

# Part 1, Skin, Bone, and Cells

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**W**ith this multipart article, I will summarize viral inactivation methods published during the last decade of the 20th century and into the year 2001.

Data were gathered from scientific journal publications and summarized by test article. In some cases, older publications were reviewed. Eliminating inactivation of bacteriophage and plant viruses as well as in vivo studies narrowed my search through the Internet Grateful Med (no longer online, incorporated into [www.ncbi.nlm.nih.gov/PubMed](http://www.ncbi.nlm.nih.gov/PubMed)). Only papers written in English were included, and the key search terms I used were *virus* and *viral inactivation*. The main intent was to retrieve those papers that provide manufacturing or final product viral inactivation methods. In most cases, the effects of various inactivation techniques on product viability were not further investigated.

Virus inactivation references are sorted by test article, which includes cells, plasma and plasma products, tissue culture media, biotechnology products, vaccines, tissues such as bone and skin, and equipment. I also looked at disinfection techniques to provide information that might be relevant for

sanitizing processing equipment or ensuring worker safety. Of approximately 460 references finally entered into the database, those dealing with plasma and plasma products are, not surprisingly, the most numerous.

## **Skin**

Table 1 summarizes the methods reported for viral inactivation in skin.

**Glycerol.** At concentrations of 70% or higher, glycerol was found to completely inactivate cell-free HIV-1 within 30 minutes at 4°C (1). Cell- or skin-associated HIV-1, however, was not totally eliminated with 70% or 85% glycerol at 4°C. Cell- or skin-associated HIV-1 was recovered after storage in 85% glycerol at 4°C for up to 72 hours, but virus isolation was infrequent after storage for more than five days. At 20°C or 37°C, either 70% or 85% glycerol could inactivate cell- or skin-associated HIV-1 within eight hours. The article reporting those results addresses the importance of washing test articles to remove glycerol that may interfere with the biological test system (1). In that article, the de Backere (2) procedure was used, except that the glycerolization procedure was performed at 37°C instead of 33°C.

Glycerol was found to be effective for inactivating HSV-1 and polio as model intracellular viruses in cadaver skin, but only under certain conditions (3). At 4°C, 85% glycerol could not fully inactivate intracellular HSV-1 or polio even after four weeks. At 20°C, 85% glycerol inactivated intracellular HSV-1 within one week but could not fully inactivate polio after four weeks. HSV-1 was inactivated by 98% glycerol within one week and polio within two weeks.

Extracellular HSV-1 was inactivated by 85% glycerol at 4°C, but a 50-day incubation was required. Polio, however, survived even after 50 days at 4°C. At 37°C, 24 hours were needed to completely inactivate polio (4,5). Experiments performed in a virus transport medium containing either 85% or 98% glycerol at 4°C, 20°C, or 37°C showed that 98% glycerol completely inactivated both HSV-1 and polio at either 4°C or 20°C (5).

Table 2 summarizes glycerol methods of viral inactivation for skin.

**Alcohols, dyes, and disinfectants.** The inactivation of HIV-1 in suspension by ethanol, isopropanol, and industrial methylated spirits was investigated to evaluate the efficacy of alcohols as skin disinfectants (6). High titers of HIV in suspension were rapidly inactivated by 70% ethanol independent of protein load (10% and 100% serum). Ethanol (70%) inactivated both cell-free and cell-associated viruses suspended or dried in 10% serum within two minutes. Cell-associated virus in neat serum was more difficult to inactivate when dry than when in suspension. When virus was dried onto a glass surface, the inactivation rate decreased when high levels of protein were present.

Inactivation of VSV in viable human foreskin fibroblasts by poly r(A-U) was potentiated by xanthene dyes, including rhodamine 123, rhodamine B, rhodamine 6G, and sulforhodamine B (7).

In a study on hand disinfection, 80% ethanol was insufficient to inactivate polio-contaminated hands (8). In a suspension study, however, 80% ethanol reduced the infectivity of polio within two minutes by three log<sub>10</sub>. Those results demonstrated that recovery of polio on the hands was reproducible from person to person. Rotavirus in suspension was inactivated within a minute by about five log<sub>10</sub> using a disinfectant called Desderman, which contains 95.3% ethanol (v/v) and 2,3,4,5-tetrabromo-6-methyl phenol. The same disinfectant, however, required five minutes for inactivation of rotavirus on hands (8).

