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Practical proof of the validity of the Target Theory by simulating cellular targets

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برهان عملي على صلاحية نظرية الهدف بأستخدام أهداف خلوية مقلدة

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الملخص: الهدف: يهدف البحث لأثبات عمليا صلاحية نظرية الهدف كنموذج رياضي موضوع لوصف وفهم آليات التأثير القاتل للأشعة المؤينة. **الطريقة:** تمت محاولة إثبات صلاحية نظرية الهدف تجريبيا باستخدام ابواغ (سبورات) بكتيريا عصيات ميغاتيريوم ، وقد تم تعريض مجاميع يحوي كل منها على 100 قنينة، احتوت قنا ني كل مجموعة على500,50,5,2,1،بوغ لكل قنينة، إلى جرع مختلفة من أشعة غاما بوجود وعدم وجود الأوكسجين. واعتبرت النسبة المئوية للقنا ني التي أظهرت نموا بكتيريا بعد حضنها لمدة ستة أيام يحتوي على بوغ واحد أو اكثر استطاع مقاومة الجرعة الإشعاعية. ولغرض التشبيه اعتبرت كل قنينة تمثل خلية حية متكاملة تحوي عددا من الأهداف (ابواغ) كل منها يحتاج إلى ضربة واحدة كي يفقد فاعليته، وقد اعتمد افتراض الحاجة إلى الضربة الواحدة هذه إلى شكل منحنى البقاء الخاص بالابواغ المستعملة حيث اتصف بعدم احتوائه على انحدار في الجرعة الإشعاعية التي قيمتها صفر. **النتيجة:** اتصفت منحنيات البقاء التي اشتقت من العمل الحالي باحتوائها على كتف يليه انحدار أسي، كان حجم الكتف يزداد بزيادة عدد الابواغ في كل قنينة ، في حين إن انحدار الجزء الأسى بقي ثابتا. **الخلاصة:** بالرغم من بعض الافتراضات التي وضعت لتسهيل عملية التشبيه فان البيانات التي حصل عليها من هذه الدراسة تتطابق جيدا مع تلك التي حسبت باستخدام النموذج الرياضي لنظرية الأهداف المتعددة ذات الضربة الواحدة التالي:
$P = 1 - (1 - e^{-KD})^N$ حيث إن P هو الجزء المتبقي ، و K هو ثابت فقدان الفاعلية الإشعاعي ، وD هي الجرعة الإشعاعية ، و N هو عدد الأهداف. إن هذا التطابق يثبت صلاحية نموذج نظرية الهدف كأداة تمكننا من فهم التأثيرات الإشعاعية المؤذية بصورة افضل.

ABSTRACT: ***Objective –*** To practically prove the validity of the target theory as a mathematical model to describe and understand the mechanisms involved in cell killing by ionising radiation. ***Method –*** Experimental validation of the target theory was attempted using *Bacillus megaterium* spores. Sets of 100 vials containing averages of 1, 2, 5, 50 and 500 spores per vial was exposed to varying gamma radiation doses in presence (oxic) and absence (anoxic) of oxygen (O_2). The percentage of the vials that exhibited bacterial growth after 6 days of incubation was taken as containing one spore or more, which survived a given dose. For the purpose of simulation each vial was considered to represent one living cell, as a unit, containing a given number of targets (spores) each of which needed a single hit to be inactivated. The need for single hit was assumed depending on the shape of the dose ln-survival curve of *B. megaterium* spores, which has a non-zero slope at zero dose. ***Result –*** The dose ln-survival curves derived from these radiation experiments are characterized by a shoulder followed by an exponential part. The size of the shoulder increases with increasing number of spores per vial. However, the slope of the exponential parts stays the same. ***Conclusion –*** Despite some assumptions imposed to easily manipulate the simulation process, the data obtained from the present study correlate well with those calculated using the multitarget single hit (MTSH) equation: $P = 1 - (1 - e^{-KD})^N$ where P is the surviving fraction, K is the radiation inactivation constant, D is the radiation dose and N is the number of target. This proves the validity of the target theory model as a tool to provide a better understanding of the observed notorious effects of radiation.

KEY WORDS: Target theory, bacterial spore, gamma radiation, simulation

It is a long time since Lea[1] proposed his interpretive models for cell killing by radiation, generally known as the "target theory of cell killing". The theory was developed using microorganism inactivation data and bioactive molecules. Although there have been debates about the validity of the Target Theory as a general model of cellular radiation inactivation that characterizes cellular death, it has wide applicability to mammalian clonogenic survival.[2,3] Target theory has taken into consideration a number of variables that can possibly modify the inactivation capacity of ionising radiation such as chemical additives, temperature, type of test organism and its physiological status, type of radiation and many others. Nevertheless, there are still insurmountable shortcomings that prevent the generalization of the applicability and validity of the theory.

