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The Bystander Effect in Radiation Oncogenesis: II. A Quantitative Model

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There is strong evidence that biological response to ionizing radiation has a contribution from unirradiated "bystander" cells that respond to signals emitted by irradiated cells. We discuss here an approach incorporating a radiobiological bystander response, superimposed on a direct response due to direct energy deposition in cell nuclei. A quantitative model based on this approach is described for α-particle-induced *in vitro* oncogenic transformation. The model postulates that the oncogenic bystander response is a binary "all or nothing" phenomenon in a small sensitive subpopulation of cells, and that cells from this sensitive subpopulation are also very sensitive to direct hits from α particles, generally resulting in a directly hit sensitive cell being inactivated. The model is applied to recent data on *in vitro* oncogenic transformation produced by broad-beam or microbeam α-particle irradiation. Two parameters are used in analyzing the data for transformation frequency. The analysis suggests that, at least for α-particle-induced oncogenic transformation, bystander effects are important only at small doses—here below about 0.2 Gy. At still lower doses, bystander effects may dominate the overall response, possibly leading to an underestimation of low-dose risks extrapolated from intermediate doses, where direct effects dominate.

INTRODUCTION

It has generally been accepted for many years that most biological damage produced by ionizing radiation occurs when the radiation acts on DNA, either by directly ionizing the DNA or through reactions with free radicals produced by the radiation in nearby water molecules (*1–4*). However, over the past decade, a number of reports have appeared describing the results of α-particle irradiations in which a larger proportion of cells showed biological damage than were estimated to have been traversed by α particles.

Reports of apparently this same bystander effect have appeared for a variety of biological end points including cell killing, micronucleus induction, mutation induction, genomic instability, stimulated proliferation, and changes in gene expression (*5–20*). Bystander effects have been inferred both in systems where irradiated cells are in contact with one another (i.e. where direct cell-to-cell communication is plausible), and also when cells are considerable distances apart from each other. In some cases (*16*), irradiated cells appear to affect other cells at quite large distances. Bystander effects have been reported after cells were subjected to γ as well as α-particle irradiation (*18–20*).

While the existence of radiobiological bystander effects now seems incontrovertible, it is clear (*20*) that there must also be a component of radiobiological damage which is "direct", in the sense that it involves damage in a cell by a radiation track which directly deposits energy in that cell nucleus (*1–3*). We discuss an approach to radiation-induced biological response, termed $\mathcal{B}a\mathcal{D}$, which incorporates both $\mathcal{B}$ystander and $\mathcal{D}$irect damage. A comparatively simple quantified form of the $\mathcal{B}a\mathcal{D}$ approach is outlined here and applied to data, described in detail in a companion paper (*9*), describing *in vitro* oncogenic transformation of C3H 10T½ cells subjected to acute α-particle irradiation.

An important tool which has facilitated the elucidation of bystander effects is the single-particle/single-cell microbeam (*21–25*), which has made it possible to define precisely which cell nuclei (and what proportion of cell nuclei) are traversed by exactly defined numbers of α particles, rather than relying on estimates of probabilities. Data using microbeam targeting of well-separated individual cell nuclei by α particles (*9, 26*) show several features which have influenced the structure of the model outlined here, as follows:

1. In experiments designed to directly probe the bystander effect, when only 1 in 10 cells on a dish has its nucleus traversed by a precisely known number of α particles and the remaining cells are not irradiated, the oncogenic response initially increases sharply with increasing α particle number, but then shows little further increase with increasing particle number (*9*). A similar result was also reported by Lehnert and Goodwin in bystander studies with sister chromatid exchanges as the end point (*12, 13*; see also refs. *14, 15, 18, 20*).