## **Bone**

As noted in the section on skin, the methods that are employed for in vitro virus inactivation are not usually as effective with organic materials, such as tissues. Gamma radiation, supercritical carbon dioxide fluid extraction, hydrogen peroxide, sodium hydroxide, ethanol, demineralization, and acid treatment have all been evaluated for virus inactivation in bone. Table 3 summarizes the methods used for viral inactivation of bone.

**Gamma radiation** with 29 kGy did not completely inactivate FeLV-infected bone (9). In another study, the effects of several different doses of gamma radiation were evaluated for inactivation of HIV in fresh-frozen, whole patellar bone ligament-bone grafts taken from an infected cadaver (10). It was determined that 20 or 25 kGy did not destroy the HIV genes as determined by PCR, but in grafts treated with 30–40 kGy DNA of HIV was not detectable (10).

In a reference on the inactivation of HIV in banked bone for human allografts 25 kGy of gamma radiation inactivated cell-free HIV and HIV-infected T-cells (10). In another set of experiments, polypropylene vials with 2-mL suspensions of chronically infected cells or cell-free supernatants were placed within the medullary canal of bone exposed to the same dose of gamma radiation. In contrast to the previous reference, the inactivation was considered complete using a radiation dose of 25 kGy (11).

The maximum amount of virus bioburden to be inactivated in a bone allograft was estimated for a study performed to determine the dose required to inactivate HIV-infected bones (12). That dose was 35 kGy. The authors, however, noted that a dose of 89 kGy would be required for a sterility assurance level of  $10^{-6}$  (for HIV), and a dose that high would destroy the allograft. They recommended disregarding gamma radiation as a significant virus inactivation method for bone allografts.

Table 4 summarizes the results of gamma irradiation used for viral inactivation in bone.

**Demineralization.** To evaluate the effectiveness of demineralization (decalcification) in HCl and treatment with a virucidal agent, two test materials were prepared (13). Human cortical bone tested free of HIV before it was spiked with  $5.26 \times 10^9$  viral particles. The second test sample came from a donor who died of AIDS. Demineralization in 0.6 N HCl with 0.025% virucidal agent (ethanol combined with a nonionic detergent) destroyed HIV.

Ethylene oxide for virus inactivation in bone was also discussed (13). Unpublished results showed that ethylene oxide (a strong alkylating agent) inactivates HIV in dense cortical bone, but ethylene oxide may significantly affect bone induction. Residual levels of ethylene oxide cause morphological changes in fibroblasts cultured with an allograft.

An author reviewing the safety of freeze-dried bone allografts noted that no case of HIV transmission from mineralized or demineralized freeze-dried bone allograft has yet been reported (14). Bone infected with HIV was treated with a virucidal agent and demineralization, but HIV replication could not be demonstrated.

The effectiveness of demineralization (using a proprietary three-step process) was evaluated in spiking studies with HIV, DHBV, BVDV, CMV, and polio (15). The process resulted in a reduction of infectivity of greater than six  $\log_{10}$  for all viruses and as much as 12  $\log_{10}$  for polio

in a 100–500  $\mu\text{m}$  bone powder (probably due to high titer in the test solutions and high sensitivity of the assay system).

**Supercritical fluid extraction, hydrogen peroxide, sodium hydroxide, and ethanol.** Supercritical fluid extraction was evaluated as a viral inactivation method for bone tissue. Hydrogen peroxide (35% w/w for two hours at 40°C), sodium hydroxide (one M for an hour at 20°C), and ethanol (95% for three hours at 20°C) treatments were also evaluated (16). When all four steps were incorporated into the viral inactivation process,  $\log_{10}$  reduction values of greater than 14.2 for HIV, greater than 18.2 for SIN, more than 24.4 for polio, and greater than 17.6 for PRV were achieved. Supercritical fluid extraction alone provided  $\log_{10}$  reduction values of greater than 4.0 for HIV, greater than 4.3 for SIN, more than 6.6 for polio, and greater than 4.0 for PRV (16).

Ethanol is also reported effective for inactivating SIV in contaminated bone (14). No monkeys receiving ethanol-treated bone

became infected. In contrast, all those receiving nonprocessed frozen bone allografts tested positive for SIV.