The physiological dormancy of bacterial spores renders them unable to perform biological activities,[4–6]

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including repair of radiation damage.[7] This could be justified on the basis that bacterial spores that survived a given radiation dose (both in presence or absence of O_2) demonstrated no change in the number of surviving spores even after a long period of holding in distilled water and in buffer prior to plating out.[8] In addition, exposure of spores to harsh conditions normally damaging to vegetative cells does not alter their viability.[9–12]

Radiation inactivation of bacterial spores in presence and absence of O_2 has been extensively studied and the shapes of the survival curves have been very well characterized.[13–16] Generally, spores irradiated in the absence of O_2 exhibited non-shoulder dose ln-survival curves with an extrapolation number (the intercept of the back extrapolated linear portion of the dose ln-surviving curve with the y-axis), *n*, of 1.0. However, the presence of O_2 slightly increased this value to about 1.2. This small increase in the value of *n* may be attributed to the dose modifying action of O_2,[17] which alters mainly the slope (*k*) of the exponential part of the survival curve. In addition, the experimental design of the dose ln-survival curve adopted in most of the previous studies was not good enough to determine the exact values of both *k* and *n;* the major part of the curve was determined by very small numbers of viable cells at higher doses.[18] Therefore, in the present investigation, the small shoulder of the oxic cells was not considered as a change in number of targets.

There has always been an application of target theory to explain the effect of radiation on biological systems.[17–26] Based on the MTSH mathematical formulation, a relationship between the number of cellular targets and the value of the extrapolation number was assumed,[17,27] i.e., as the value of extrapolation number increases, the shoulder of the survival curve widens. Such increase in width can also be obtained if the number of targets per cell (values of *N*) is increased. Therefore, spores exhibiting a value of extrapolation number close to 1.0 can be assumed to represent cells that have one target which requires a single hit to be inactivated. It should be noted, however, that this assumption could not be extended to fully describe the effects of radiation on other biological systems such as mammalian cells. Some of these systems respond to radiation in a multiphase fashion and the relationship between cell survivors and radiation dose deviates from the usual trend, i.e., a shoulder followed by an increasingly curving exponential part.[3] The relationship is rather well described by a linear quadratic model which assumes two components of cell killing by radiation, one proportional to the dose and the other proportional to the square of the dose.[2]

Based on the above assumption, it was hoped in the present investigation to study the possibility of using vials containing bacterial spores, with a particular experimental design, to simulate cells with varying number of targets. Each spore was considered as a single target that required a single hit to become inactivated.

METHOD

TEST MICROORGANISM AND GROWTH MEDIA

Spores of *Bacillus megaterium* ATCC 8245 were suspended in distilled water to make a stock suspension of 2×10^7 spores/ml. The spores were prepared and washed as previously described.[28] In order to activate the spores and to kill the vegetative cells, the spore suspension was heat shocked at 80°C for 15 minutes. Plating efficiency of the nutrient medium (nutrient broth and agar; Oxoid, Hampshire, UK) was determined to be 100% by comparing the total number of spores (counted with the aid of a platelet counting chamber) with the number of spores capable of forming visible colonies on the nutrient agar. The latter (viable count) was done weekly to follow the degree of spore batch stability during storage at 4°C.

The selection of spores as the biological indicator was made on the basis of their physiological dormancy. This characteristic allowed their storage in distilled water for a long period without noticeable loss of viability, which permitted the use of the same spore suspension over the entire experimentation period.

IRRADIATION TECHNIQUE

For the purpose of determining the regular oxic and anoxic radiation response, spore suspensions were exposed at 24°C to ^{137}Cs gamma radiation (Nordion International Inc., Ontario, Canada), at a dose rate of 0.035 kGy/minute in the presence and absence of O_2. For anoxic experiments, 5 ml of spore suspension (10^5 spores/ml) was purged with nitrogen (N_2) (99.995%; British Oxygen Co.) for 10 minutes prior to the commencement of irradiation and then vials were held tightly closed during the irradiation periods. However, for the oxic experiment, vials were irradiated in equilibrium with air. Caps were kept slightly loosened to allow for air equilibration. The oxygen enhancement ratio (OER) was determined to be 2.16.

