ELSEVIER

Antiviral Research 52 (2001) 261–273

Antiviral Research

www.elsevier.com/locate/antiviral

Comparison of gamma and neutron radiation inactivation of influenza A virus

R. Joel Lowy *, Gerard A. Vavrina, David D. LaBarre

Armed Forces Radiobiology Research Institute/RPT, 8901 Wisconsin Avenue, Bethesda, MD 20889-5603, USA

Received 13 March 2001; accepted 9 July 2001

Abstract

Radiation inactivation of viral pathogens has potential application in sterilization and in the manufacture of biological reagents, including the production of non-infectious viral antigens. Viral inactivation by gamma radiation has been extensively investigated, but few direct comparisons to other qualities of radiation have been explored. Experiments were designed to examine direct radiation damage by both gamma photons (γ) and neutrons (n) while minimizing methodological differences. Frozen samples of influenza A X31/H3N2 and PR8/H1N1 were exposed to γ and n at doses between 0 and 15.6 kGy. Other experimental parameters, including dose-rate, were not varied. Virus titers were determined by tissue culture infectious dose ($TCID_{50}$) and plaque forming unit (PFU) assays. D_{10} values, kGy per log reduction, were calculated from these assays. PR8 D_{10} values based on PFU assays were approximately 2 and 5 kGy for γ and n exposures, respectively, and those based on $TCID_{50}$ were approximately 6 and 14 kGy. Similar results were obtained for the A/X31 strain. The data demonstrate that γ was 2–3-fold more effective than n, with a relative biological effectiveness (RBE) range of 0.43–0.65. These neutron results are likely the first reported for a medically relevant virus. PAGE analysis of viral proteins and RNAs failed to show macromolecular damage. D_{10} values were found to be similar to a broad summary of previously reported gamma inactivation values for other virus types. The dependence of the magnitudes of D_{10} on titer assay in this study suggests that more than one titer method should be used to determine if complete inactivation has occurred. Published by Elsevier Science B.V.

Keywords: Ionizing radiation; Gamma photons; Neutrons; Influenza A virus

1. Introduction

Since the 1940s experiments have been conducted to examine the ability of ionizing radiation to inactivate viruses (Barendsen, 1994; Ward, 1988). A number of studies determined the appropriate total gamma photon radiation dose necessary to inactivate a broad number of virus types in materials such as food, serum, sewage, or as positive control reagents in viral assays (Daley et al., 1998; Gamble et al., 1980 Gruber, 1971; House et al., 1990; Sullivan et al., 1971; Thomas et al., 1981, 1982; White et al., 1990). A major advantage of ionizing radiation, compared with ultraviolet light or chemical agents, is its high penetration into and through most biological and

* Corresponding author. Tel.: +1-301-295-1790; fax: +1-301-295-0313.

E-mail address: lowy@afrri.usuhs.mil (R.J. Lowy).

0166-3542/01/$ - see front matter Published by Elsevier Science B.V.

PII: S0166-3542(01)00169-3

non-biological materials. Therefore, all portions of the sample receive the desired radiation dose. This is one reason ionizing radiation is used in commercial applications for sterilization or pasteurization of laboratory and clinical supplies, biological reagents and, increasingly, for food (Bidawid et al., 2000; Daley et al., 1998; Farkas, 1998; House et al., 1990; Hubbard, 1998; Thomas et al., 1981; White et al., 1990). In laboratory settings, it has been used to prepare non-infectious antigen for vaccine studies and positive controls for antibody based assays (Elliott et al., 1992; Gamble et al., 1980; Gruber, 1971; Pang et al., 1992; Sun et al., 1978). In some cases, the doses necessary for virus inactivation has been re-visited when a particular hazardous virus, such as Ebola, was newly introduced into the research or clinical laboratory setting (Elliott et al., 1992; Lupton, 1981). Viruses have also been used as a model system to understand the mechanism of radiation damage (Megumi et al., 1993; Rosen et al., 1987; Singh et al., 1990; Toyoshima et al., 1980).

The vast majority of these studies used gamma photons. To the best of our knowledge, only one study (Singh et al., 1990) examined the effect of neutrons, but with a non-medically relevant virus (bateriophage M13). The goal of this investigation was to make a direct empirical comparison between the effects of neutrons and gamma photons. The expected benefit of this comparison was to establish specific recommendations as to the quality of radiation best suited for virus inactivation. Potential problems for comparing radiation inactivation values from differing laboratories include the titer method selected, the inherent variability of viral quantal and quantitative assays, and differences in the exposure conditions. The physical experimental setup can greatly alter the radiation dose actually delivered and/or the amount of direct versus secondary free radical damage to the sample. Having all the necessary radiation sources on site provided a unique ability to conduct experiments with differing types of ionizing radiation under nearly identical circumstances with internally consistent dosimetry measurements. A priori arguments for the relative biological effectiveness (RBE) of neutrons relative to gamma photons based on radiation-biology studies can come to very different and opposite conclusions. Radiation damage to DNA in vitro and in vivo and disruption of eukaryotic and prokaryotic cell replication using both high linear energy transfer (LET) radiation, such as neutrons, and low LET radiation, such as gamma photons, have been extensively studied. The RBE tends to be less than one when considering DNA damage to molecules in vitro and greater than one when considering reproductive death of cells (Barendsen, 1994; Goodhead, 1994; Mustonen et al., 1999; Ward, 1988). The presence of free radical scavengers, as either small exogenously added chemicals or as associated biological molecules, such as histones, alters the effects of neutrons on DNA in vitro and the RBE approaches one (Stankus et al., 1995).

The most striking result of this study is that neutrons were much less effective than gamma photons in inactivating influenza virus replication as demonstrated by a RBE of approximately 0.5. The D_{10} (total radiation dose per one log titer reduction) values were titer-assay dependent, suggesting that care should be used in evaluating the effectiveness of inactivation treatments. The gamma photon D_{10} of 3 kGy was similar to previously reported values, which implies that for multi-log reductions high total doses would be needed. The neutron D_{10} values based on 50% tissue culture infective dose ($TCID_{50}$) were at the upper limit of reported values, suggesting that even higher total doses of neutrons are required to inactivate infectious particles. The technical problems of high radiation dose exposure of viral material within the various source facilities have been solved and are described briefly.

