G₂-phase radiation response in lymphoblastoid cell lines from Nijmegen breakage syndrome

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Abstract. The relationship between G₂-phase checkpoint activation, cytoplasmic cyclin-B1 accumulation and nuclear phosphorylation of p34^{CDC2} was studied in Nijmegen breakage syndrome cells treated with DNA damaging agents. Experiments were performed on lymphoblastoid cell lines from four Nijmegen breakage syndrome patients with different mutations, as well as on cells from an ataxia telangiectasia patient. Lymphoblastoid cell lines were irradiated with 0.50–2 Gy X-rays and the percentage of G₂-phase accumulated cells was evaluated by means of flow cytometry in samples that were harvested 24 h later. The G₂-checkpoint activation was analysed by scoring the mitotic index at 2 and 4 h after treatment with 0.5 and 1 Gy X-rays and treatment with the DNA double-strand break inducer calicheamicin- γ 1. Cytoplasmic accumulation of cyclin-B1 was evaluated by means of fluorescence immunostaining or Western blotting, in cells harvested shortly after irradiation with 1 and 2 Gy. The extent of tyrosine 15-phosphorylated p34^{CDC2} was assessed in the nuclear fractions. Nijmegen breakage syndrome cells showed suboptimal G₂-phase checkpoint activation respect to normal cells and were greatly different from ataxia telangiectasia cells. Increased cytoplasmic cyclin-B1 accumulation was detected by both immunofluorescence and immunoblot in normal as well as in Nijmegen breakage syndrome cells. Furthermore, nuclear p34^{CDC2} phosphorylation was detected at a higher level in Nijmegen breakage syndrome than in ataxia telangiectasia cells. In conclusion, our data do not suggest that failure to activate checkpoints plays a major role in the radiosensitivity of Nijmegen breakage syndrome cells.

INTRODUCTION

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disorder, characterized by such clinical features as progressive microcephaly, growth retardation, typical facial

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appearance and immunodeficiency (The International NBS Study Group 2000). NBS as well as ataxia telangiectasia (AT) are chromosomal instability syndromes that share a high predisposition to malignancies and many cellular and cytological features (Weemaes *et al.* 1981; Shiloh 1997; Digweed *et al.* 1999). *Nbs1*, the gene responsible for NBS is located on chromosome 8q21 (Saar *et al.* 1997) and has been recently cloned (Varon *et al.* 1998). The encoding protein, named nibrin, is absent in most NBS patients. Nibrin is part of the human Rad50-complex which is involved in DNA double-strand break (dsb) repair (Carney *et al.* 1998; Digweed *et al.* 1999; Petrini 1999). The hRad50-complex has been proposed to act as a DNA damage sensor, and a linking function between DNA repair and checkpoint pathways has been proposed (see Shiloh 1997; Digweed *et al.* 1999). Stewart *et al.* (1999) reported that the hRad50-complex and AT are involved in the same DNA damage response pathway. This is in agreement with previous cell fusion data (Stumm *et al.* 1997). Very recently, an ataxia telangiectasia mutated gene (ATM)-dependent phosphorylation of nibrin, in response to radiation exposure, has been reported (Gatei *et al.* 2000; Lim *et al.* 2000; Petrini 2000; Zhao *et al.* 2000; Wu *et al.* 2000).

Cell cycle checkpoint defects were recognized in fibroblasts and lymphoblastoid cell lines (LCL) established from AT patients. Irradiated AT cells fail to activate checkpoints at G₁/S and G₂/M boundaries as well as when traversing S-phase (Beamish & Lavin 1994). Since AT has been established to show only a subtle defective repair of dsbs (Foray *et al.* 1997), the radiation hypersensitivity in this disorder has been linked to checkpoint defects (for review see Shiloh 1997). However, recently published results on AT (Jeggo *et al.* 1998) and NBS fibroblasts (Girard *et al.* 2000) seem to indicate that defects of cell cycle checkpoints are not responsible for the radiosensitivity observed in these syndromes.

