Radioresensitivity in Nijmegen Breakage Syndrome Cells Is Attributable to a Repair Defect and not Cell Cycle Checkpoint Defects


ABSTRACT

Cells derived from Nijmegen Breakage Syndrome (NBS) patients display radioresensitivity and cell cycle checkpoint defects. Here, we examine whether the radiosensitivity of NBS cells is the result of a repair defect or whether it can be attributed to impaired checkpoint arrest. We report a small increased fraction of unjoined double strand breaks and, more significantly, increased chromosome breaks in noncycling NBS cells at 24 h after irradiation. One of the NBS lines examined (347BR) was atypical in showing a normally near checkpoint response. In contrast to the mild checkpoint defect, 347BR displays marked γ-ray sensitivity similar to that shown by other NBS lines. Thus, the γ-ray sensitivity correlates with the repair defect rather than impaired checkpoint control. Taken together, the results provide direct evidence for a repair defect in NBS cells and are inconsistent with the suggestion that the radiosensitivity is attributable only to impaired checkpoint arrest. 347BR also displays elevated spontaneous damage that cannot be attributed to impaired G2-M arrest, suggesting a function of Nbs1 in decreasing or limiting the impact of spontaneously arising double strand breaks.

INTRODUCTION

IR induces a spectrum of lesions in DNA of which a DNA DSB is the most biologically significant. A range of damage response mechanisms operate after radiation exposure, including mechanisms of DNA repair such as base excision repair, single strand break repair, and DSB repair. In addition, IR induces arrest at a number of cell cycle checkpoints including G1-S, G2-M, and a replication-associated S-phase checkpoint. It is widely assumed that these cell cycle checkpoints function to delay cell cycle progression until the DNA damage has been repaired or critical metabolic results, such as replication and chromosome segregation, have been completed. Studies in yeast provide evidence for this concept because defects in cell cycle checkpoint genes can confer increased sensitivity to DNA-damaging agents and increased genomic instability. Checkpoints also operate in mammalian cells. The G1-S phase checkpoint is a p53-dependent process involving activation of p21waf1cip1, an inhibitor of cyclin-dependent kinases. The replication-associated S-phase checkpoint is a p53-independent process that involves phosphorylation of Chk2, the mammalian homologue of the Saccharomyces cerevisiae Rad53 and Saccharomyces pombe Cds1 protein kinases (1, 2).}

Mutant cell lines defective in these damage response mechanisms are important in evaluating their function. Rodent lines defective in the NHEJ mechanism of DNA DSB repair are exquisitely sensitive to IR but are able to effect cell cycle checkpoint arrest normally (3). Cell lines derived from patients with A-T display pronounced radioresitivity and cell cycle checkpoint defects including an inability to effect G1-S and S-phase checkpoint arrest (4, 5). A-T cell lines in G2 at the time of irradiation also fail to arrest at a G2-M checkpoint (6, 7). ATM is a member of the phosphatidylinositol kinase-related kinase family, and the prevailing evidence suggests that ATM functions as an upstream sensor of DNA damage and activates, by phosphorylation, at least two signal transduction pathways, one involving p53, leading to G1-S arrest, another involving Chk2, and possibly a third involving Chk1 (2, 8). However, indirect evidence suggests that the γ-ray sensitivity of A-T cells is separable from these checkpoint defects (see Refs. 9–12 for further discussion). Intriguingly, A-T cell lines have only a subtle defect in DNA DSB repair, leaving open the basis underlying the radioresensitivity of A-T cells (13).

NBS is another rare autosomal recessive disorder associated with clinical radioresistance (14, 15). Like A-T, NBS patients also show immunodeficiency and developmental delay but do not display ataxia or telangiectases. In contrast to A-T patients, they have associated microcephaly and a characteristic facial appearance (16). Furthermore, they have a significantly elevated cancer incidence when compared with A-T patients (4). The cellular phenotypes of A-T and NBS cell lines overlap because NBS cells also display radioresitivity and cell cycle checkpoint defects (17–20). Indeed, the phenotype of RDS, which represents a failure to arrest replication after irradiation, is used in the diagnosis of NBS. Recently the gene defective in NBS, NBS1, was cloned, and the encoded protein, termed Nbs1, nibrin, or p95, was shown to interact with hMre11p and hRad50p (21, 22). Nuclear foci involving these three proteins form after radiation treatment, but this is not observed in NBS cell lines (21). hMre11 and hRad50 are homologues of yeast proteins that coassociate with a third protein, Xrs-2p (23). Nbs1 appears to be a functional homologue of Xrs-2p, although the two proteins share only limited sequence homology. Recently, the link between A-T and NBS was strengthened by the finding that one class of A-T variant patients have mutations in hMre11 (24). Yeast mre11, rad50, and xrs-2 null mutants display a pleiotropic phenotype, and it has been suggested that the proteins function in both homologous recombination and NHEJ (25–29). The pleiotropic phenotype of NBS cell lines leaves open the function of Nbs1 in damage response mechanisms.