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2. When 1 in 10 of the cell nuclei on a dish are exposed to exactly defined numbers of α particles and the rest are not irradiated, the frequency of induced oncogenic transformation is not less than when *all* the cell nuclei on the dish are exposed to the same number of α particles (*9*); for the case when 1 in 10 of the cell nuclei on a dish is exposed to exactly one α particle, the resulting frequency of induced oncogenic transformation appears greater than when all the cell nuclei on the dish are exposed to exactly one α particle (*9*).
3. When all the cell nuclei on a dish are exposed to exactly one α particle, the oncogenic transformation frequency is significantly lower than when the cell nuclei are exposed to a mean of one α particle but with a Poisson-distributed number of traversals through individual nuclei (*26*); for higher fluences, however, there is no significant difference between the effects of an exact number of α particles traversing all the cell nuclei and the effects of a Poisson-distributed number of α-particle traversals (*26*).

Further observations from earlier data that have influenced the development of this model are:

4. At intermediate doses (above a few tenths of a gray), the oncogenic transformation frequency induced by exposure to conventional (broad-beam) high-LET radiation increases approximately linearly with increasing dose (*27, 28*).
5. There is a well-documented inverse dose-rate effect at low doses of high-LET (and in some cases low-LET) radiations, in which protracting the dose rate can result in an increase in oncogenic transformation frequency (*29–31*).

These observations suggest that:

1. An irradiated cell can indeed send out a signal which can lead to an oncogenic response in a bystander cell, i.e. a cell whose nucleus is not hit.

For α-particle-induced *in vitro* oncogenic transformation, these observations also suggest that:

2. In this case, the bystander effect is likely to be a binary ''all or nothing'' effect, in which a signal is sent out by cells traversed by one or more α particles, but more signal does not lead to any increase in oncogenic response; and
3. the cell population may contain, at any given time, a small subpopulation that is hypersensitive to oncogenic transformation by the bystander signal.

The binary nature of the bystander process is suggested by the apparent saturation of the bystander response at low fluences (*9*). Such saturation may also occur for other end points (*12, 13*; and see also refs. *14, 15, 18, 20*). The rationale for suggesting the existence of a sensitive subpopulation that is highly responsive to bystander-induced oncogenesis is that the two phenomena of (a) an initial steep rise of response at low acute doses followed by a plateau in response and (b) an inverse dose-rate effect at low doses are both characteristics of the existence of a subpopulation of cells that is, at least at the time of irradiation, hypersensitive or already partially damaged (*32–36*).

A model, based on the $\mathcal{BaD}$ approach, which quantifies these considerations for acute doses of high-LET radiation, will be described in the Methods section. It is our hope that the quantitative approach illustrated here will facilitate improvements to our understanding of the bystander phenomenon that will no doubt become possible and necessary as additional experimental evidence becomes available.

METHODS

The $\mathcal{BaD}$ approach assumed here is that the overall response comes from both a bystander response in unhit cells and a direct response in hit cells. Based on the considerations outlined above, the quantitative model outlined here for the bystander component is that, at any given instant, there is a subpopulation of cells which are hyper-responsive to oncogenic transformation by the bystander signal emitted from directly irradiated cells. The oncogenic bystander response to this signal is a binary ''all or nothing'' effect; i.e., at least under the conditions of the experiments analyzed here, subjecting the irradiated cells to larger doses does not lead to any increase in the observed effect. In addition, we shall assume that the response of a cell whose nucleus was directly traversed by an α particle will be dominated by the effects of that direct hit; i.e., bystander effects in hit cells will be neglected compared to the direct effects.

Although the motivation of this work is to understand induced oncogenic transformation frequencies, it is also necessary to analyze cell killing effects in bystander cells compared to directly traversed cells. This is because only transformed cells that survive are relevant, and cells transformed by a bystander signal might be expected to be inactivated at a lower rate than cells transformed by a direct α-particle traversal.