2. Methods and materials

2.1. Virus stocks

Large single lots of concentrated influenza virus strains A/X31/H3N2 and A/PR8/H1N1 were purchased from SPFAS (Specific Pathogen Free Avian Supply, Storrs, CT). The virus was grown in eggs and concentrated by density centrifugation

and resuspended in HEPES buffered saline. Viral stocks were thawed as described for the titer assay, below, and each individual experimental sample of 200 μl was placed in pre-sterilized, O-ring sealed-1.5 ml centrifuge tubes. Sample concentration was 1 mg/ml protein, as determined by SPFAS. The measured average titers per milliliter sample were 5×10^8 plaque forming units (PFU) and a $TCID_{50}$ of 3.6×10^3. Virus stocks and samples were stored at -80 °C between all experimental manipulations.

2.2. Sample handling for radiation exposure

All samples were irradiated while frozen to minimize the effects of secondary hydrated free radical damage. Developing a simple system for maintaining frozen samples during irradiation was technically challenging especially for the highest doses, which required a 6-h irradiation time. The system could not affect dosimetry measurements, nor contain materials that would activate in the reactor, but needed to be compact enough for the reactor's rapid sample transport system. This transport system allowed removal of samples from the reactor exposure room prior to radiation levels decreasing to safe levels for personnel to enter, which for the highest doses was approximately three days. A passive dry ice cooled insulated box constructed entirely of Styrofoam was developed which met these criteria. Preliminary trials demonstrated that the samples were held at less than -40 °C for at least 8 h. As the samples were not in contact with the dry ice, but cooled by the vapor phase, there was no effect of changing amounts of dry ice on the dose received by the samples. The box is effective and simple in design; a similar system could readily be adapted to other facilities. Two separate boxes were used for all experiments, one for the irradiated samples and one for the mock exposed controls. Each box contained samples of both virus strains with the position of all samples explicitly known. Multiple paired control samples were included in the experimental design as a precaution against uncontrolled changes in the virus sample from storage and asynchronous titer assays. Samples were necessarily assayed over several months due to the number of samples, the use of two different titer assays, and the need for high radiation doses. For scheduling and safety reasons, the exposures necessary to complete the inactivation curves took several weeks to complete.

2.3. Radiation exposure and dosimetry

All exposures were completed at the Armed Forces Radiobiology Research Institute (AFRRI, Bethesda, MD) Cobalt-60 facility and at the TRIGA Mark-F research reactor facility. The gamma doses were given bilaterally while the neutron flux was unilateral. For neutron exposures, measurements were performed in an exposure room designed to absorb thermal neutrons and equipped with an extractor tube system for rapid insertion and withdrawal of the virus sample array. Exposures occurred in a bismuth cave with average fluence-weighted neutron energy of 0.71 MeV.

A constant 65 Gy/min dose rate was used, which was the highest practical dose rate at the Cobalt-60 facility. An average reactor power of 746 ± 15 kW produced the required 65 Gy/min for the series of irradiation experiments. The measured dose values were in kGy: 1.3, 2.6, 3.9, 5.2, 6.5, 7.8, 9.1, 10.4, 13.0 and 15.6. Coefficients of variation (CV) at all the doses being equal to or less than 2.2 and 5.6% for the gamma photon and neutron exposures respectively. The total dose was controlled by the time of exposure using the dose rate. Field uniformity was determined for both sources. The array was within a constant dose region. Loss of dry ice was determined to make no more than a 4% difference in the dose delivered.

Preliminary experiments were used to calibrate the radiation sensors for use with experimental sample holder array and to establish the source operational conditions to accurately produce the desired dose within the experimental samples. Check calibration runs were also performed prior to every set of virus exposures and rates were monitored during the experiments. Calibration was accomplished by preliminary runs without virus, but with samples containing the same volume and media and the remaining sample posi-

tions occupied by an appropriate dosimetry-measuring device. The dose measured within the sample array was calibrated relative to the doses measured by detectors external to the array but at a known standard positions. These same external detectors were then used to monitor the actual virus exposures during the experiment. The internal and external monitoring detectors for the gamma photon experiments were 0.5 cm^3. Exradin air-filled ionization chambers composed of A-150 plastic. Calibration of these ionization chambers was performed at AFRRI and is traceable to the National Institute of Standards and Technology (NIST; Gaithersburg). Absorbed-dose rates in tissue were calculated according to the TG21 protocol (American Association of Physicists in Medicine AAPM Task Group 21, 1983). A two-dosimeter method was used to measure the neutron and gamma tissue-dose rates inside the virus array. A pair of ionization chambers with differential sensitive to both neutrons and gamma combined or sensitivity to gamma alone was used to determine the total dose-rate within the array, to separate the neutron and gamma components and to determine the gamma dose rate. Dosimetry using these paired-ionization chambers was based on a semi-annual calibration and traceable to the NIST. An average neutron-to-total dose rate of 0.97 ± 0.07 was determined for this series of experiments. The external monitors, calibrated relative to the array, were two additional chambers mounted outside the bismuth cave near the ceiling of the exposure room.

2.4. Virus titer assays

Titer determinations for each radiation dose and type were performed using paired control and irradiated samples that were assayed simultaneously to minimize experimental variability. Both PFU and $TCID_{50}$ assays were performed on all the samples. For each titer determination of each sample the within assay replication was three or four for the PFU assays and six replicates for the $TCID_{50}$ assays. The two assays were based on standard methods for influenza virus, with modifications as described below (Barrett and Inglis, 1985; Burleson et al., 1992; Patterson and Lamb, 1993; Rodriguez-Boulan, 1983).

Virus samples were transferred from the −80 °C Revco freezer to ice (4 °C) and shielded from light during all manipulations. After 10 min samples were thawed quickly at 37 °C and returned to ice during serial dilution. The medium for virus dilution and cell infections was Hanks Basic Saline prepared in house (HBS; Freshney, 1983) supplemented with 25 mM HEPES Buffer and 4 mM sodium bicarbonate, pH 7.3. All culture reagents unless otherwise noted were from Gibco-BRL (Gaithersburg). Stock cultures of MDCK cells used for both titer assays were maintained in T-75 Corning flasks with Dulbecco's Minimal Essential Media (DMEM) media containing 10% fetal bovine serum (Hyclone, Logan, UT), 4.5 mg/ml glucose, L-glutamine, and penicillin-streptomycin. Cell passage was accomplished with the standard method of using calcium magnesium free phosphate buffered saline (PBS) and trypsin-EDTA (Quality Biofluids Gaithersburg, MD).

