation, leading to less damage of polymers when carried out in air, due to there being less time for chemical reactions, but this difference produces less depyrogenation by electron beams than by gamma rays at the same total dose for much the same reasons.

Another problem with electron beam irradiation is that, because of the strong absorption of electrons, material with the density of water (such as pre-saturated wipers) can only be approximately uniformly irradiated to a few centimeters depth from one side and about twice this depth if the irradiation is from two sides. The pattern of dose versus equivalent depth (g/cm^2, water equivalent = cm) is one that first increases, then falls off. If the energy, E (MeV), and material (e.g., polystyrene equivalent), and thickness Z (cm) are chosen to give a dose at the exit that is equal to the dose at the entrance, Z=0.40(E-0.1), the dose-vs.-depth curves show that such a choice produces about 50 percent higher dose at the maximum in the interior. At 10MeV, Z=4cm (=$4g/cm^2$). Also, Z is 0.47(E-0.5) (=4.5 at 10MeV) if the criterion is that the exit dose be 50 percent of the maximum dose.[24]

Gamma irradiation produces doses versus equivalent thickness, z, that are proportional to exp(-kz), and a max/ min ratio < 1.5 has been readily achieved in sterilizing wipers this way, where z is over ten centimeters in depth, for cobalt-60 gamma rays (1.17 and 1.33 MeV). Gamma irradiation has been well described along with irradiation equipment, product qualification and dose-setting,

process qualification and validation and control.[25] Power utilization efficiency is optimized for densities near that of water or most plastics, but uniformity improved as density is decreased.[25]

Just as the sterility of sterile products should be monitored, recognizing that there is always a possibility of an occurrence of non-sterility, so should the residual levels of pyrogens be monitored, recognizing that no material that has been in contact with Gram-negative bacteria or their endotoxins is likely to be "pyrogen-free," an absolute term of rare applicability. As discussed next, the most common of such assays is the "LAL assay."

Pyrogen Assays

Since pyrogen means "fever-producing," the classic test has been by measuring the change in body temperature of rabbit (e.g., a 1.5°C rise), an expensive and relatively slow procedure.

A widely used "pyrogen" (endotoxin, actually) assay is based on biological fluid extracted from the Horseshoe crab. The Limulus Amebocyte Lysate (LAL) test depends on the tendency of the amebocyte material derived from the crab to clot when in the presence of endotoxins. [The solution also becomes more turbid, the basis of an alternative method.] The LAL assay's use in monitoring ultra-pure water was described as having the kind of sensitivity appropriate for evaluating cleanroom wipers and cleaning liquids.[14] As outlined in the USP 23 (Supplement #1, <161> "Transfusion and Infusion Assemblies and Similar Medical Devices"), the devices (typically three to 10) are each treated with 40mL of extraction liquid, LAL Reagent Water, then this liquid is sampled, and tested with the standard LAL reagent. If there are fewer than 20 endotoxin units from the device, it passes. [The FDA considers 1ng of E. coli endotoxin as similar to 5EU of the USP Endotoxin Reference Standard.] The reagent itself is tested against an endotoxin standard, with serial dilutions, and the extracts are tested to assure they do not inhibit or enhance the formation of the gel when the endotoxins are present ("gel-clot" technique). Tests for sensitivity, inhibition and enhancement are part of the standard operating procedure for such assays. Glassware used for these tests is either certified pyrogen-free or heated at 180°C for three hours.

Tests carried out by Health Sciences Laboratories of Riverdale, NJ, using the materials and associated LAL Pyrotell™ procedures from Associates of Cape Cod, Inc. (Woods Hole, MA) gave the following results for a total of 50 sterile wipers:

- 10 of 10 polypropylene/cellulose wipers had <20EU/wiper: "Non-Pyrogenic"
- 10 of 10 hydroentangled polyester/cellulose wipers had <20EU/wiper: "Non-Pyrogenic"
- 10 of 10 knitted polyester wipers had <<20EU/wiper: "Non-Pyrogenic"
- 10 of 10 polypropylene wipers in 70 percent IPA/30 percent water had <20EU/wiper: "Non-Pyrogenic"
- 10 of 10 cellulose/polyester wipers in 70 percent IPA/30 percent water had <20EU/wiper: "Non-Pyrogenic"

If the true fraction (in the total population from which the sample is obtained) that would not pass a test is p, then the probability of getting all n of n of these to pass the test is (1-p) to the nth power. Thus, if p were actually as high as 0.1, all 50 of 50 would pass in only 0.5 percent of the instances in which 50 were tested. Thus, wipers like these can be made sufficiently clean to meet the 20EU/device endotoxin levels specified for medical devices by USP23 <161> (Supplement 1).

Conclusion

Clean zones help prevent product contamination. They need regular cleaning with materials, such as wipers and cleaning liquids, that are themselves clean. Cleanliness means the negligibly small presence of harmful chemicals, particles, microorganisms and endotoxins (pyrogens). Control of raw materials, the processing of these materials, and their packaging, and final treatment (if any) can produce sterile cleaning materials with levels of endotoxins lower than the level required of medical devices that come in contact with the blood circulating in a patient. Such low levels

should help assure the quality of the products made in the clean zones these materials are used to clean and disinfect.

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