Analyzing Survival

The three experiments (*9, 26*) pertinent to the bystander effect that we will analyze here are microbeam experiments in which all the cell nuclei are exposed to known exact numbers of α particles, microbeam experiments in which 1 in 10 cell nuclei is exposed to known exact numbers of α particles, and conventional ''broad-beam'' experiments in which populations of cells are exposed to Poisson-distributed numbers of α particles. The appropriate survival formula for each of these cases is:

1. For the microbeam experiment where 100% of the cell nuclei are hit by an exact number of α particles, the usual assumption for high-LET radiation that one-track action is responsible for lethality would imply that the surviving fraction, SF, is (*17*)

$$\mathrm{SF} = q^N, \tag{1}$$

where q is the probability of surviving a single α-particle traversal and N is the number of α-particle traversals per nucleus. From an analysis of the microbeam survival data of Sawant *et al.* (*9*), $q \approx 0.80$.

2. For the microbeam experiments in which 1 in 10 cells has its nucleus traversed with an exact number of α particles and the remaining cells are not hit, from our assumption above that the direct response in a cell whose nucleus is hit will dominate any bystander response, the surviving fraction will be

$$\mathrm{SF} = 0.1\, q^N + 0.9\, F(N), \tag{2}$$

where the first term refers to direct effects and the second to bystander-mediated cell killing. Here $F(N)$ is the fraction of bystander cells that

survive in this experiment, where 1 in 10 cells has its nucleus traversed with exactly N α particles and the remaining cells are not hit. Having already estimated q, we can empirically estimate $F(N)$ by subtraction, using Eq. (2) and the microbeam survival data of Sawant *et al.* (*9*). Using this technique, a reasonable fit to $F(N)$ is given by

$$F(N) \approx \exp(-0.0034N - 0.0027N^2), \ (0 \leq N \leq 8). \quad (3)$$

Equation (3) is a purely empirical function to enable us to analyze bystander-related transformation frequencies per surviving cell. Of interest here is that bystander-mediated cell killing is not a binary ''all or nothing'' effect as we have suggested for bystander-mediated oncogenic transformation; i.e., in the fluence range $0 < N \leq 8$, more signal results in more bystander-mediated cell killing. As expected, the inactivation rate in bystander cells is not, at least under the conditions of the present experiment, as large as for hit cells. For example, for $N = 2$, Eq. (3) predicts a survival rate in bystander cells of about 98%, whereas the survival rate for hit cells is only about 64% (Eq. 1).

3. For the conventional ''broad-beam'' experiments in which cells are exposed to a Poisson-distributed number of α-particle nuclear traversals with a mean of $\langle N \rangle$, there would be different mix of direct and bystander effects:

$$\mathrm{SF} = \exp[-(1 - q)\langle N \rangle] - \exp(-\langle N \rangle)\,[1 - G(\langle N \rangle)], \quad (4)$$

where $G(\langle N \rangle)$ is the fraction of bystander cells which survive under the conditions of the broad-beam experiment. The derivation of this equation from Poisson statistics is as follows: First, suppose that all bystander cells survive; based on Poisson statistics and using Eq. (1), the fraction of all cells that survive is

$$\exp(-\langle N \rangle)\,[1 + q\langle N \rangle + (q\langle N \rangle)^2/2! + \ldots]$$
$$= \exp[-(1 - q)\,\langle N \rangle]. \quad (5)$$

The proportion of bystander cells is $\exp(-\langle N \rangle)$, and subtraction thus gives Eq. (4). As with $F(N)$ and Eq. (2), having estimated q, the function $G(\langle N \rangle)$ can be evaluated using Eq. (4) and the experimental survival data for broad-beam irradiation as reported by Miller *et al.* (*26*). Based on this methodology, we obtain

$$G(\langle N \rangle) \approx 1; \ (0 \leq \langle N \rangle \leq 8); \quad (6)$$

i.e., there is little evidence of bystander-mediated cell killing for the case of broad-beam irradiation. For $\langle N \rangle$ significantly larger than 1, Eq. (6) represents only a crude estimate because there are so few bystander cells, but, by the same token, this paucity of bystander cells implies that only a crude estimate for $G(\langle N \rangle)$ is required.