Two sets of experiments were carried out with vials (capacity 2 ml) containing 1 ml of spore suspension: one set was purged with N_2 for one minute only prior to irradiation (to deoxygenate suspension), and the other set was irradiated in equilibrium with air. Equilibrium with air was maintained without any additional air flow or stirring. No direct measurement of the concentration of O_2 was performed. However, the efficiency of deoxygenation was predicted simply by comparing the OER value (2.21) obtained from these experiments with that derived from the regular survival curves (2.16).

Diluted spore suspension was prepared to have an average number of 1, 2, 5, 50 and 500 spore/ml of nutri-

ent broth. Aliquots of 1 ml of the diluted suspension were introduced into 100 screw capped, 2 ml capacity vials so that each vial contained the given average number of spores. Test of spore distribution among vials was performed. Vials containing average of 1 and 2 spores per vial exhibited only 71 and 90% growth, respectively, i.e. the number of vials which exhibited growth among the 100 vials used in each test. Vials containing average of more than 2 spores each exhibited 100% growth. For ease of comparison, percentage of the vials containing 1 and 2 spores per vial was made up to 100%.

Radiation exposure was carried out by arranging vials in a beaker and exposing the beaker to gamma radiation. To ensure homogeneous exposure, the beaker was rotated throughout the irradiation period. No specific radiation dosimetry was performed. Dose rate calculation was performed using the theoretical decay scheme of ^{137}Cs and the isodose distribution provided by the manufacturer.

Irradiated vials were then incubated at 37°C for 6 days. The presence of growth, which was detected by the presence of turbidity, was used as an indication of the presence of one or more spores surviving the given radiation dose. In each experiment, two vials containing the same number of spores, but without irradiation, were used as controls.

RESULTS

Figure 1 shows typical dose ln-survival curves for spores in 5 ml suspensions irradiated in presence and absence of O_2. The calculated values of k and n were 7.30 $\pm$ 0.22 $\times$ 10^{-4} Gy^{-1} and 1.03 $\pm$ 0.027 for anoxic spores and

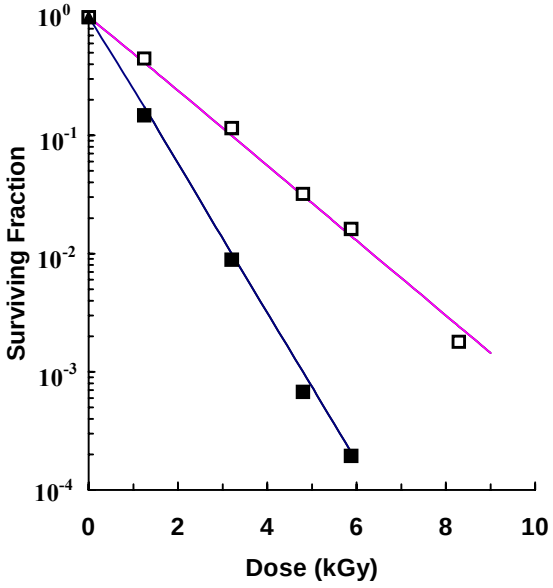

FIGURE 1. *Typical dose ln-survival curves for spores irradiated in presence (closed squares) and in the absence of (open squares) of O_2.*

17.30 $\pm$ 0.38 $\times$ 10^{-4} Gy^{-1} and 1.24 $\pm$ 0.028 for oxic spores, respectively. These values were derived from 6 replicated experiments.

Using the value of k given above (7.30 $\times$ 10^{-4} Gy^{-1}) as the inactivation constant, K, and varying values of extrapolation number, N, as the number of target, to range between 0–500 and radiation doses, D, to range between 0–12 kGy, a set of theoretical survival curves for anoxic condition was constructed using the MTSH equation:

$$P = 1 - (1 - e^{-KD})^N$$

as depicted by the solid lines in Figure 2. These curves are characterized by the same slope at the exponential regions and increasing size of shoulders and hence increasing extrapolation numbers. Similar theoretical set of curves was also constructed for spores irradiated in the presence of O_2 using a value of k derived from the typical oxic survival curve (17.30 $\times$ 10^{-4} Gy^{-1}), as depicted by the solid line in Figure 3. Again all curves exhibited the same slope but increasing size of shoulder.