So far however, the cell cycle regulation and the induction/modulation of related proteins still remain poorly investigated in NBS cells and are mainly restricted to the analysis of the G₁/S response. Early papers have shown that both NBS and ATM deficiencies resulted in an impaired ability to accumulate p53 and the cyclin-dependent kinase inhibitor p21/WAF1 after irradiation (Kastan *et al.* 1992; Jongmans *et al.* 1997). In contrast, more recent papers have demonstrated that these events occur in NBS cells with an extent, which is comparable or slightly reduced/delayed with respect to that of normal cells (Matsuura *et al.* 1998; Yamazaki *et al.* 1998; Antoccia *et al.* 1999; Stewart *et al.* 1999). These findings indicate that the radiation response is not totally overlapping in NBS and AT. Furthermore, only few data are available on checkpoint activation leading to G₂-delay in NBS cells.

A G₂ checkpoint activation appears to be the result of multiple and complex mechanisms which include transcriptional and post-transcriptional regulation of cyclin-B1, inhibitory phosphorylation of p34^{CDC2}, ATM-chk2/cds1-mediated inhibitory phosphorylation of cdc25C and subcellular localization of key regulator proteins to cytoplasm or nucleus (see Shackelford *et al.* 1999). In particular, the formation of an active cyclin-B1/p34^{CDC2} complex is recognized as a central downstream component in progression through G₂ to mitosis. The activity of such a protein complex is regulated by both cyclin-B1 availability and specific phosphorylation of p34^{CDC2}. Cyclin-B1 levels rise dramatically during the G₂-phase when the protein tends to be cytoplasmic until late G₂/prophase when it is found in the nucleus (Pines & Hunter 1991). Inhibition of p34^{CDC2} kinase occurs through phosphorylation at either threonine 14 or tyrosine 15 (see Hwang & Muschel 1998).

The present paper was aimed at analysing the G₂-phase checkpoint in NBS cells in more detail. Inhibition of mitotic entry, G₂-phase accumulation, the expression of cyclin-B1 and the phosphorylation status of the p34^{CDC2} kinase were evaluated after X-ray irradiation in LCL established from four NBS patients with different mutations.

MATERIALS AND METHODS

Cell lines

LCL were derived from normal individuals (1228; 614), from four NBS patients and from an AT patient. LCL from NBS were characterized by different mutations: ZRA, with common *Nbs1* founder mutation (Varon *et al.* 1998), 2345, with 742-743insGG (Maraschio *et al.* 1986, Varon personal communication), 668, with 900del25 in *Nbs1* (Maraschio *et al.* 2001) and 96B453 without a detectable mutation in the *Nbs1* gene. The mutation in the AT line was not known.

LCL were cultured in RPMI-1640 (Gibco BRL, Milano, Italy) supplemented with 15% heat-inactivated fetal calf serum (HyClone), 2 mM L-glutamine, antibiotics and 0.5% non-essential amino acids, at 37 °C in a 5% CO₂ atmosphere.

Irradiation and calicheamicin treatment

Cells in exponential phase of growth were irradiated at room temperature with X-rays delivered by a Gilardoni MGL 300/6-D apparatus (Gilardoni, Mandello Lario, Italy), operating at a dose rate of 0.53 Gy/min (250 kV, 6 mA, Cu filter). Based on pilot experiments on LCL cell cycle length, cells harvested 2–4 h from irradiation were assumed to be in the G₂-phase at the time of treatment. In order to evaluate the inhibition of mitotic entry, cells were irradiated with doses of 0.5 and 1 Gy and harvested 2 and 4 h later. In the flow cytometry assay, cells were treated with 0.5, 1 and 2 Gy and harvested after 24 h. To determine the induction and the localization of cyclin B1 and p34^{CDC2} kinase, cells were exposed to 1 and 2 Gy of X-rays and harvested 2 and 4 h later.

The antitumoral antibiotic calicheamicin- γ 1, which specifically introduces DNA dsbs at the TCCT/AGGA recognition sequence (Zein *et al.* 1988), was kindly provided by Dr C. A. Ellestad (American Cyanamid Co., Pearl River, NY, USA). Analysis of inhibition of mitotic entry was performed using doses of 4 and 8 pg/ml in cells treated for 2 and 4 h.