Analyzing Transformation

Again, the three experiments (*9, 26*) pertinent to the bystander effect that we will analyze here are microbeam experiments in which all the cell nuclei are exposed to exact numbers of α particles, microbeam experiments in which 1 in 10 cell nuclei is exposed to exact numbers of α particles, and conventional ''broad-beam'' experiments in which cells are exposed to Poisson-distributed numbers of α particles. The appropriate formula for each of these cases is:

1. For the case where 100% of cell nuclei are microbeam-irradiated with exact numbers (N) of α particles, since the transformation frequency at intermediate doses appears to increase linearly in a variety of experiments with high-LET radiation (*27, 28*), we use a conventional linear expression for the response (TF = transformants per surviving cell). Thus the fraction of cells that respond and survive is

$$\mathcal{D} = \nu\, N\, q^N, \quad (7)$$

and the number of transformants per surviving cell is

$$\mathrm{TF} = \nu N, \quad (8)$$

where the adjustable constant, ν, is the slope of the linear dose–response relationship.

2. For the case that 10% of the cell nuclei are hit by an exact number of α particles, under our assumptions, the fraction of cells at risk which are transformed and survive is

$$\mathcal{D} + \mathcal{B} = 0.1\, \nu\, N\, q^N + 0.9\, \sigma\, F(N), \quad (9)$$

where σ is a constant. Equation (9) is the crux of our approach. The reasoning behind it is the following: The 10% of the cells whose nuclei are hit respond, according to our assumptions above, just as do the hit cells in the case where 100% are hit; hence the first term $\mathcal{D}$. For the bystander cells which contribute the second term, $\mathcal{B}$, as discussed above, it is assumed that there is a small hypersensitive fraction, σ, of cells which are transformed if they are bystander cells and receive any bystander signal at all, whereas other bystander cells are not transformed.

It should be noted in Eq. (9) that the bystander killing term, $F(N)$, originally derived from analyzing killing in all bystander cells, is applied here to the hypersensitive subpopulation of bystander cells that are transformed. It could be that these hypersensitive cells are also more sensitive to bystander-mediated killing. In such a case, assuming the surviving fraction for the hypersensitive cells is decreased to $SF(N)$, where $S < 1$ and S is independent of N, this would simply mean that the quantity σ in Eq. (9) would be underestimated by the factor S, but would not otherwise alter the formalism or its predictions. The response, expressed as transformants per surviving cell, is then given by Eqs. (2) and (9) as

$$\mathrm{TF} = [0.1\nu\, N\, q^N + 0.9\, \sigma\, F(N)]/[0.1\, q^N + 0.9\, F(N)]. \quad (10)$$

3. For broad beams, our assumptions imply that the number of surviving transformants is

$$\mathcal{D} + \mathcal{B} = \nu\, q\langle N \rangle \exp[-(1 - q)\,\langle N \rangle] + \sigma \exp(-\langle N \rangle). \quad (11)$$

The proof, which again involves Poisson statistics, is similar to the proof of Eq. (4). Using Eq. (11) and Eq. (4) with $G = 1$, the predicted frequency of transformants per surviving cell is

$$\mathrm{TF} = \nu\, q\, \langle N \rangle + \sigma \exp(-q\ \mathrm{k}N\mathrm{l}). \quad (12)$$

When $\langle N \rangle$ is large, $\exp(-q\ \langle N \rangle)$ is negligible and Eq. (12) reduces simply to a linear response $\mathrm{TF} = \nu\, q\, \langle N \rangle$ or, equivalently, $\mathrm{TF} = \alpha D$, since $\langle N \rangle$ and D are proportional. When $\langle N \rangle$ is small, Eq. (12) describes a response greater than $\nu\, q\, \langle N \rangle$, corresponding to an increase in transformation at low doses by an amount ≤σ, the fraction of cells that are hypersensitive to transformation by the bystander signal.