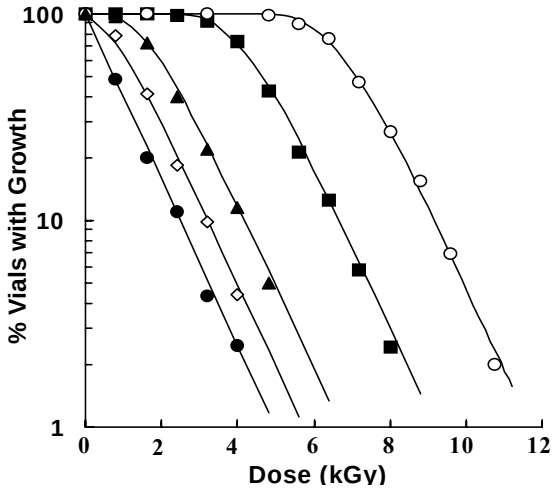

FIGURE 2. *The numbers of vials that exhibited growth after gamma irradiation in the absence of O_2.*

Symbols represent vials contained averages of 1 (closed circles), 2 (open diamonds), 5 (Closed triangles), 50 (closed squares) and 500 (open circles) spores/ vial. Solid lines represent curves derived from the MTSH formula in which the values of N varied, in multiple fashion, according to the corresponding value of extrapolation number of the dose ln-survival curve.

Survival curves derived from the present investigation in which vials containing varying number of spores and exposed to gamma radiation in the absence of O_2 are presented as symbols in the composite Figure 2. For clarity purposes and to avoid overlapping of data, and to allow easy comparison the lines are not shown. Curves are also characterized by having the same slope at the exponential regions and varying size of shoulder corresponding to the number of spores in each vial in a manner similar to the theoretical ones.

Similarly, data derived from oxic experiments are given as symbols in Figure 3. Again the slopes of the curves at the exponential regions are the same and the shoulders increase in size with increasing the number of spores per vial. Apparently, data derived from these experiments agree totally with the theoretically calculated.

DISCUSSION

Assuming *B. megaterium* spore as a cell having a single target that requires a single hit to be inactivated based on the value of extrapolation number (*n)* derived from the survival curves reported previously by many investigators.[9,12,15] Such a characteristic was considered in the present attempt to simulate a living cell by a vial containing nutritive medium in which varying numbers of spores were introduced to represent the bioactive molecules. The number of spore in each vial represents the number of target (target multiplicity), each of which requires a single hit to be inactivated.

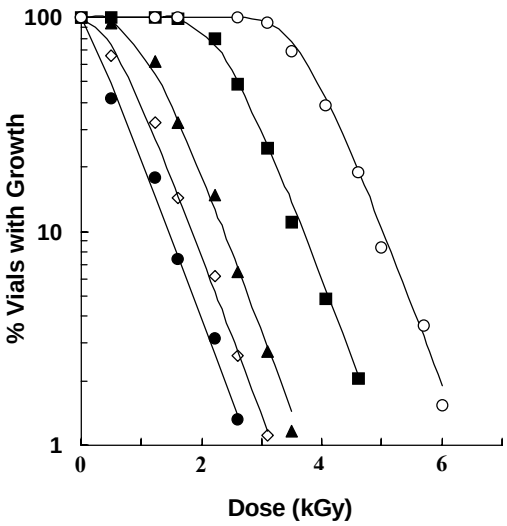

FIGURE 3. *Numbers of vials exhibited growth after gamma irradiation in the presence of* O_2.

Symbols represent vials contained averages of 1 (closed circles), 2 (open diamonds), 5 (Closed triangles), 50 (closed squares) and 500 (open circles) spores/vial. Solid lines represent curves derived from the MTSH formula in which the values of N varied, in multiple fashion, according to the corresponding value of extrapolation number of the dose ln-survival curve.

In order to test the validity of the present assumption theoretical data were obtained by substituting the value of *N* in the MTSH formula by the numbers 1, 2, 5, 50 and 500 for both oxic and anoxic condition. When the value of the extrapolation numbers, 1.03, and its multiples was used for anoxic spores, a set of curves is obtained (solid lines in Figures 2). However, in this set it is apparent (as it should be) that the change in the value of *N* influenced the size of the shoulders without changing the slope of the exponential part of the survival curves.

Similar findings were obtained when the number of spore in each set of vials was changed. The survival curves derived from anoxic experiments exhibited changes in the shoulders comparable with those of the theoretical survival curves and the values of extrapolation number correspond to their values of *N*. The two sets of survival curves superimpose, as shown in Figure 2.