For PFU assays, just-confluent cultures were prepared in 6-well plates infected for 2 h, washed and then overlaid with 2% agarose (FMC Corp, Rockland, ME) prepared in DMEM containing 2 μg/ml acetyl-trypsin (Sigma, St. Louis, MO). After plaques had formed, 72 h post infection (pi), they were fixed with 4% fomalin–PBS, pH 7.4, the agarose removed, and the cell layer stained with crystal violet dye. The plaques were then manually counted using a binocular dissecting microscope. For the $TCID_{50}$ assay 96-well plates, containing 1×10^5 MDCK cells per well, were infected for 1 h, washed, and re-fed with culture media. No trypsin was added to this media, making the assay effectively a single cycle of infection assay. At 72 h pi, the 96-well plates were washed twice to remove dead cells and then treated with 1% methylene blue dye in 50% ETOH to fix and stain the remaining live cells. The cell layers were solubilized in 1% SDS and the optical density (OD) of the wells was determined on a multichannel absorbance plate reader (Multiskan MCC/340, Flow Laboratories, McLean VA) using a wavelength of 620 nm. Each 96-well plate contained both uninfected wells used to determine the maximum OD, and blank wells for background subtraction. Calculation of titer values

from the plaque counts and OD values was based on standard techniques but used modified statistical methods that provided the ability to calculate both the titer and a standard error for the titer estimate. The D_{10} values were estimated from the PFU per ml and $TCID_{50}$ data by first normalizing them with their respective paired control titer values. The titer-radiation dose relationship was fitted using inverse variance weighted single exponential equations constrained to pass through the point at zero radiation and 100% survival (LaBarre and Lowy, 2001).

2.5. PAGE analysis of RNA and proteins

Viral RNA and protein were extracted from control and irradiated samples for polyacrylamide gel electrophoresis (PAGE) analysis of molecular structure. All reagents were RNAase free, nucleic acid grade and/or diethylpyrocarbonate ('DEPEC') treated, from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Briefly, 100 μl virus aliquots were rapidly thawed and adjusted to 250 μl total volume and held on ice. TRIZOL LS (Gibco BRL Life Technologies, Gaithersburg) reagent was used to simultaneously extract viral RNA and proteins following the manufacture's instructions. After phase separation by addition of chloroform, as recommended by the manufacturer, 5–10 μg of RNAase free glycogen (Gibco BRL Life Technologies) was added as a carrier to the aqueous RNA containing fraction.

RNA was precipitated using purified isopropyl alcohol and centrifugation. The pellets were washed in 75% RNAase free ethanol–water, air dried and re-dissolved in water. The RNA concentration was checked using a GeneQuant II micro-spectrofluorometer (Pharmacia Biotech (Biochrom) Ltd, Cambridge, England) and samples having an OD of less than 1.6 were rejected. Gel electrophoresis was done using Novex 6% 15-well TBU gels (Novex, San Diego, CA). Fifteen microliter samples in Bio-Rad TBE-Urea sample buffer (Bio-Rad, Hercules, CA) containing approximately 50 μg RNA/μl used as RNA standards were run on each gel consisting of TRIZOL LS extracted XJ744 cellular RNA and an RNA ladder of 0.24–9.5 kb (Gibco BRL Life Technologies, Gaithersburg). Gels were electrophoresed at constant voltage in a Novex XCell II (Novex, San Diego, CA) minicell, fixed, washed, and visualized with Bio-Rad Silver Stain Plus (Bio-Rad, Hercules, CA).

Viral proteins were precipitated from the phenol–ethanol supernatant with purified isopropyl alcohol and sedimented by centrifugation. The pellets were washed three times with a solution of 0.3 M guanidine hydrochloride in 95% ethanol. The pellets were dried and then dissolved in 1% SDS by pipetting. As a check on the extraction method, viral proteins from control samples were dissolved in 2 × Laemmli buffer containing 1 mg/ml DTT. No differences in samples with or without TRIZOL extraction were observed. All samples were boiled for 10 s in DTT/SDS buffer and chilled immediately. Then, 10–15 μl samples containing not less than 100 μg/μl protein were loaded on Bio-Rad 10–20% gels 1.0 mm (10-wells) in Bio-Rad Tris–Glycine/SDS buffer (Bio-Rad, Hercules, CA). Bio-Rad broad range protein standards were run on each gel. The gels were electrophoresed in a minicell, fixed, washed, and visualized with either GelCode Blue stain (Pierce Chemical Company, Rockford, IL) or Biorad Silver Stain Plus.

Protein and RNA gels were trans-illuminated and their images captured using video and digital imaging hardware and soft ware from Universal Imaging Corp (West Chester, PA). Densitometry was performed on the RNA and on the protein bands and the irradiated sample densities were compared with those for control samples extracted and electrophoresed in parallel on the same gel.

3. Results and discussion

3.1. Virus inactivation curves

Fig. 1 shows the titer values obtained for the control and exposed A/PR8 and A/X31 samples for all of the radiation and-titer assay combinations used in this study. Fig. 2 shows the titer data normalized by the respective paired control values, for both influenza virus strains. The absolute

titer data for both strains were very similar in magnitude and essentially identical in the pattern of response, for each quality of radiation, gamma photons and neutrons, and for the two different titer the assay methods. The inactivation curves

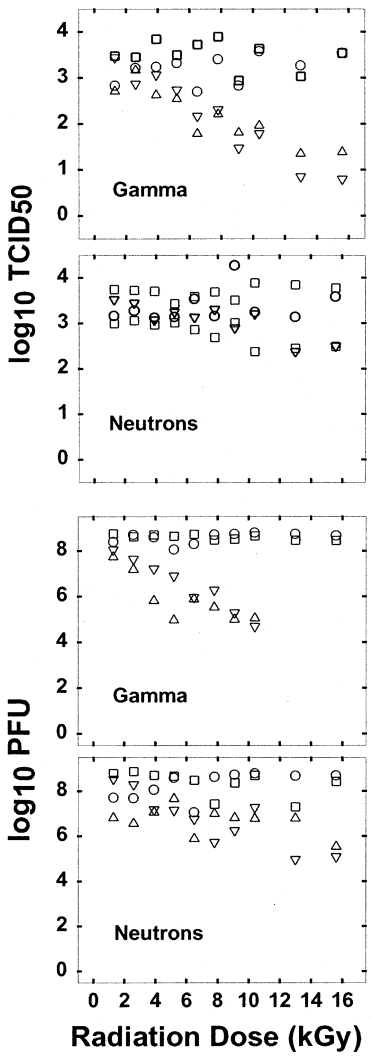

Fig. 1. Comparison of titer values for control and irradiated A/PR8 and A/X31 virus samples. Control (circles and squares) and irradiated (triangles) influenza virus samples (A/X31, circles and upright triangle; A/PR8, squares and inverted triangles) exposed to either gamma photons or neutrons. The titer values for the plaque forming unit (PFU per ml) and tissue culture infectious dose ($TCID_{50}$) assays are shown for both irradiated and the paired control samples. Data shown are means without error limits. Symbols were generally much larger than the S.E.M. values.