### Cells (Excluding Plasma and RBCs)

Platelets and red blood cells (RBCs) will be discussed in Part 2 of this multipart article. This section reviews virus inactivation in other types of cells.

**Lymphocytes.** HIV-1 infected T lymphocytes can be inactivated in vitro by nonoxynol-9 (N-9) using a surface active agent (C31G) or by sodium dodecyl sulfate (SDS) at concentrations of 0.05% and greater (17). At lower concentrations, the various HIV-1 strains appear to have different sensitivity to microbicides. In FeLV-infected T-cells, photodynamic inactivation of FeLV was achieved with a benzoporphyrin derivative and red light (600–700 nm) (18). That preliminary work led to further investigations on the use of photodynamic inactivation for whole blood (discussed in Part 2).

**Table 1.** Viral inactivation methods for skin

| Test Article                        | Viruses          | Methods                     | References |
|-------------------------------------|------------------|-----------------------------|------------|
| Split skin grafts                   | HIV              | Glycerol                    | 1          |
| Skin allografts, dermal fibroblasts | HSV-1, polio     | Glycerol                    | 3,4        |
| Skin, banked                        | HSV-1, polio     | Glycerol                    | 2,5        |
| Skin                                | HIV-1            | Ethanol, isopropanol        | 6          |
| Foreskin                            | VSV              | Xanthene dyes and poly(r-U) | 7          |
| Skin                                | polio, rotavirus | Ethanol, Desderman          | 8          |

**Table 2.** Glycerol used as a viral inactivation method for skin

| Test Article                   | Glycerol Concentration <sup>a</sup> | Temperature <sup>b</sup> | Time     | Inactivation Results  | Reference |
|--------------------------------|-------------------------------------|--------------------------|----------|-----------------------|-----------|
| Cell-free HIV-1                | 70                                  | 4                        | 30 min.  | Complete              | 1         |
| Cell- or skin-associated HIV-1 | 85                                  | 4                        | 72 hours | Incomplete            | 1         |
| Cell- or skin-associated HIV-1 | 85                                  | 4                        | 75 days  | A few virus recovered | 1         |
| Cell- or skin-associated HIV-1 | 70 or 85                            | 20 or 37                 | 8 hours  | Complete              | 1         |
| Intracellular HSV-1            | 85                                  | 4                        | 4 weeks  | Incomplete            | 3         |
| Intracellular HSV-1            | 85                                  | 20                       | 1 week   | Complete              | 3         |
| Intracellular HSV-1            | 98                                  | 20                       | 1 week   | Complete              | 3         |
| HSV-1                          | 85                                  | 4                        | 50 days  | Complete              | 5         |
| HSV-1                          | 85                                  | 37                       | 2 hours  | Complete              | 5         |
| HSV-1                          | 98                                  | 4 or 20                  | 0 days   | Complete              | 5         |
| Polio                          | 85                                  | 4                        | 4 weeks  | Incomplete            | 3         |
| Polio                          | 85                                  | 4                        | 50 days  | Incomplete            | 4         |
| Polio                          | 85                                  | 20                       | 4 weeks  | Incomplete            | 3         |
| Polio                          | 85                                  | 37                       | 24 hours | Complete              | 5         |
| Polio                          | 98                                  | 20                       | 2 weeks  | Complete              | 4         |

<sup>a</sup>Concentration expressed as a percentage

<sup>b</sup>Temperature in degrees Centigrade (°C)

**Table 3.** Viral inactivation methods for bone

| Test Article   | Viruses                              | Methods   | References |
|----------------|--------------------------------------|---|------------|
| Bone tissue    | HIV-1, SIN, polio, PRV               | Supercritical carbon dioxide fluid extraction, hydrogen peroxide, sodium hydroxide, and ethanol | 16         |
| Banked bone    | HIV-1                                | Gamma radiation   | 10,11,12   |
| Bone allograft | HIV, DHBV, HAV, polio, HBV, HCV, BVD | Deminerlization   | 13,14,15   |