Equations (9) and (11) represent the basic model described here. An assumption implicit in these equations merits explicit mention, namely that cells which are hypersensitive to oncogenic transformation by the bystander signal are also hypersensitive to direct α-particle hits and are thus highly likely to be inactivated by a direct hit on the cell nucleus. In such a situation, a cell which is hypersensitive to the bystander signal would make no contribution to the observed transformation rate if it were directly hit.[2]

RESULTS

Figure 1 shows earlier experimental results (*26*) on microbeam irradiation either with an exact number of α particles per cell nucleus, or with a broad beam giving the same average number of α particles per cell nucleus. These

[2] Technically, under these assumptions, Eq. (1) should be replaced by $\mathrm{SF} = (1 - \sigma)q^N$ for $N \geq 1$, and appropriate modifications need to be made in the subsequent equations. Here, however, in our case, $\sigma \ll 1$ (see below), so all the corresponding corrections are negligible.

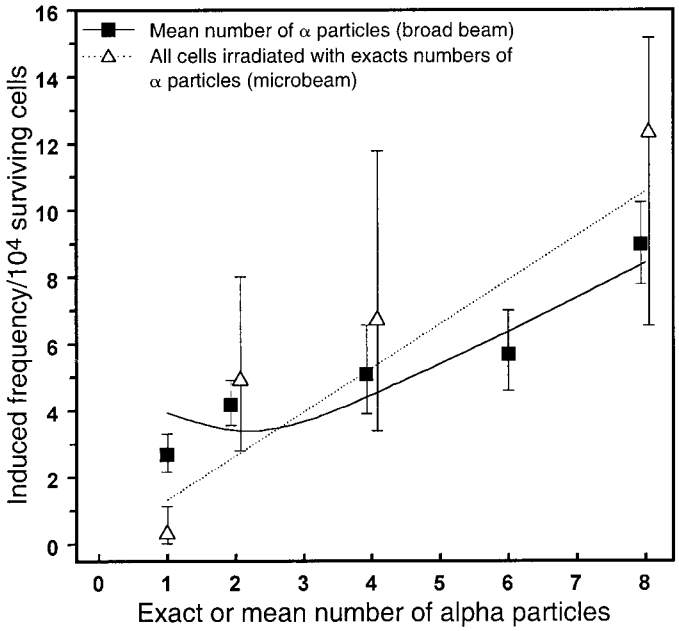

FIG. 1. Triangles show measured (*26*) induced transformation frequencies (with 68% confidence limits) per 10^4 surviving cells for microbeam irradiation of all cell nuclei with exact numbers of α particles. Squares show corresponding measured data (*26*) for broad-beam irradiations with doses corresponding to the given average numbers of α particles per cell nucleus. The dotted curve and solid curve give the fits of the model for the microbeam and broad-beam data, respectively. Note that the predictions for the microbeam irradiations (dotted lines) were made only at integer values of the α-particle number, but have been connected to guide the eye.