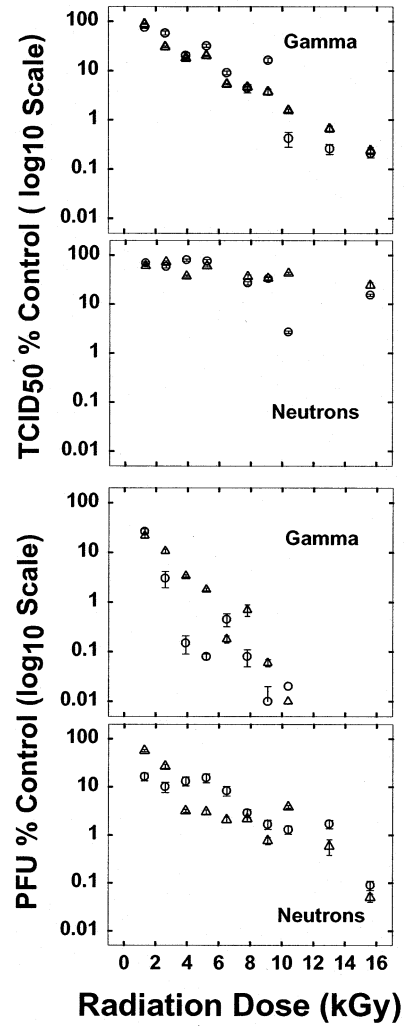

Fig. 2. Comparisons of percent of control values for radiation inactivation of A/PR8 and A/X31 influenza virus. Titer values for A/PR8 (triangles) and A/X31 (circles) were normalized by the paired control values. The data is shown on a semi-log axis with the same scale for all titer method-radiation combinations to facilitate comparison of the results. Data are mean ± S.E.M. plotted as open symbols to display the error estimates. D_{10} values, the radiation doses necessary for a unit $\log_{10}$ reduction, were determined from these data and are presented in Table 1.

from the normalized data were used to determine the D_{10} values. The D_{10} is the radiation dose in kGy necessary for a one-log reduction in titer, and is effectively the slope parameter of these survival curves. D_{10} values are a convenient

means for cross comparisons within this study and for other studies using a single parameter and are presented in Table 1. Table 1 also shows the RBE, the proportion of neutron inactivation (efficacy) relative to gamma photons.

The control titer values do not change appreciably (Fig. 1) with radiation dose, indicating that there were no large systematic errors in the titer assays. This is despite the assays for different strains and radiation doses being spread over several months (Section 2). However, with the exception of a few determinations, all the values, whether considering control or experimental samples, lay reasonably well on the same trend lines. Also, the errors for each titer determination were small, and generally were within the dimensions of the symbols for Fig. 1. Fig. 2 has been graphed using open symbols so the error magnitudes can be observed.

The survival curves (Figs. 1 and 2) are log-linear. Such single exponential inactivation curves were observed for a wide variety of virus types and reported in the majority of the literature. No plateau of incomplete inactivation at high doses was observed, indicating a refractory response to increasing radiation, as has been reported for cells, cell lines, and bacteria. Such plateaus are generally observed after very large log-reductions, e.g. 6–8 log cycles, in survival. Inactivation plateaus, if they exist, would be hard to demonstrate due to the extremely low titers that would need to be measured with high accuracy and the need for total doses of 50–100 kGy.

3.2. Neutrons compared with gamma photons

The most striking result is that neutrons were much less effective in inactivating influenza A virus in comparison to gamma photons. This result is most readily illustrated for the $TCID_{50}$ titer values, in Fig. 1, as for both virus strains the neutron values decrease much less rapidly with dose, remaining in magnitude close to the control values. The differences in radiation effects measured by the PFU titer data are easier to observe in Fig. 2. The slope of these survival curves is greater for gamma than neutron exposures, another indication of the greater efficacy of gamma photons. The qualitative impressions from inspection of the data curves in Figs. 1 and 2 are confirmed by the magnitudes of the D_{10} values, as shown in Table 1. The D_{10} values for neutron exposure are consistently greater than those for gamma, independent of either virus strain or titer method. The relative effective dose of neutrons compared with photons was between 0.4 and 0.6. These RBE values did not appear to be assay or strain dependent.

3.3. Comparison of D_{10} to previously reported values

Data from the literature on radiation inactivation of viruses are summarized in Table 2. In many reports, the exact exposure conditions were not completely described. Notably, unless authors stated that frozen samples were used, it was as-

Table 1
Summary of D_{10} values for radiation inactivation

Virus strain	Titer method	$D_{10} \pm$ S.E.				RBE
		Gamma		Neutron		
X31	$TCID_{50}$	7.08	0.427	15.07	1.944	0.47
X31	PFU	2.46	0.152	5.49	0.267	0.45
PR8	$TCID_{50}$	5.77	0.204	14.21	1.330	0.41
PR8	PFU	2.82	0.094	4.89	0.535	0.58

D_{10}, radiation dose in kGy required to cause a 1 $\log_{10}$ reduction in virus titer. D_{10} values were determined from single inverse variance weighted exponential fits of the normalized titers versus radiation dose curves for each combination of the influenza virus strain, radiation quality and titer determination method. The standard error (S.E.) is based on inverse error estimation. RBE, relative biological effectiveness of neutrons compared with gamma photons.

sumed that only unfrozen material was irradiated. No reports were found using dried material. Radiation doses and quality in some cases may not be strictly comparable to those used in this study. For example, some viruses were exposed to gamma radiation in metal containers for safety. However, whether dose corrections were done for attenuation and the change in radiation quality from gamma to secondary X-rays was unclear. Many investigators reported D_{10} values, but in some instances estimates of D_{10} were made from the published inactivation curves or by conversions from D_0 (37% inactivation).