Flow cytometric analysis

Cells were harvested, washed with cold phosphate-buffered saline (PBS) and fixed with a 1 : 1 mixture of PBS : methanol for 10 min. Then DNA was stained by addition of 0.05 ml propidium iodide solution (250 μ g/ml propidium iodide, 2.5 μ g/ml RNase, 100 μ g/ml Triton-X-100, in PBS) to 0.2 ml of cell suspension for 20 min at 37 °C. To determine the DNA content of each sample, 10 000 cells were analysed in three repeated experiments, using a Cell Sorter FACStar Plus (Becton Dickinson, San Jose, CA, USA). Percentages of cells in G₁, S and G₂/M-phases were established using the Multicycle AV software (Version 2.5-Phoenix Flow System, San Diego, CA, USA) and the statistical analysis was performed using the χ^2 -test.

Mitotic index

Irradiated or calicheamicin-treated cells were harvested and washed with cold PBS. The resulting cell pellets were resuspended for 7 min in 75 mM KCl at room temperature (RT). Then the samples were transferred on ice and fixed with a mixture of methanol/acetic acid (3 : 1) for 1 h. Cellular suspensions were seeded on slides and stained in 3% Giemsa. The mitotic index was evaluated in 1000 cells/sample in repeated experiments and the statistical analysis was performed using the χ^2 -test.

Immunofluorescence staining

Four hours after irradiation with 1 Gy X-rays, cells were washed with cold PBS, cell pellets resuspended in PBS and cytocentrifuged for 6 min at 600 r.p.m. (Cytospin-3, Shandon). Cells

were fixed for 10 min in -20°C , following by 1 min in a 1 : 1 mixture of methanol : acetone at -20°C . Slides were air-dried and then immediately processed. Cells were rinsed in PBS and blocked for 40 min in 3% bovine serum albumin (BSA; Sigma, Milan, Italy) in PBS. Afterwards, the cells were incubated overnight at 4°C with polyclonal anticyclin-B1 antibody (H-433, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 10 in 2% BSA/PBS. An extensive wash was performed in 1% BSA/PBS and then, the cells were incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (ICN) diluted 1 : 50 in 2% BSA/PBS. Slides were washed in 1% BSA/PBS and PBS, and then mounted in a 1 : 1 mixture of 4', 6-diamidino-2-phenylindole (DAPI) and antifade (Vector Laboratories, Burlingame, CA, USA). Cells showing bright green staining of cyclin-B1 in the cytoplasmic compartment were classified as G_2 -phase cells. DAPI staining of DNA assured the identification of cells in the field under scoring. One thousand cells per experiment were analysed with an Axiophot microscope (Zeiss) and the statistical analysis was performed using the χ^2 -test. Images were captured with a CCD camera (Spot2). Experiments were repeated twice.

Protein extraction and immunoblotting

Cells were washed in cold PBS and treated for 15 min on ice in 0.5 ml hypotonic buffer (10 mM Hepes pH 7.9, 10 mM KCl, 2 mM MgCl_2 , 1 mM DTT and 1 mM PMSF). After centrifugation (5 min, 3000 r.p.m.), cells were lysed on ice for 1 min with 0.1 ml lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 2 mM MgCl_2 , 0.2% NP-40, 1 mM dithiothreitol (DTT), 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 mM orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF)). Samples were centrifuged (300 g, 5 min) and supernatants containing the cytoplasmic extracts were collected. The pellets were lysed for further 30 min on ice with 0.1 ml nuclear lysis buffer (50 mM Hepes pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 mM orthovanadate, 1 mM PMSF). Twenty-five μg and 10 μg of cytoplasmic and nuclear proteins, respectively, were separated on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore, Immobilon-P, Bedford, MA, USA). To verify equal loading of proteins and blotting efficiency membranes were stained with Ponceau red solution (Sigma), then destained in a basic solution, washed and blocked at RT in 3% non-fat milk pH 7.4 (Sigma). Filters were then incubated with either 1 $\mu\text{g}/\text{ml}$ anticyclin-B1 monoclonal antimouse antibody (#sc-752, Santa Cruz Biotechnology), or 1 $\mu\text{g}/\text{ml}$ tyrosine 15-phosphorylated $p34^{\text{CDC}2}$, polyclonal antirabbit antibody (#9111S BioLabs Inc, New England BioLabs Inc, Beverly, MA) or 1 $\mu\text{g}/\text{ml}$ $p34^{\text{CDC}2}$ anti-mouse (Signal Transduction, Lexington, KY) overnight at 4°C in 5% milk in PBS or 5% BSA in TTBS, respectively. Furthermore, the membranes were incubated with the diluted (1 : 2000) secondary antibodies for 1 h at RT. Membranes were washed with TTBS and the antibody reaction was visualized using an enhanced chemiluminescence system (ECL, Amersham). Relative protein levels were determined using Fluor-S Multimager (Bio-Rad). Experiments were repeated at least three times and statistical differences in the intensity of the bands were evaluated by means of Student's *t*-test.