Support to the above findings comes from the work done by Khan and Tallentire[29] in their attempt to verify a model relating frequency of contaminated items and increasing radiation dose. Theoretical (using MTSH mathematical model) and practical (using *B. pumilus* spores and *Serratia marcescens* cells) findings agree. This adds more to the present assumption in that these two microorganisms and *B. megaterium* spores behaved similarly regarding the validity and applicability of the MTSH model.

Typically, the presence of O_2 seems to have no effect on the observed values of *n* although the initial value of extrapolation number as calculated from the regular survival curve (1.24) was higher than that in the absence of O_2 (1.03). This relatively high value of *n* seemed to also affect the observed values of extrapolation number in a multiple fashion, i.e. the values of the extrapolation numbers increased by a factor of about 1.25 multiplied by the number of spores per vial.

The present findings support very well the role of targets in cell killing by radiation. However, the generalization of the present validity of the simulation is subject to a lot of questioning regarding the structure of the cell and the use of a vial, of a completely different composition, to represent the cell. But spore(s) present in a vial, no matter what the surrounding medium is, needed to be inactivated for the vial to show no growth. In addition, the large distances separating spores (targets) in the vial cannot, by no means, be compared with that in the cell. And the nature of the cellular targets vary from one cell to the other. Nevertheless, these shortcomings cannot be considered disproving factors for the simulation purpose since the ultimate end point is the same, i.e. the inactivation of the cell as the usual parameter and the absence of growth in the vial as the present investigation parameter.

The most encouraging aspect of the present finding is the similarity between the shapes of the theoretical survival curves and those derived from the present investigation. Similarly, the values of extrapolation number and slope derived from the present work correspond with those theoretically calculated. A major limitation reported earlier in the target theory[17] was that it has never been possible experimentally to obtain a zero slope at zero dose. In the present investigation, however, a zero slope was obtained even at high doses, particularly when larger numbers of spores per vial were used. The latter adds

one more support to the validity of the present experimental design, which substantiates our findings and supports the simulation process.

Based on the current molecular understanding of subcellular structures and the size of the elements affecting cellular radiation response,[30] target theory seems more complicated than can simply be justified by a simple simulation process. More work is needed to draw a solid conclusion about its general applicability. Hyperthermia,[2,31] radiation dose rate,[32] repair of radiation damage[23,33] and presence of chemical additives[17] have been so far studied and their effects on the validity of target theory were considered. Yet a generalized formula is far from being produced due to the diversity of factors involved in the modification of radiation damage.

Moreover, systems other than microorganisms, such as mammalian cells, impose difficulties in applying the target theory to describe the effect of radiation despite that the survival curves derived from mammalian cells experiments are characterized by having an initial shoulder followed by a portion that tends to become straight. However, for some cell lines the survival curve appears to bend continuously so that the conventional target theory model cannot be applied and therefore, the linear quadratic relationship is a better fit and the extrapolation number (*n*) has no meaning.[2]

CONCLUSION

The present data coincide very well with those calculated using the MTSH formula. This implies that the simulation of cell by vial containing a given number of spores to represent the bioactive molecules (targets), needed to be inactivated for the cell to die, is valid.