The D_{10} values from this study for gamma inactivation all fall within the range of reported values. As frozen samples reduce attack by hydrated free radicals, exposure even at ice temperatures was expected to result in very different sensitivities to radiation, possibly even order of magnitude changes. The aggregate data shows no such effect, the range of values being very similar for the frozen and unfrozen data. However, two sets of experiments made direct comparisons between frozen and ice temperature samples for Lassa and Marburg ss RNA viruses (Elliott et al., 1992) and HSV a ds DNA virus (Zamansky and Little, 1982). The D_{10} values differed by factors of 1.6–1.8 for the ss RNA viruses and approximately 2.8 for the ds DNA virus with, as expected, the unfrozen material requiring the lower radiation dose. Taking this 2–3-fold factor into account, the D_{10} values of 2 and 3 based on PFU assay data correspond reasonably well to previously reported values of 4.9 kGy for unfrozen influenza A virus (Sullivan et al., 1971).

The neutron data from this study is at the upper range of the D_{10} values reported, those determined from the $TCID_{50}$ titers being particularly high. Only one other study using neutrons (Singh et al., 1990) is for the ss DNA bacteriophage M13m11. Comparison to gamma photons was also performed with a RBE of 6. Therefore, contrary to the results reported here, their findings show gamma photons being less effective than neutrons for inactivating the virus. No exposure temperature was given, but samples were in a buffered saline, apparently at near ambient temperature, and air cooled for the neutron exposures to prevent excessive heating. One concern in interpreting these findings is that the D_{10} values reported were 1–2 orders of magnitude lower than other reported values being 15 Gy (neutrons) and 90 Gy (gamma). Genome target size is unlikely to be an explanation for the low D_{10} values as it is one of the smaller viruses, having a molecular weight lower than influenza virus.

The magnitude of the D_{10} values in this study was dependent on the titer method used, with those for the PFU assay being consistently smaller. It is important to note that differences between D_{10} values are not dependent on the absolute magnitudes of raw titer data as the D_{10} values are derived from normalized proportional survival curves. Most titers from the literature were determined by PFU assays, but in two reports, $TCID_{50}$ assays were used (Table 2 Elliott et al., 1992; House et al., 1990). Unfortunately, no other studies, for radiation inactivation, were found which directly compared titer methods. The aggregate means for the ss RNA data does show a slight upward trend for the D_{10} determined from $TCID_{50}$ (3.4 ± 0.52) versus PFU (1.5 ± 0.21) data for unfrozen samples, but little difference for the frozen material, 3.0 ± 0.45 and 3.3 ± 0.60, $TCID_{50}$ and PFU data, respectively. The possibility that D_{10} values differ depending on the titer assay method has important implications in considering the total gamma dose necessary to inactivate viruses. For example, using the gamma D_{10} values from this study, 25 kGy would result in a 10–12-log reduction in titer, as predicted by the PFU assay based D_{10}, but only a 3–4-log reduction in titer as predicted by the $TCID_{50}$ assay based D_{10}. Therefore, in studies where demonstrating essentially complete loss of virus viability by radiation is important, such as the preparation of biologicals or antigen for vaccination, a single type of titer assay may not be stringent enough.

3.4. Mechanism of radiation inactivation

Assays for gross molecular damage were done for the four major structural protein bands and six RNA bands of influenza virus that can be readily separated by PAGE (Fig. 3). The protein bands are for the structural proteins; RNA depen-

Table 2
Summary of previously reported D_{10} values

	Genome	Titer assay	Temperature media (°C)	Radiation source	D_{10} (kGy)	Literature citation
Adenovirus	ds DNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.1–4.9	Sullivan et al., 1971
Coxsackievirus	ss RNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.1–4.8	Sullivan et al., 1971
Echovirus	ss RNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.4–5.1	Sullivan et al., 1971
Herpes simplex virus	ds DNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.8–5.2	Sullivan et al., 1971
Influenza A	ss RNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.9	Sullivan et al., 1971
Newcastle Disease	ss RNA	Plaque	RT? 2% serum/MEM	Cobalt 60	5.2	Sullivan et al., 1971
Poliovirus	ss RNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.9–5.2	Sullivan et al., 1971
Reovirus I	ds DNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.2	Sullivan et al., 1971
Simian virus	ds DNA	Plaque	RT? 2% serum/MEM	Cobalt 60	4.5	Sullivan et al., 1971
Coxsakievirus	ss RNA	Plaque	RT? Water	Cobalt 60	1.2	Sullivan et al., 1971
Echovirus	ss RNA	Plaque	RT? Water	Cobalt 60	1.4	Sullivan et al., 1971
Influenza A	ss RNA	Plaque	RT? Water	Cobalt 60	1	Sullivan et al., 1971
Poliovirus	ss RNA	Plaque	RT? Water	Cobalt 60	1.1	Sullivan et al., 1971
Psuedorabies	ss RNA	TCID	−70 °C	Cobalt 60	5	Sun et al., 1978
Ebola Zaire	ss RNA	Plaque	Frozen	Cobalt 60	2.3	Lupton, 1981
Akabane	ss RNA	Plaque	RT?	Cobalt 60	<2.0	Thomas et al., 1981
African Swine Fever	ds DNA	HA	RT?	Cobalt 60	<2.0	Thomas et al., 1981
Avian Adenovirus	ds DNA	Plaque	RT?	Cobalt 60	4.8	Thomas et al., 1981
Avian pox	ds DNA	Plaque	RT?	Cobalt 60	2.2	Thomas et al., 1981
Bovine Diarrhea Virus.	ss RNA	Plaque	RT?	Cobalt 60	<2.0	Thomas et al., 1981
Infect. bovine rhino	ds DNA	Plaque	RT?	Cobalt 60	<2.0	Thomas et al., 1981
Bluetongue	ds RNA	Plaque	RT?	Cobalt 60	<2.0	Thomas et al., 1981
Maedi-visna	ss RNA	Plaque	RT?	Cobalt 60	3.5	Thomas et al., 1981
Newcastle disease	ss RNA	Eggs	RT?	Cobalt 60	2	Thomas et al., 1981
Porcine parvovirus	ss DNA	HA	RT?	Cobalt 60	4	Thomas et al., 1981
Pseudorabies	ds DNA	Plaque	RT?	Cobalt 60	<2.0	Thomas et al., 1981
Swine vesc. Disease	ss RNA	Plaque	RT?	Cobalt 60	5.5	Thomas et al., 1981
Teschen	ss RNA	Plaque	RT?	Cobalt 60	2.8	Thomas et al., 1981
Transm. gastroent.	ss RNA	Plaque	RT?	Cobalt 60	<2.0	Thomas et al., 1981
Ebola	ss RNA	TCID	−60 °C/culture media?	Cobalt 60	2.2	Elliott et al., 1992
Ebola	ss RNA	TCID	4 °C/culture media?	Cobalt 60	1.4	Elliott et al., 1992
Lassa	ss RNA	TCID	−60 °C/culture media?	Cobalt 60	3.1	Elliott et al., 1992