RESULTS

Flow cytometric analysis of long-term post-irradiated cells

Figure 1 shows the percentages of G_2/M accumulated cells in samples harvested 24 h after exposure to 0.50–2 Gy X-rays. Since the fraction of G_2/M -phase cells was about 15% in all untreated

Figure 1. Percentage of G₂/M accumulated cells harvested 24 h after irradiation. Bars: SE.

LCL (not shown), the analysis of cells treated with such low doses and harvested 1 day later, was expected to be mainly restricted to cells in G₁ or S-phase at time of irradiation.

Irradiation determined the accumulation of G₂/M phase cells in a dose dependent manner, in all LCL tested. Normal and NBS cells showed a similar accumulation at all three doses. The highest fraction of G₂ accumulation was obtained at 2 Gy: 15.1, 22.3 and 19.8% for normal, ZRA and 96B453, respectively, after subtraction of G₂ values observed in untreated cultures. The extent of accumulation in AT cells was considerably greater than that observed in normal and NBS cells, in fact percentages of G₂ accumulated cells ranged from 28.4 to 40.7% compared with untreated cultures. No statistical differences were detected between normal and NBS cells; on the contrary, the percentage of G₂-phase accumulated cells in AT displayed high significant differences compared to the remaining LCL ($P < 0.01$; Fig. 1).

Mitotic index analysis of short-term post-irradiated cells

Figure 2 shows the modifications of the mitotic index in cells treated with X-rays or calicheamicin- γ 1 and harvested 2 and 4 h later, that is in the G₂ phase at time of treatment.

Following irradiation, normal and NBS cells were strongly impaired in their progression from G₂ to M phase. Nevertheless, the suppression of progression was less pronounced in NBS in comparison to control cells ($P < 0.05$ normal vs. NBS at 2 h with 0.5–1 Gy). The differences between NBS and AT were more evident. In particular at the first harvesting time the reduction in mitotic index of NBS cells compared to AT ranged from 2.0- to 3.5-fold at 0.5 Gy (Fig. 2a) and from 2.2- to 4.0-fold at 1 Gy (Fig. 2b; $P < 0.01$ NBS and normal vs. AT).

Similar modifications of the G₂/M progression were observed in cells treated with the dsb-inducer calicheamicin. The percentage of mitotic cells was reduced from 1.7- to 2.3-fold in NBS compared to AT, 2 h after incubation with the low dose of drug (Fig. 2c). Such reduction in mitotic index ranged from 1.8- to 5.6-fold in cells scored 2 h after the highest treatment (Fig. 2d; $P < 0.01$ NBS vs. AT). The response of NBS was intermediate between that observed in normal and AT cells.

Immunofluorescence analysis of cyclin-B1

Figure 3(a) shows the modifications in the number of cells displaying cytoplasmic accumulation of cyclin-B1 observed 4 h after irradiation with 1 Gy. The controls and two NBS LCL reacted

Figure 2. Kinetics of delay into mitosis following exposure to X-rays (0.5 Gy (a); 1 Gy (b)) or calicheamicin- γ 1 treatment [4 pg/ml (c); 8 pg/ml (d)]. The mitotic index (MI) of treated cells is represented as a percentage of the mitotic index of sham-treated cells. 1228 (open square), ZRA (open triangle), 96B453 (open circle, grey), AT (filled circle). Bars: SE.

similarly, with a cyclin-B1 accumulation ranging from 1.5- to 2.1-fold increase. In contrast NBS LCL 668 and the AT LCL showed a lower accumulation of G₂-phase cells, with a maximum of 1.2-fold and 0.9-fold, respectively ($P < 0.05$ 668 or AT vs. the remaining LCL). An example of G₂-cells with a high level of cyclin-B1 expression in the cytoplasmic compartment is reported in Fig. 3(b,c), showing, respectively, the localization of antibody reaction and the morphology of cellular nuclei (DAPI staining). The staining with DAPI, together with localization of antibody reaction ensured that highly stained cells were not mitotic cells.