Our aim here was to investigate the basis underlying the radioresitivity of NBS cells and to assess whether they have a repair defect or whether the radioresitivity could be attributed to a defective checkpoint response. We show that two NBS lines have a small but reproducible defect in DNA DSB rejoining as well as an elevated level of chromosome breaks after radiation exposure. From an analysis of three NBS lines, the γ-ray sensitivity is shown to correlate with...
the repair defect rather than impaired S-phase arrest. Finally, one of the lines studied, 347BR, has a nearly normal G<sub>1</sub>-S, S, and G<sub>2</sub>-M checkpoint response. Taken together, our data suggest that NBS cells have a repair defect and are inconsistent with the suggestion that the γ-ray sensitivity of NBS cells is the result of failed checkpoint arrest. We also show that 347BR has an elevated level of spontaneous abnormalities that cannot be attributed to aberrant G<sub>2</sub>-M arrest.

**MATERIALS AND METHODS**

**Cells and Cell Culture Conditions.** 1BR3 are untransformed fibroblasts derived from a normal patient. 851129W and 87RD102 are untransformed skin fibroblast lines derived from NBS patients kindly sent to us by Dr. K. Jaspers (Frasinus University, Rotterdam, The Netherlands). CzD82CH, another NBS cell line, was kindly provided by Drs. J. Hall and K. Chrzanowska (Poland Children’s Memorial Health Institute, Warsaw, Poland). 347BR, an untransformed fibroblast line, was derived from a skin biopsy from a patient with a diagnosis of common variable immunodeficiency. The patient displayed elevated chromosome aberrations, microcephaly, and primary ovarian failure, all features of NBS, and developed a midline granuloma, a rare tumor type, that has not been described previously in NBS patients. AT5B1, AT2BR, and AT1BR are primary fibroblasts derived from A-T patients. Cells were cultured in MEM supplemented with 15% FCS, penicillin, and streptomycin as described previously (30). Survival after exposure to γ-rays, UV, or MMC was carried out as described previously (30).

**Immunoblotting.** Whole-cell extracts were prepared by the method of Scholer et al. (31). For analysis of p53 and p21, whole-cell extracts were boiled in SDS-PAGE loading buffer and separated in 12% SDS-PAGE, and the proteins were transferred to nitrocellulose using a wet-blotting apparatus. The anti-p53 antibodies were a kind gift from Dr. P. Hall (Dundee University, Dundee, Scotland). Ku antibodies (Ku80-4 from Serotec, Oxford, United Kingdom) and PCNA antibodies (PC10 mAb from Santa Cruz Biotechnology, Santa Cruz, CA) were used as loading controls. Immunoblot analysis using hMre11, Rad50, and Nbs1 antibodies was as described previously (21). For the analysis of Chk2, nuclear extracts were used, and the proteins were separated in 10% SDS-PAGE. Chk2 antibodies were as described previously (2).

**Cell Cycle Analysis Using FACS.** A detailed description and analysis of the FACS procedure used to assay G<sub>1</sub>-S and G<sub>2</sub>-M checkpoint arrest has been described elsewhere (32). In brief, cells were labeled for 30 min with 10 nM bromodeoxyuridine, washed, irradiated, and incubated for varying times. Each sample was harvested by trypsinization and subjected to immunostaining and treatment with propidium iodide as described previously (33). Samples were analyzed using a Coulter Epics Elite, ESP flow cytometer (Coulter Electronics Ltd., Luton, United Kingdom). Cells were separated on the basis of DNA content using propidium iodide and bromodeoxyuridine incorporation to measure cell size and FITC signal. On this basis, cells were divided into gates defined as labeled or nonlabeled G<sub>1</sub>, S, and G<sub>2</sub>. Figures showing typical separation and analysis carried out under identical conditions can be seen in Badie et al. (32).

**Analysis of G<sub>2</sub>-M Checkpoint by Estimation of the Mitotic Index.** For these experiments, actively growing cells from a partly confluent flask were used. T25 flasks were inoculated with 10<sup>5</sup> cells, 40 h before irradiation. After irradiation with 2 Gy, the medium was changed, 0.15 mg/ml Colcemid was added, and the cells were reincubated for the times shown. Cells were harvested by trypsinization, resuspended in 10 ml of 0.075 M KCl, and incubated at 37°C for 20 min. Cells were fixed in methanol:acetic acid (3:1) overnight at 4°C, spread on slides, and stained with Hoescht, and the mitotic index was estimated by scoring at least 1000 cells. The results represent the mean mitotic indices for three replicate samples at each time point.

**Estimation of RDS.** The protocol used was followed as described previously (34). In brief, actively growing cells were labeled with [<sup>3</sup>H]thymidine for 3 days, then irradiated with the doses shown, and incubated in fresh medium containing [<sup>3</sup>H]thymidine for 4 h prior to fixation and estimation of the label incorporated.