Table 2 (*Continued*)

	Genome	Titer assay	Temperature media (°C)	Radiation source	D_{10} (kGy)	Literature citation
Lassa	ss RNA	TCID	4 °C/culture media?	Cobalt 60	1.9	Elliott et al., 1992
Marburg	ss RNA	TCID	−60 °C/culture media?	Cobalt 60	2.1	Elliott et al., 1992
Marburg	ss RNA	TCID	4 °C/culture media?	Cobalt 60	1.2	Elliott et al., 1992
HSV	ds DNA	Plaque	−75 °C 5% serum/MEM	Cobalt 60	1.0	Zamansky and Little, 1982
HSV	ds DNA	Plaque	4 °C 5% serum/MEM	Cobalt 60	0.4	Zamansky and Little, 1982
HSV 1 Theta	ds DNA	Plaque	−80 °C/water	Cobalt 60	3.3	Rosen et al., 1987
HSV 1 Ang	ds DNA	Plaque	−80 °C/water	Cobalt 60	4.3	Rosen et al., 1987
HSV 1 Kos	ds DNA	Plaque	−80 °C/water	Cobalt 60	2.7	Rosen et al., 1987
HSV 1 Muller	ds DNA	Plaque	−80 °C/water	Cobalt 60	4.3	Rosen et al., 1987
Akbane	ss RNA	TCID	−68 °C	Cobalt 60	2.5	House et al., 1990
Aino	ss RNA	TCID	−68 °C	Cobalt 60	3.5	House et al., 1990
Bovine ephem. fever	ss RNA	TCID	−68 °C	Cobalt 60	2.9	House et al., 1990
Bluetongue	ds RNA	TCID	−68 °C	Cobalt 60	8.3	House et al., 1990
Foot-mouth disease	ss RNA	TCID	−68 °C	Cobalt 60	5.3	House et al., 1990
Hog Cholera	ss RNA	TCID	−68 °C	Cobalt 60	5.5	House et al., 1990
Minut. virus of mice	ss DNA	TCID	−68 °C	Cobalt 60	10.7	House et al., 1990
Swine vesicular fever	ss RNA	TCID	−68 °C	Cobalt 60	5	House et al., 1990
Bacteriaphage M13	ss DNA	Plaque	RT?	Gamma-particle accelerator	0.090	Singh et al., 1990
Bacteriaphage M13	ss DNA	Plaque	RT?	Neutron-particle accelerator	0.015	Singh et al., 1990
Hepatitis A	ss RNA	Plaque	RT	Cobalt 60	2.9	Bidawid et al., 2000

Data are grouped first by author chronologically and then by virus alphabetically. Exposure conditions are provided when and as possible from the published methods. MEM, minimal essential tissue culture media. RT, ambient temperature conditions reported. RT?, no temperature conditions were described, but no means of holding temperature below ambient were reported. In a few cases, D_{10} values were calculated from either D_{37} values or estimated from slopes of published survival curves.

dent transcriptase (polymerase) proteins, P, nucleoprotein, NP, matrix protein, M, and the combined co-migrating neuramininadase, N, and hemagglutinin, HA0 band. The RNA bands are for the respective P, HA, NP, NA, M and nonstructural NS genes (Lamb and Krug, 1996). The P band consists of the three unresolved viral transcriptase complex genes. Densitometry failed to find any large or systematic changes in the bands from the irradiated virus in comparison to the controls. Nor were changes in molecular weights, accumulation of small molecular species at the gel front or increased smearing along the length of the gels observed for either RNA or protein. This suggests that gross structural changes did not occur in the majority of the viruses. Even at the highest radiation doses PAGE would not likely reveal single hits in any one genomic segment against the large background of undamaged RNA from viruses with lesions in one of the other eight genes. Therefore, based on our results, the damage site cannot be unequivocally defined for either quality of radiation. Defining the precise damage the site(s) will likely require use of functional assays for each stage of the replication process.

Several observations suggest that RNA rather than protein is likely to be the primary target for radiation damage. First, neutrons were consider-