Analysis of cell cycle protein induction by Western blotting

Figure 4 shows the induction of cyclin-B1 and the extent of accumulation of tyrosine 15-phosphorylated p34^{CDC2} in cells treated with 1 and 2 Gy and harvested 2 or 4 h later. The accumulation was evaluated by Western blot analysis in cytoplasmic and nuclear protein extracts. An immunoblot from a representative experiment is shown in Fig. 4(a,b). To ascertain the efficiency of the extraction procedure 20 μ g of the samples were immunoblotted with anti-I κ B antibody (a cytoplasmic protein) or poly (ADP-ribose) polymerase (PARP; a nuclear protein) (Fig. 4d).

Normal cells treated with 1 Gy showed about 2-fold accumulation of cyclin-B1 at 2 h and 3.5–4-fold accumulation at 4 h (Fig. 5a). In contrast, NBS and AT cells showed similar lower

Figure 3. G₂-phase accumulation in cells irradiated with 1 Gy X-rays and harvested 4 h later (a). The increase in the fraction of cells in the G₂ phase (y-axis) is obtained as the ratio between the frequency of labelled cells in treated and untreated cells. Those cells showing a bright green signal in cytoplasm after staining with an anticyclin-B1 antibody were classified as G₂ cells. An example of two G₂-phase cells is shown in b; (c) the same field with interphase nuclei counterstained with DAPI. Bars: SE.

protein accumulation between 1.3- and 2.3-fold at 2 h (NBS mean: 1.64; $P < 0.05$ normals vs. NBS; $P < 0.01$ normals vs. AT; $P < 0.05$ 2345 vs. AT) (Fig. 5a). At 4 h, NBS behaved intermediately between normal and AT cells (normals mean: 3.7; NBS mean 2.6; AT 1.3-fold; $P < 0.05$ normals vs. NBS; $P < 0.01$ normals and NBS vs. AT) (Fig. 5a).

Cells treated with 2 Gy X-rays and harvested 2 h later showed a picture similar to that observed at 1 Gy, with the highest cyclin-B1 accumulation in normal cells (normals mean 3.4; NBS mean: 1.5; $P < 0.01$ normals vs. NBS and AT; Fig. 5b). At 4 h, cyclin accumulation was different and NBS cells showed the strongest cyclin-B1 accumulation (Fig. 5b). In contrast, no cyclin-B1 modulation was observed in AT cells ($P < 0.001$ normals and NBS vs. AT).

The nuclear accumulation of tyrosine 15-phosphorylated p34^{CDC2} in cells treated with 1 Gy radiation and harvested 2 h later did not show marked differences among the investigated normal cells and NBS lines (normals mean 1.7; NBS mean: 1.7, Fig. 5c). At 4 h, an increase in the fraction of phosphorylated p34^{CDC2} was observed in normals, 2345 and 96B453. A similar result was observed in cells irradiated with 2 Gy, and in particular at the time harvest of 4 h, normals and NBS statistically differed from AT (normals mean: 2.3; NBS mean: 1.9; AT: 1.0; $P < 0.01$ normals and NBS vs. AT; Fig. 5d). Interestingly, we frequently observed a high level of basal p34^{CDC2} phosphorylated protein (not shown) and no modulation in the fraction of the phosphorylated protein in AT cells (Fig. 5c,d).