REFERENCES

1. **Kiefer J.** *Biological Radiation Effects.* Springer-Verlag, Berlin 1990.
2. **Hall EJ.** *Radiobiology for the Radiologist*. (4th ed.), Lippincott, Philadelphia 1994.
3. **Nias AHW**. *An Introduction to Radiobiology* 2nd ed., Wiley, Chichester 1998.
4. **Hopson JL, Wessells NK**. *Essentials of Biology*, McGraw-Hill Publishing Co. New York 1990.
5. **Popham DL, Gilmore ME, Setlow P.** Roles of low molecular weight penicillin-binding proteins in *Bacillus subtilis* spore peptidoglycan synthesis and spore properties. *J Bacteriol* 1999, **181**, 126–32.
6. **Atrih A, Foster SJ**. The role of peptidoglycan structure and structural dynamics during endospore dormancy and germination. *Antonie Van Leeuwenhoek* 1994, **75**, 299–307.
7. **Tallentire A**. Symposium on bacterial spores: XI. Radiation resistance of spores. *J Appl Bacteriol* 1970, **33,** 141–6.
8. **Salih FM**. Modification of liquid holding recovery of gamma irradiated *Bacillus megaterium* cells by chemicals and heat. *J Biol Sci Res* 1986, **17**, 127–46.
9. **Tallentire A, Jones AB.** Radiosensitization of bacterial spores by potassium permanganate. *Int J Radiat Biol* 1973, **24,** 345–54.
10. **Tallentire A, Jacobs GP**. Radiosensitization of bacterial spores by ketonic agents of differing electron affinities. *Int J Radiat Biol* 1972, **21**, 205–13.
11. **Tallentire A, Schiller NL, Powers EL**. 2,3-butanedione, an electron-stabilizing compound, as a modifier of sensitivity of *Bacillus megaterium* spores to X-rays. *Int J Radiat Biol* 1968, **14,** 397–402.
12. **Salih FM**. Radioensitization of *Bacillus megaterium* spores by combinations of oxygen and misonidazole. *J Radiat Res* 1984, **25,** 160–9.
13. **Stratford IJ, Maughan RL, Michael BD, Tallentire A**. The decay of potentially lethal oxygen-dependent damage in fully hydrated spores exposed to pulsed electron irradiation. *Int J Radiat Biol* 1977, **32**, 447–55.
14. **Stratford IJ, Tallentire A**. A comparative study of the gamma radiation responses of *Bacillus megaterium* spores suspended in aqueous solutions of ethanol and ethylene glycol. *J Pharm Phamacol* 1974, **26**, 103.
15. **Purdie JW, Ebert M, Tallentire A**. Increased response of anoxic *Bacillus megaterium* spores to radiation at high dose rate. *Int J Radiat Biol* 1974, **26,** 435–43.
16. **Stratford IJ, Tallentire A**. Inactivation by gamma-rays of *Bacillus megaterium* spores suspended in aqueous solutions of ethanol, acetone and 1,4-dioxane of widely varying concentrations. *J Pharm Pharmacol* 1973, **25,** 130.
17. **Alpen EL**. *Radiation Biophysics*. (2nd ed.), Academic Press San Diago 1998.
18. **Chadwick KH, Leenhouts HP**. A molecular theory of cell survival. *Phys Med Biol* 1973, **18,** 78–87.
19. **Kiefer J**. Target theory and survival curves. *J Theor Biol* 1971, **30,** 307–17.
20. **Alper T**. Mechanisms of cell killing in the light of formal target theory. *Br J Radiol* 1975, **48,** 415–26.
21. **Gilbert CW**. Target-type models for survival curves. *Br J Radiol* 1975, **48,** 1045–56.
22. **Domon M**. A biological variability model of cell survival curves. *Radiat Res* 1980, **82,** 611–5.
23. **Louw WK, van Rensburg EJ, Izatt H, Engelbrecht RI**. Nucleoid sedimentation analysis of DNA superstructure, gamma radiation induced damage and repair in human and chacma baboon (*Pupia ursinus*) peripheral lymphocytes. *Int J Radiat Biol* 1991, **59**, 951–62.
24. **Katz R, Zachariah R, Cucinotta FA, Zhang C**. Survey of cellular radiosensitivity parameters. *Radiat Res* 1994, **140**, 356–65.
25. **Deshpande A, Goodwin Eh, Baily SM, Marrone BL, Lehnert BE**. Alpha particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extra nuclear target. *Radiat Res* 1996, **145**, 260–7.
26. **Briden PE, Holt PD, Simmons JA**. The track structures of ionizing particles and their application to radiation biophysics, I. A new analytical method for investigating two biophysical models. *Radiat Environ Biophys* 1999, **38**, 175–84.
27. **Tallentire A, Dwyer J, Ley FT**. Microbiological quality control of sterilized products: evaluation of a model relating frequency of contaminated items with increasing ra-

diation treatment. *J Appl Bacteriol* 1971, **34,** 521–34.
28. **Powers EL, Ehret CF, Bannon A**. The membrane filter technique in radiation studies of spores of *Bacillus megaterium*. *Appl Microbiol* 1957, **5,** 61–4.
29. **Khan AA, Tallentire A, Dwyer J**. Quality assurance of sterilized products: verification of a model relating frequency of contaminated items and increasing radiation dose. *J Appl Bacteriol* 1977, **43**, 205–13.
30. **Savage JR**. Update on target theory applied to chromosomal aberrations. *Environ Mol Mutagen* 1993, **22**, 198–207.
31. **Petin VG, Komarov VP**. Mathematical description of synergistic interaction of hyperthermia and ionizing radiation. *Math Biosci* 1997, **146**, 115–30.
32. **Goodhead D**. Track structure considerations in low dose and low dose rate effects of ionizing radiation. *Adv Radiat Biol* 1992, **16**, 7–44.
33. **Gordon AT, McMillan TJ**. A role for the molecular biology in radiotherapy. *Clin Oncol* 1997, **9**, 70–78.