**Table 4.** Gamma radiation as an enveloped virus inactivation method for bone

| Test Article  | Gamma Radiation (kGy) | Inactivation Results                  | Reference |
|---|-----------------------|---------------------------------------|-----------|
| FeLV-infected bone  | 29                    | Incomplete                            | 9         |
| HIV in bone grafts  | 20 or 25              | Incomplete                            | 10        |
| HIV in bone grafts  | 30–40                 | HIV DNA undetectable                  | 10        |
| HIV in banked bone  | 25                    | Cell-free HIV inactivated             | 11        |
| HIV in banked bone  | 25                    | HIV from infected T cells inactivated | 11        |
| HIV-infected T cells in bone                              | 25                    | HIV inactivated                       | 11        |
| Cell-free supernatants from infected cells placed in bone | 25                    | HIV inactivated                       | 11        |
| Bone allograft  | 35                    | HIV inactivated                       | 12        |

In CD4+ lymphocytes (SupT cells) and two CD4-B cell lines (Ramos and Raji), HIV-1 lost infectivity when treated with trypsin (19). With trypsin at low concentrations that are nontoxic to cells, this method should completely inactivate HIV-1 in cell cultures.

**Mouse embryo fibroblasts** were infected with murine CMV. Hypericin augmented with light inhibited the CMV replication cycle in that study (20).

**Baby hamster kidney (BHK) cells.** Recombinant vaccinia virus present in BHK cells was inactivated by paraformaldehyde (PFA) and binary ethylenimine (BEI) (21).

**Murine cell line.** The poxviruses ectromelia and vaccinia, along with HSV-1, was inactivated by inducible nitric oxide in murine macrophage-derived cells (cell line RAW 264.7) and in bystander cells of epithelial and fibroblast origin (22).

**Cells used in laboratory analysis.** Unlike the vaccinia virus tested in BHK cells, cell-associated HIV was not quickly inactivated by the 1% PFA used to fix cells (phytohemagglutinin-stimulated cells and a subclone of H9 cells chronically infected with HIV) (23). Infectious virus were detectable in specimens for as long as 18 hours after fixation. Levels were higher in those treated for up to three hours at 4°C. Increasing temperature increased the effectiveness of PFA. Temperatures of 22°C and 37°C were more effective than 4°C.

HIV-1 was rapidly inactivated in infected cells using psoralen (AMT) and UVA light (24). Chronically infected U937 cells and human T-lymphoblastoid lines CEM and HUT-78 were used to evaluate the effectiveness of that viral inactivation procedure.

Sodium lauryl sulfate (SLS) inactivated HIV-1 in cultured Ig5 cells (25). Pretreating HIV-1 with SLS decreased infectivity more than pretreating the cells did. VSV, which infects cells by a different mechanism than that of HIV-1, was not affected by SLS.

(Lymphocytes are discussed in the “Other Cells” section. See reference 19).

**References**

- (1) Cameron, P.U. et al., “Efficacy and Kinetics of Glycerol Inactivation of HIV-1 in Split Skin Grafts,” *J. Med. Virol.* 60(2), 182–188 (2000).
- (2) de Backere, A.C., “Euro Skin Bank: Large-Scale Skin-Banking in Europe Based on Glycerol Preservation of Donor Skin,” *Burns* 20(1), S4–S9 (1994).
- (3) Marshall, L. et al., “Effect of Glycerol on Intracellular Virus Survival: Implications for the Clinical Use of Glycerol-Preserved Cadaver Skin,” *Burns* 21(5), 356–361 (1995).
- (4) Ghosh, M.M. and Freedlander, E., “Inactivation of Viruses by Glycerol as Used in Allograft Skin Preservation” (letter, comment), *Burns* 20(5), 476–477 (1994).
- (5) Van Baare, J. et al., “Virucidal Effect of Glycerol as Used in Donor Skin Preservation,” *Burns* 20(1), S77–S88 (1994).
- (6) Van Bueren, J., Larkin, D.P., and Simpson, R.A., “Inactivation of Human Immunodeficiency Virus Type 1 by Alcohols,” *J. Hosp. Infect.* 28(2), 137–148 (1994).