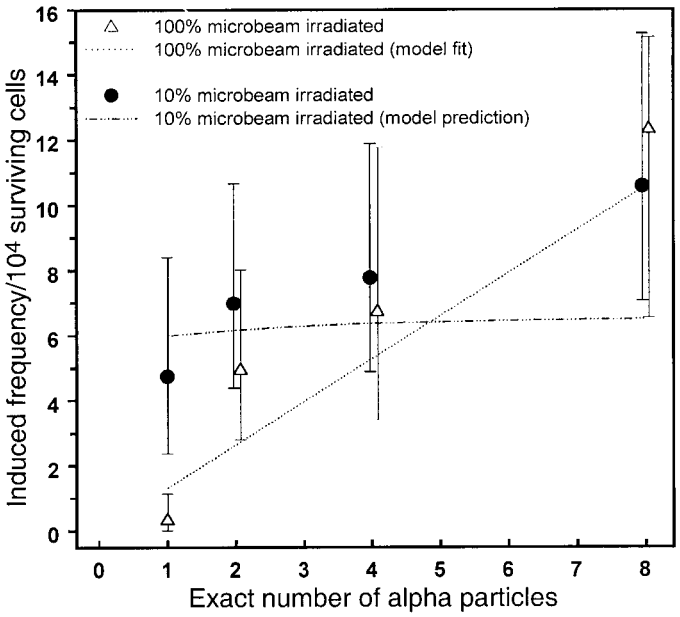

FIG. 2. The triangles and corresponding dashed theoretical curve (repeated from Fig. 1 for comparison) refer to microbeam irradiation of all cell nuclei with a given number of α particles. The circles show the measured (*9*) induced transformation frequencies (with 68% confidence limits) per 10^4 surviving cells in a microbeam experiment where only 10% of the cell nuclei are irradiated with exact numbers of α particles. The dot-dash line is the theoretical prediction for this case, based on the two parameters obtained from the fit to the data in Fig. 1.

data were fitted, using standard maximum likelihood techniques, to the model described above, Eqs. (8) and (12), adjusting the two parameters, σ (relating to the bystander phenomenon) and ν (relating to the direct effect). The resulting fits are also shown in Fig. 1. The two parameters of the model, adjusted for optimal fit to the data, are $\sigma = 6.4 \times 10^{-4}$ and $\nu = 1.3 \times 10^{-4}$. A reasonable fit of the model to the data is seen, with the increased effect for a mean of one α particle compared to exactly one α particle being well reproduced, as well as the reversal of this difference with increasing numbers of α particles. Within the current approach, the putative reason for the difference in the observed transformation rate from one α particle is the presence in the case of broad-beam irradiation (Poisson) of cells which are not directly hit (some of which are hypersensitive to the bystander signal), whereas when all the cell nuclei are irradiated, those cells which are hypersensitive to the bystander signal are inactivated. As the fluence increases, however, the number of bystander cells in the case of broad-beam irradiation becomes small.

With the two model parameters fixed by these data (*26*), we can also determine from the model the predictions (Eq. 10) for the microbeam experiment (*9*), where only 10% of the cells are irradiated with exact numbers of α particles. It is seen in Fig. 2 that the predictions of the model reproduce the trend of the data; in particular, the predictions are consistent with the *prima facie* anomalous observation that irradiation of 10% of cells with exactly one α particle appears to result in a larger effect than irradiation of 100% of cells with exactly one α particle. Analogous to the case for broad-beam irradiation, the putative reason for the different response to one α particle is the survival, when only 1 in 10 cells is directly irradiated, of hypersensitive cells which can contribute a bystander oncogenic response.

DISCUSSION

We have presented a quantitative model, based on the $\mathcal{B}a\mathcal{D}$ approach, incorporating radiobiological damage both from a bystander response to signals emitted by irradiated cells, and also from direct traversal of ionizing radiations through cell nuclei. In the current model, no detailed signaling mechanisms were hypothesized, so the approach could, in principle, apply to the situation where cells are in direct contact with one other, as well as to the situation where the cells are further apart.

In essence we have assumed, at least for high-LET radiation, that the oncogenic bystander phenomenon is a binary ''all or nothing'' response to the bystander signal in a small sensitive subpopulation of cells; we assume that cells from this sensitive subpopulation are also very sensitive to direct hits from α particles, generally resulting in a directly hit sensitive cell being inactivated.