Figure 6 shows a Western blot of a representative qualitative experiment obtained by treating a LCL with 2 Gy and harvesting them 24 h later. This blot demonstrates that 24 h after irradiation, the extent of tyrosine 15-phosphorylation of p34^{CDC2} occurred in all the LCL investigated

Figure 4. Accumulation of cyclin-B1 in cytoplasmic extracts (a) and tyrosine 15-phosphorylated p34^{CDC2} in nuclear extracts (b) in cells treated with 1 and 2 Gy X-rays and harvested 4 h later. Alpha-tubulin signals as evaluated after stripping cyclin-B1 antibody and reprobing the membrane (c). Cytoplasmic and nuclear protein extracts were immunoblotted with an anti-IkB antibody and PARP to ascertain the validity of the extraction procedures (d). Bars: SE.

Figure 5. Induction of cytoplasmic cyclin-B1 as evaluated in Western blot of cells treated with 1 Gy (a) and 2 Gy (b) and harvested at 2 and 4 h. Induction of nuclear tyrosine 15-phosphorylated p34^{CDC2} as evaluated in Western blot of cells treated with 1 Gy (c) and 2 Gy (d) and harvested at 2 and 4 h. Bars: SE.

Figure 6. Accumulation of tyrosine 15-phosphorylated p34^{CDC2} as evaluated in nuclear extracts (a) of cells treated with 2 Gy X-rays and harvested 24 h later. Parallel samples were immunoblotted with an anti-p34^{CDC2} antibody (b).

(Fig. 6a), whereas it was less visible for NBS shortly after treatment at 2 Gy or in AT at both times of harvesting (Fig. 5c,d). It should be observed that the total nuclear p34^{CDC2} protein did not vary in amount after treatment (Fig. 6b). Differences between untreated and treated samples were not ascribed to variations in the total level of p34^{CDC2}.

DISCUSSION

In the present paper we have investigated the G₂-radiation response in four NBS LCL and compared it to AT and normal cells. Moreover, a possible correlation between genotype and cellular phenotype has been attempted.

In agreement with data gained on other NBS lines (Antoccia *et al.* 1997; Maraschio *et al.* 2001), the present results indicate that NBS cells harvested 24 h after irradiation, showed a markedly reduced G₂-phase accumulation. In fact, in all the five NBS LCL so far investigated, this delay was similar to that found in the control cell lines, at least at the lower doses, and greatly different from that of AT-cells. Nevertheless, this difference is most probably not a direct G₂ checkpoint effect, since more than 85% of cells were in G₁ and S-phase at moment of irradiation. In fact, it is assumed that this difference may be the result of a less disturbed irradiation-induced G₁/S checkpoint activation in NBS in comparison to AT, resulting in a lower number of damaged cells which reach the next G₂ phase. In contrast, AT cells which fail to undergo post-irradiation delay at the G₁/S phase border accumulate strongly in the next G₂ phase (Beamish & Lavin 1994; Shiloh 1997). These results are also in agreement with previous reports on correct G₁/S phase checkpoint activation and a proficient radiation-induced p53 accumulation in NBS cells (Yamazaki *et al.* 1998; Antoccia *et al.* 1999; Stewart *et al.* 1999).

Also, with respect to the response to G₂-phase irradiation, that is shortly after treatment, NBS cells greatly differ from AT (present data, Antoccia *et al.* 1997). Both ZRA (657del5 *Nbs1*) and 96B453 (no detectable *Nbs1* mutations), similarly to LCL 7078 (675del5 *Nbs1*; Varon *et al.* 1998) and 1548 (835del4 *Nbs1*; Varon *et al.* 1998), showed a normal or close-to-normal inhibition of cells to enter mitosis, as comparable to the control (present data, Antoccia *et al.* 1997). In contrast, the AT cells showed a reduced G₂ delay shortly after irradiation. These observations are confirmed by data obtained from G₂-treatment with the specific DNA dsb inducer calicheamicin- γ 1.

In contrast to the results observed in the other NBS LCL, the early G₂ checkpoint post-irradiation response in LCL 668 cells (900del25 *Nbs1*) was similar to that of AT, and intermediate with calicheamicin treatment (Maraschio *et al.* 2001), indicating also some modifier

effects, probably caused by different genetic backgrounds. The more pronounced calicheamicin sensitivity points also to a relationship between specific DNA dsb lesion types and a specific checkpoint activation.