- (7) Jamison, J.M. et al., “Potentiation of the Antiviral Activity of Poly r(A-U) by Xanthene Dyes,” *Cell Biol. Int. Rep.* 14(12), 1075–1084 (1990).
- (8) Eggers, H.J., “Experiments on Antiviral Activity of Hand Disinfectants: Some Theoretical and Practical Considerations,” *Zentralbl. Bakteriol.* 273(1), 36–51 (1990).
- (9) Withrow, S.J. et al., “Evaluation of the Antiretroviral Effect of Various Methods of Sterilizing/Preserving Corticocancellous Bone,” *Orthopaedic Res. Soc.* 76A, 1032–1035 (1990).
- (10) Fideler, B.M. et al., “Effects of Gamma Irradiation on the Human Immunodeficiency Virus: A Study in Frozen Human Bone–Patellar Ligament–Bone Grafts Obtained from Infected Cadavers,” *J. Bone Joint Surg. Am.* 76(7), 1032–1035 (1994).
- (11) Salai, M. et al., “Human Immunodeficiency Virus (HIV) Inactivation of Banked Bone by Gamma Irradiation,” *Ann. Transplant.* 2(1), 55–56 (1997).
- (12) Campbell, D.G. and Li, P., “Sterilization of HIV with Irradiation: Relevance to Infected Bone Allografts,” *Aust. NZ J. Surg.* 69(7), 517–521 (1999).
- (13) Mellonig, J.T., Prewett, A.B., and Moyer, M.P., “HIV Inactivation in a Bone Allograft,” *J. Periodontol.* 63(12), 979–983 (1992).
- (14) Mellonig, J.T., “Donor Selection, Testing, and Inactivation of the HIV Virus in Freeze-Dried Bone Allografts,” *Pract. Periodontics Aesthet. Dent.* 7(6), 13–22, Quiz 23 (1995).
- (15) Scarborough, N.L. et al., “Allograft Safety: Viral Inactivation with Bone Demineralization,” *Contemp. Orthop.* 31(4), 257–261 (1995).
- (16) Fages, J. et al., “Viral Inactivation of Human Bone Tissue Using Supercritical Fluid Extraction,” *ASAIO J.* 44(4), 289–293 (1998).
- (17) Krebs, F.C. et al., “Inactivation of Human Immunodeficiency Virus Type 1 by Nonoxynol-9, C31G, or an Alkyl Sulfate, Sodium Dodecyl Sulfate,” *Antiviral Res.* 43(3), 157–173 (1999).
- (18) North, J. et al., “Photodynamic Inactivation of Retrovirus by Benzoporphyrin Derivative: A Feline Leukemia Virus Model,” *Transfusion* 32(2), 121–128 (1992).
- (19) Tang, S.B. and Levy, J.A., “Inactivation of HIV-1 by Trypsin and its Use in Demonstrating Specific Virus Infection of Cells,” *J. Virol. Methods* 33(1–2), 39–46 (1991).
- (20) Hudson, J.B., Lopez-Bazzocchi, I., and Towers, G.H.N., “Antiviral Activities of Hypericin,” *Antiviral Res.* 15, 101–112 (1991).
- (21) Hulskotte, E.G. et al., “Chemical Inactivation of Recombinant Vaccinia Viruses and the Effects on Antigenicity and Immunogenicity of Recombinant Simian Immunodeficiency Virus Envelope Glycoproteins,” *Vaccine* 15(17–18), 1839–1845 (1997).
- (22) Karupiah, G. and Harris, N., “Inhibition of Viral Replication by Nitric Oxide and its Reversal by Ferrous Sulfate and Tricarboxylic Acid Cycle Metabolites,” *J. Exp. Med.* 181(6), 2171–2179 (1995).
- (23) Aloisio, C.H. and Nicholson, J.K., “Recovery of Infectious Human Immunodeficiency Virus from Cells Treated with 1% Paraformaldehyde,” *J. Immunol. Methods* 128(2), 281–285 (1990).
- (24) Watson, A.J., Klaniecki, J., and Hanson, C.V., “Psoralen/UV Inactivation of HIV-1-Infected Cells for Use in Cytologic and Immunologic Procedures,” *AIDS Res. Hum. Retroviruses* 6(4), 503–513 (1990).
- (25) Bestman-Smith, J. et al., “Sodium Lauryl Sulfate Abrogates Human Immunodeficiency Virus Infectivity by Affecting Viral Attachment,” *Antimicrob. Agents Chemother.* 45(8), 2229–2237 (2001). **BPI**