The model was applied to a series of experiments on

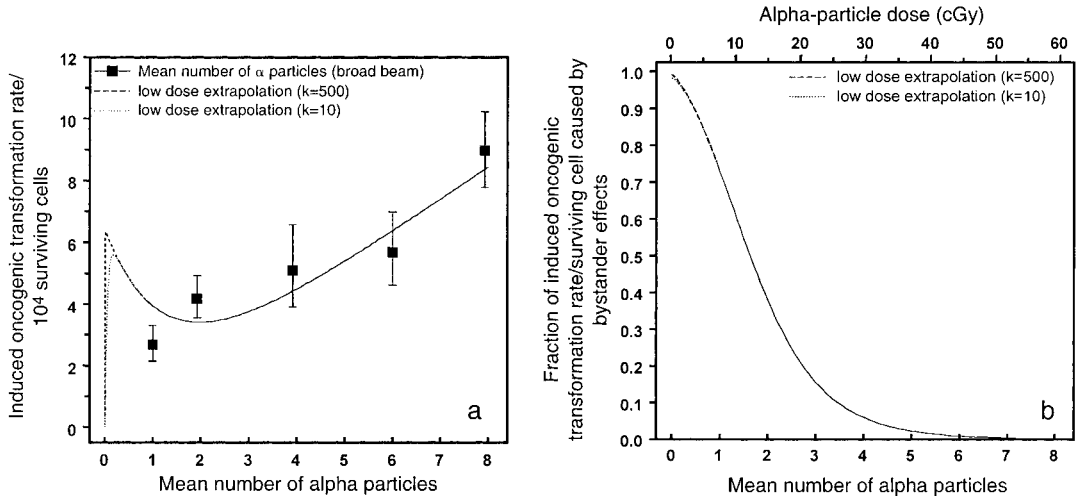

FIG. 3. Panel a: Possible extrapolations (see Eq. 13) of the fit of the model to the broad-beam data (Fig. 1) to very low doses. Panel b: Estimated proportion of total induced oncogenic transformation frequency per surviving cell resulting from bystander effects for broad-beam α-particle exposure.

α-particle-induced *in vitro* oncogenic transformation with a single-cell/single-particle microbeam, as well as with broad-beam irradiation. It was able to reproduce the main features of the data, for both single and larger numbers of α particles.

We have referred to the minority of bystander cells that are sensitive to signal-mediated transformation as a sensitive subpopulation. It is possible that their sensitivity could occur by virtue of their geometric location (i.e. unusually near a hit cell) rather than by virtue of their biological status. In other words, a cell which is very close to a hit cell would receive an extremely large bystander signal. Although their results were for end points other than oncogenic transformation, the type of data that would argue against this interpretation is the apparently very long range of the bystander signal (hundreds of micrometers) established by Prise *et al.* (*16*) in microbeam studies of micronuclei and of apoptosis. Again, although the data are for other end points, the type of data that would argue for a geometric interpretation of the sensitive subpopulation comes from low-dose broad-beam α-particle studies of gene expression in confluent cells (*7*), where bystander-mediated changes in gene expression tended to occur in geometric clusters. To help elucidate this issue for oncogenic transformation, experiments are under way in which exponentially growing cells at differing densities are irradiated with very low doses of α particles.

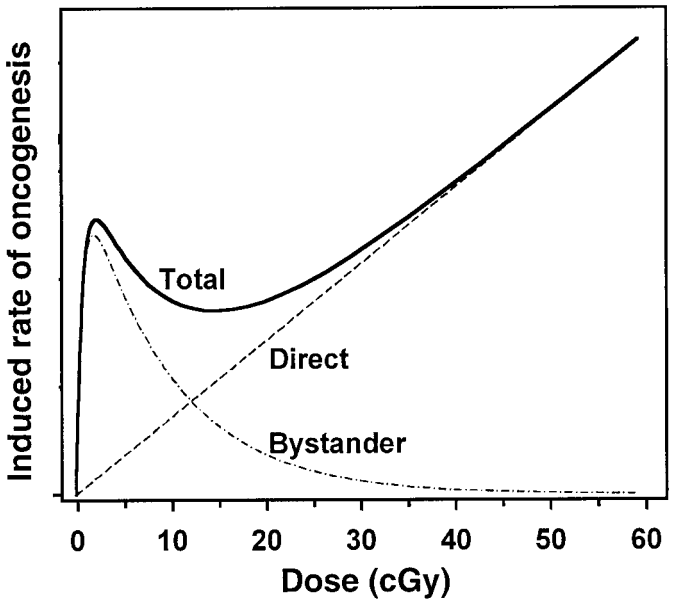

FIG. 4. Schematic of the relative patterns with dose of the direct and bystander phenomena, as modeled here.