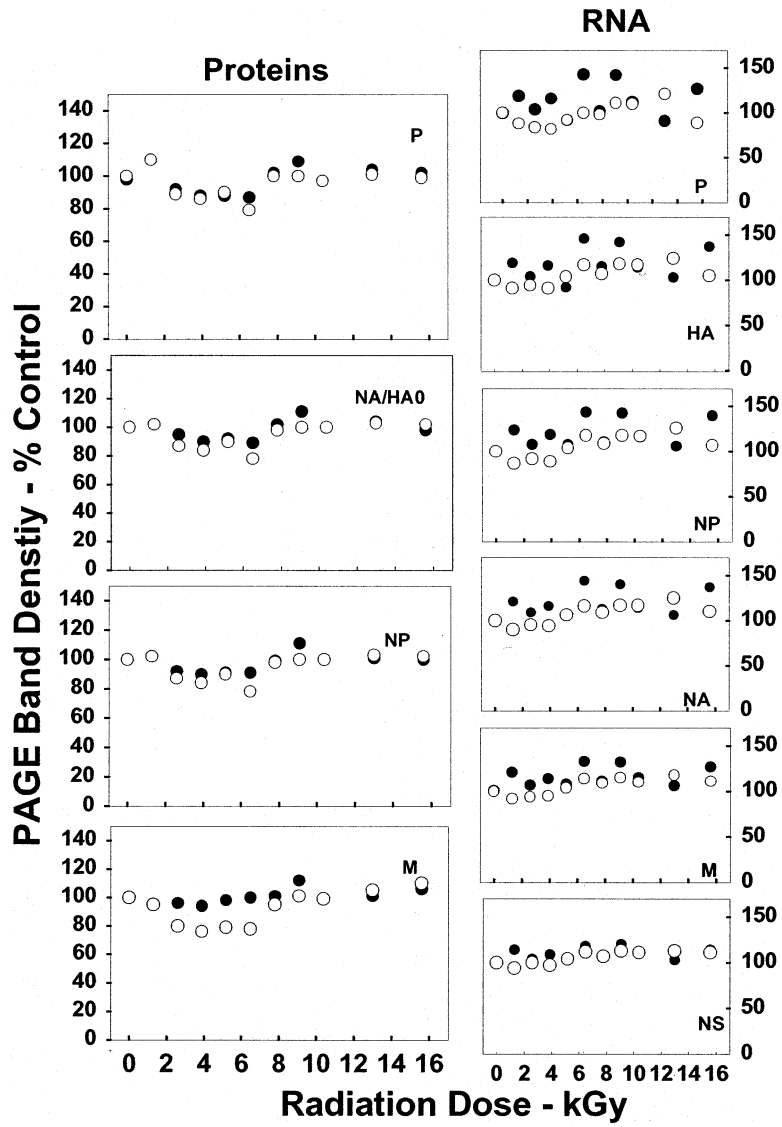

Fig. 3. Normalized PAGE band density of A/PR8 proteins and RNA. Viral protein and RNA were extracted from irradiated virus samples and an unirradiated control sample. The control and all radiation doses were run on a single gel. Band densities for the major structural proteins and genomic RNA that can be resolved by PAGE were determined and normalized by the density of the respective band from the control sample. Neutron irradiated (open symbols) and gamma irradiated (closed symbols). Data is from a representative gel, from two to three replicate gel analysis experiments. Results for A/X31 samples were nearly identical (data not shown).

ably less effective than gamma photons in causing damage. This is the same RBE pattern that is observed in studies of direct high (neutron) and low (gamma) LET radiation damage by 'frank' direct strand breaks to DNA molecules in vitro (Barendsen, 1994; Goodhead, 1994; Mustonen et al., 1999; Ward, 1988). RBEs of less than 1 for neutrons are consistently observed and appears to be independent of the form of the DNA having been observed for both circular, linear, and oligonucletides (Stankus et al., 1995; Swenberg and Speicher, 1995; Swenberg et al., 1997). Litera-

ture reports for RNA molecules were not found. Secondly, the radiation doses were lower than the 100 kGy per 0.5 log reduction shown necessary for damage of influenza virus hemmaglutination and membrane fusion, which are envelope protein dependent processes (Bundo-Morita et al., 1988; Gibson et al., 1986). This is consistent with data from other viruses where radiation doses causing loss of replication were consistently lower than those that alter these protein dependant activities such as virus electron microscopy profiles, antigenicity, and hemagglutination (DeFlora and Badolati, 1973; Elliott et al., 1992; Gamble et al., 1980; Pang et al., 1992; White et al., 1990). Thirdly, the single exponential inactivation curves in this study are generally interpreted to indicate a single hit process of radiation damage. Single hit kinetics have been usually observed for viruses and several authors have concluded that nucleic acids are the primary target as D_{10} values tended to correlate with genome size (House et al., 1990; Lupton, 1981; Thomas et al., 1982). Influenza RNA is single stranded and there is no known repair or re-ligation activity known for the influenza transcriptase complex (Lamb and Krug, 1996). Therefore, single hits resulting in single strand breaks, in any one of the genes, would be lethal unless complimented by a second virus. Proteins as radiation targets cannot be excluded completely (Megumi et al., 1993). Studies with influenza virus demonstrated that manifestation of radiation damage to proteins was temperature dependent; −80 °C storage had no effect on hemagglutination but storage longer than 3 months at 4 °C or greater caused significant decreases (DeFlora and Badolati, 1973).

In summary, the broad outlines of inactivating viruses by ionizing radiation have been shown for both gamma and neutron inactivation. This study suggests that gamma radiation should be used when the goal of inactivation is sterilization. All reported D_{10} values are generally in the range of 1–10 kGy, suggesting that total doses in the range of 10–100 kGy are needed for multi-log reductions in viral load. A number of the studies cited demonstrated that viral material could be rendered non-infectious by radiation without major losses in antigencity. Radiation is potentially a particularly advantageous method of inactivating viral pathogens for the preparation of vaccines or positive controls for analytical methods. The possibility of ancillary protein damage combined with unwanted residual immunlogical activity needs to be carefully monitored for some applications.

Acknowledgements

Contributions of the Radiation Sciences and Safety and Health Departments are gratefully recognized. The Nuclear, Biological, Chemical Interactions and Countermeasures program within the Armed Forces Radiobiology Research Institute supported this work.

References

American Association of Physicists in Medicine AAPM Task Group 21, 1983. A protocol for the determination of absorbed dose from high-energy photon and electron beams. Med. Phys. 10, 741–771.

Barendsen, G.W., 1994. The relationship between RBE and LET for different types of lethal damage in mammalian cells: biophysical and molecular mechanisms. Radiat. Res. 139, 257–270.

Barrett, T., Inglis, S.C., 1985. Growth, purification and titration of influenza viruses. In: Mahy, B.W.J. (Ed.), Virology A Practical Approach. IRL Press, Washington, DC.

Bidawid, S., Farber, J.M., Sattar, S.A., 2000. Inactivation of hepatitis A virus (HAV) in fruits and vegetables by gamma irradiation. Int. J. Food Micro. 57, 91–97.

Bundo-Morita, K., Gibson, S., Lenard, J., 1988. Radiation inactivation analysis of fusion and hemolysis by vesicular stomatitis virus. Virology 163, 622–624.

Burleson, F.G., Chambers, T.M., 1992. In: Wiedbrauk, D.L. (Ed.), Virology: a Laboratory Manual, first ed. Academic Press, New York.

Daley, J.P., Danner, D.J., Weppner, D.J., Plavisic, M.Z., 1998. Virus inactivation by gamma irradiation of fetal bovine serum. Focus (Life Techology) 20 (3), 86–88.