Despite the absence of Nbs1 mutations, patient 96B453 showed the typical NBS cardinal symptoms, marked chromosomal radiosensitivity (see Barbi *et al.* 1991; Antoccia *et al.* 1999, Antoccia & Stumm unpublished data) and proficient checkpoint activation. Results on checkpoint activation in these cells indicate that the presence of nibrin fails to counteract the typical cellular NBS features supporting the hypothesis that at least one further gene may be involved in the NBS phenotype. Very recently it has been shown that an alternative mode of translation permits the production of a 70-kDa variant NBS1 protein physically associated with the MRE11 complex in NBS patients (Maser *et al.* 2001). In this context, one can speculate that NBS retains some of the normal cellular functions (p53 induction, G₂-radiation response), whereas others are strongly compromised (radiosensitivity, radioresistant DNA synthesis).

Beside the mitotic inhibition, we analysed the cytoplasmic accumulation of cyclin-B1 as a measure of G₂-phase cell accumulation post-irradiation. The data obtained at 'the single cell level' by means of immunocytochemical staining analysis, revealed an increase in the number of cells with cytoplasmic accumulation of cyclin after irradiation and were in line with the mitotic index analysis, also supporting the cell cycle differences between NBS and AT cells. This technique appeared to be particularly sensitive when the effect of 'low, biologically significant doses of radiation' was assessed; on the other hand, the analysis 'at the whole cell population level', though less sensitive, allowed the radiation response of a higher number of cells to be analysed.

In immunoblot evaluations of cyclin-B1, differences were detectable between normal and NBS cells, as a function of the harvesting times. At both irradiation doses the response appeared delayed in NBS compared with normal cells. It cannot be excluded that such result might be mediated by the suboptimal response of the signal pathway responsible for checkpoint activation or by differences in the length of the G₂-phase between normal and NBS, as reported in lymphocytes from an NBS patient (Pincheira & Bravo 1998).

In AT, cells the absence of cyclin-B1 accumulation, as a function of the harvest times and irradiation dose is in agreement with data provided from an AT fibroblast strain (Paules *et al.* 1995), and also confirms the documented defect of the G₂-phase checkpoint in this syndrome (Scott & Zampetti-Bosseler 1982; Beamish & Lavin 1994).

Radiation has been demonstrated to result in a transiently decreased p34^{CDC2}/cyclin-B1 kinase activity mediated by the inhibitory phosphorylation of p34^{CDC2} (Metting & Little 1995). Recently, in HeLa cells the p34^{CDC2} kinase activity has been shown to be excluded from the nucleus while tyrosine-phosphorylated p34^{CDC2} was predominantly localized in the nucleus during the radiation-induced G₂ arrest (Kao *et al.* 1999). Despite the radiation-induced G₂ delay observed in normal and NBS cells, the modulation in the extent of p34^{CDC2} phosphorylation appeared less dose- and time-dependent than that observed when using cyclin-B1 antibody. Furthermore the radiation-induced p34^{CDC2} phosphorylation as observed in NBS appeared delayed compared to that of normal cells at least at the highest dose.

Interestingly, actively growing, untreated AT lymphoblastoids as well as fibroblasts (Paules *et al.* 1995) showed high levels of phosphorylated p34^{CDC2}, which did not affect the progression of these cells from G₂ to M phase. In addition, no modulation of p34^{CDC2} kinase phosphorylation was detected in our AT cells nor in fibroblasts (Paules *et al.* 1995). The observation that at a long time from treatment, phosphorylation of p34^{CDC2} was detected in both AT and NBS, suggests that the mechanism leading to the inhibition of the kinase activity of p34^{CDC2} might be delayed in these syndromes.

In conclusion, the present paper reports marked differences in radiation-induced cell cycle perturbations between NBS and AT cells, whereas the differences with normal cells demonstrating a nearly proficient or suboptimal G₂/M checkpoint activation in NBS cells. Furthermore, our results indicate that a defect in DNA dsb repair pathway, as proposed for NBS cells, is not necessarily linked directly to cell checkpoint defects. Moreover, we can speculate that the NBS radiosensitivity is more linked to a DNA repair defect rather than being the consequence of a cell cycle checkpoint defect.

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