While some of the details of the model could change, some of its essential features currently seem quite constrained by the available data. Various different experiments on the bystander effect do seem to suggest a rapid rise to a plateau at low doses, with little further dose dependence[3] (*9, 12–15, 18, 20*). Sensitive subpopulations characteristically produce such plateaus (*37*), though other phenomena, such as indirect, multistage pathways (*38, 39*) or radiation-induced adaptive responses (*32*), can also produce similar dose–response relationships. The existence of an inverse dose-rate effect in other experiments is also suggestive of a cell subpopulation which is hyper-responsive to a bystander signal: Typically, if such a subpopulation has a saturated response for acute irradiation but is restored by endogenous processes during prolonged irradiation, inverse dose-rate effects can result (*33–35*), which are indeed observed at low doses of high-LET radiation (*29–30*).

[3] Two of these bystander-related end points, induction of reactive oxygen species (*14*) and induction of the TGFB1 (*15*), have been interpreted (*14, 15*) as bystander signaling agents, rather than responses to bystander signals. It is not clear how a bystander signal (as opposed to the response to the signal) could saturate at very low α-particle doses, and such is not assumed in the current approach.

According to the picture presented above, the bystander effect is important primarily at low doses, at least for *in vitro* oncogenic transformation induced by high-LET radiation. As illustrated in Fig. 3b, based on the fits in Fig. 1, the bystander component contributes only 6% of the total transformation rate for a broad-beam irradiation with a mean of four α particles [corresponding to a mean dose of 0.3 Gy; see ref. (*40*)], increasing to 38% for a mean of two α particles (mean dose 0.15 Gy) and to 73% for a mean of one α particle (mean dose 0.074 Gy).

At still lower doses, a reasonable extension of the current approach would be that any nucleus that is directly hit by one or more α particles is capable of sending out a bystander signal to k unirradiated neighbor cells (of which a small fraction will be sensitive to the signal); here k might be as large as the total number of unirradiated cells on an irradiated dish or, in other situations, perhaps as small as around 10. Then the probability that none of the k cells are directly hit is $\exp(-k\langle N\rangle)$, so the probability that at least one is hit is $1 - \exp(-k\langle N\rangle)$, and Eq. (12) would become

$$\mathrm{TF} = \nu\, q\, \langle N\rangle + \sigma\,[1 - \exp(-k\langle N\rangle)]\exp(-q\langle N\rangle). \quad (13)$$

This extrapolation is shown in Fig. 3a for $k = 10$ and $k = 500$. In this extrapolation, the slope of the dose–response relationships near zero dose is $\sigma k + \nu q$, which is dominated by the bystander term σk (see Fig. 3b), since $\sigma > \nu$, $k > 1$, and $q \leq 1$.

If the mechanisms postulated here were applicable *in vivo*, the consequences for low-dose risk estimation might be major. For example, examination of Fig. 4 shows that a linear extrapolation of risks from intermediate doses (where the bystander effect might be negligible) to very low doses (where the bystander effect may dominate) could underestimate the risk at very low doses—an issue of considerable relevance in risk estimation for domestic radon. It is stressed, however, that the low-dose extrapolations in Eq. (13) and illustrated in Fig. 4, while motivated by the bystander model described here (which, as discussed, has some support at experimentally accessible doses), are in a dose region where data on oncogenic transformation are not easy to acquire.

The $\mathcal{B}a\mathcal{D}$ approach, applied here to *in vitro* oncogenic transformation by acute doses of α particles, may be applicable to a more general model describing different end points, radiation qualities, and dose rates. In the current work, we have used some of the specific features of high-LET α-particle radiation to generate a fairly simple, preliminary model, to explore the fundamental trends without excessive parameterization.

Our understanding of the bystander phenomenon is preliminary in nature, and the applicability of conclusions derived from *in vitro* studies to the *in vivo* situation is quite uncertain. It seems clear that as additional experimental evidence becomes available, modification of the models describing the effect will become possible and necessary. It is our hope that the quantitative approach illustrated here will facilitate these developments.

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