DeFlora, S., Badolati, G., 1973. Thermal inactivation of untreated and gamma-irradiated A2/Aichi/2/68 influenza virus. J. Gen. Virol. 20, 261–265.

Elliott, L.H., McKormick, J.B., Johnson, K.M., 1992. Inactivation of lassa, marburg, and ebola viruses by gamma irradiation. J. Clin. Micro. 16 (4), 704–708.

Farkas, J., 1998. Irradiation as a method for decontaminating food. Int. J. Food Micro. 44, 189–204.

Freshney, R.I., 1983. Culture of Animal Cells. Alan R. Liss, New York, NY.

Gamble, W.C., Chappell, W.A., George, E.H., 1980. Inactivation of rabies diagnostic reagents by gamma radiation. J. Clin. Microbiol. 12 (5), 676–678.

Gibson, S., Jung, C.Y., Takahashi, M., Lenard, J., 1986. Radiation inactivation analysis of influenza virus reveals different target sizes for fusion, leakage and neuriminidase activities. Biochemistry 25, 6264–6268.

Goodhead, D.T., 1994. Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. Int. J. Radiat. Biol. 65 (1), 7–17.

Gruber, J., 1971. Immunogenicity of purified venezuelan equine encephalitis virus inactivated by ionizing radiation. Infect. Immun. 3 (4), 574–579.

House, C., House, J.A., Yedloutschnig, R.J., 1990. Inactivation of viral agents in bovine serum by gamma irradiation. Can. J. Micro. 36, 737–740.

Hubbard, W.K., 1998. Q5A. Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. Federal Register 63 (185), 51075–51083.

LaBarre, D.D., Lowy, R.J., 2001. Improvements in methods for calculations virus titer estimates from $TCID_{50}$ and plaque assays. J. Virol. Methods 96, 107–126.

Lamb, R.A., Krug, R.M., 1996. Orthomyxoviridae: The viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Field Virology, third ed. Lippencott-Raven, Philadelphia.

Lupton, H.W., 1981. Inactivation of Ebola virus with 60Cobalt irradiation. J. Infect. Dis. 143 (2), 291.

Megumi, T., Fujita, S.I., Iwai, Y., Ito, T., 1993. The effect of gamma-ray-induced radicals on activities and membrane structure of sendai virus in aqueous solutions. Radiat. Res. 134, 129–133.

Mustonen, R., Bouvier, G., Wolber, G., Stohr, M., Peschke, P., Bartsch, H., 1999. A comparison of gamma and neutron irradiation on Raji cells: effects on DNA damage, repair, cell cycle distribution and lethality. Mutat. Res. 429, 169–179.

Pang, G.T., Clancy, R.L., O'Reilly, S.E., Cripps, A.W., 1992. A novel particulate influenza vaccine induces long-term and broad-based immunity in mice after oral immunization. J. Virol. 66 (2), 1162–1170.

Patterson, R.G., Lamb, R.A., 1993. The molecular biology of influenza viruses and paramyxoviruses. In: Davison, A.J., Elliot, R.M. (Eds.), Molecular Virology: a Practical Approach, first ed. IRL Press, New York.

Rodriguez-Boulan, E., 1983. Polarized Assembly of enveloped viruses from cultured epithelial cells. Methods Enzymol. 98, 486–502.

Rosen, A., Taylor, D.M., Darai, G., 1987. Influencing of ionizing radiation on herpes simplex virus and its genome. Int. J. Radiat. Biol. 52 (5), 795–804.

Singh, S.P., Cohen, D., Dytlewski, N., Houldsworth, J., Lavin, M.F., 1990. Neutron and gamma-irradiation of bateriophage M13 DNA: use of standard neutron irradiation facility (SNIF). J. Radiat. Res. 31, 340–353.

Stankus, A.A., Xapsos, M.A., Kolanko, C.J., Gerstenberg, H.M., Blakely, W.F., 1995. Energy deposition events produced by fission neutrons in aqueous solutions of plasmid DNA. Int. J. Radiat. Biol. 68 (1), 1–9.

Sullivan, R., Fassolitis, A.C., Larkin, E.P., Read Jr, R.B., Peeler, J.T., 1971. Inactivation of thirty viruses by gamma radiation. Appl. Micro. 22 (1), 61–65.

Swenberg, C.E., Speicher, J.M., 1995. Neutron and gamma-radiation sensitivity of plasmid DNA of varying superhelical density. Radiat. Res. 144, 301–309.

Swenberg, C.E., Vaishnav, Y.N., Li, B., Tsao, H., Mao, B., Geacintov, N.E., 1997. Single-strand breaks in oligodexoyribonucleotides induced by fusion neutrons and gamma radiation measured by gel electrophoresis: protective effects of aminothiols. J. Radiat. Res. 38, 241–254.

Sun, I.L., Gustafson, D.P., Sherba, G., 1978. Comparison of pseudorabies virus inactivation by bromo-ethylene-imine, 60Co-irradiation, and acridine dye in immune assay systems. J. Clin. Microbiol. 8 (2), 604–611.

Thomas, F.C., Davies, A.G., Dulac, G.C., Willis, N.G., Papp-Vid, G., Girard, A., 1981. Gamma ray inactivation of some animal viruses. Can. J. Micro. 45, 397–399.

Thomas, F.C., Ouwerkerek, T., McKercher, P., 1982. Inactivation by gamma irradiation of animal viruses in simulated laboratory effluent. Appl. Environ. Micro. 43 (5), 1051–1056.

Toyoshima, K., Niwa, O., Yususdo, M., Sugiyama, H., Tahara, S., Sugahara, T., 1980. Sensitivity to gamma rays of avian sarcoma and murine leukemia viruses. Virology 105, 508.

Ward, J.F., 1988. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanism of formation, and reparability. Prog. Nucl. Acid Res. Mol. Biol. 35, 96–125.

White, L.A., Freeman, C.Y., Hall, H.E., Forrester, B.D., 1990. Inactivation and stability of viral diagnostic reagents treated by gamma radiation. Biologics 18 (4), 271–280.

Zamansky, G.B., Little, J.B., 1982. Survival of 60 Co-irradiated herpes simplex virus in 15 human diploid fibroblast cell strains. Mutat. Res. 94, 245–255.