**Measurement of DNA DSBs.** The DNA DSB-rejoining protocol was as described previously (35). Briefly, experiments have been performed on plateau phase cells (92–99% in G<sub>0</sub>-G<sub>1</sub>). Cells were labeled for 3 days with [3H]thymidine and then held for 5 days to reach plateau phase. The cells were irradiated on ice with a dose of 30 Gy from a 137Cs source (1 Gy min<sup>−1</sup>) and incubated for the time periods indicated. Agarose plugs were prepared as described previously, and DNA fragments were separated by pulsed field gel electrophoresis using a CHEF DRIII (Bio-Rad, Hercules, California). The FAR was determined by estimating the radioactivity present in the lane divided by the total activity in the well plus the lane. The FAR for unirradiated cells was subtracted prior to these estimations. The results are presented as FAR remaining at the specified time compared with the FAR at time 0.

**Measurement of Interphase Chromosome Break Using PCC.** The procedure was followed essentially as described previously (36). In brief, density-inhibited cells were irradiated with 6 Gy and sampled immediately or after various times of incubation at 37°C to allow repair. The cells were then induced to undergo premature condensation by fusion with mitotic CHO cells (37). Interphase cells were scored for chromosome breaks by estimating the frequency ofacentric fragments in standard metaphase preparations. The data presented represent the percentage of fragments remaining at 24 h relative to the fragments induced after irradiation. There was no significant difference between the level of spontaneous ECFs for any of the lines. The results represent the analysis of at least 30 independent fusion events for each sample.

**Micronucleus Formation.** Cells were plated at 5 × 10<sup>4</sup> cell/dish, left overnight, and irradiated with the relevant dose. Samples were taken immediately and cytochalasin B (1 µg/ml) added to the remainder. Cells were subsequently fixed and stained with 16% Giemsa at daily intervals for 7 days, and micronucleus formation was estimated in binucleate cells. A time course for micronucleus formation was undertaken in initial experiments and shown to reach a plateau between 2 and 5 days after irradiation. For the data shown, samples were taken 3 days after irradiation. A minimum of 200 binucleate cells were scored in each experiment.

**Chromosomal Aberration Analysis.** Cells were cultured in Dulbecco/Ham's F-12 with Ultrasor (1:1 Dulbecco’s MEM/Ham’s F-12 with 2% Ultrasor, 2.5% L-glutamine) medium supplemented with 15% FCS. Exponentially growing cells were irradiated using an X-ray therapeutic radiation source (dose rate, 1.5 Gy/min). Colcemid (0.06 µg/ml) was added after 2 h, and the cells were harvested 2 h later. Chromosome preparations were obtained and stained by Giemsa using standard methods. The ratio of aberrations/cell was calculated from the number of chromatid and chromosome breaks (counted as one breakage event), dicentric chromosomes, translocations, ring chromosomes, and chromatid exchange figures (counted as two breakage events).

**RESULTS**

**Characterization of the NBS Defective Cell Lines.** We have used primary skin fibroblast cell lines for this study because transformed or immortalized lines frequently have abnormalities in cell cycle checkpoint control. 347BR was derived from a skin biopsy from an atypical NBS patient (see “Materials and Methods”). Analysis of 347BR cell extracts by Western immunoblotting showed that the cells failed to express Nbs1, whereas hRad50 and hMre11 were expressed at normal levels (Fig. 1). Sequence analysis by reverse transcription-PCR revealed a homozygous 5-bp deletion at position 657 (657del5), which was confirmed by sequence analysis of genomic DNA. This is a common mutation found in NBS patients from Central Europe (22).
ATM was expressed at normal levels in 347BR, and no mutational changes were detected in ATM using the Restriction Endonuclease Fingerprinting method (data not shown). 5 347BR cells had normal levels of Ku end-binding activity and normal DNA-PK activity (data not shown). Previous studies have shown that hMre11, hRad50, and Nbs1 colocalize in nuclear foci after irradiation and that such foci do not form in NBS cells (21). hMre11-dependent foci also failed to develop in 347BR cells (data not shown). Thus, 347BR has a common mutation in NBS1 that results in loss or severely impaired Nbs1 function. For much of this study, 347BR has been compared with a “classical” NBS line, 87RD102. Like many untransformed NBS fibroblasts, 87RD102 grows poorly, and we were unable to complete all of the analyses with this line. 851129W, another untransformed NBS line, has also been used for some analyses. The 657del5 mutational change is also present in both alleles of 87RD102 and 851129W. 6

Radiosensitivity of 851129W and 347BR Cells. We compared the NBS lines for their survival response to ionizing radiation. 347BR and 851129W showed marked γ-ray sensitivity, slightly less than that shown by a typical A-T cell line (Fig. 2). This is similar to the level of sensitivity shown by other NBS cell lines (for an example, see Ref. 18). Unfortunately, we have been unable to carry out full survival analysis with 87RD102 because of its low plating efficiency and/or early senescence. However, in a single experiment, the survival after 2 Gy of irradiation was 11.5%, similar to the survival level shown by 347BR and 851129W. 347BR showed no marked sensitivity to either MMC treatment or UV irradiation (data not shown).

DSB Rejoining in 347BR and NBS Cells. We next examined whether the NBS cells displayed evidence of a repair defect. Cell lines defective in NHEJ have a marked defect in their ability to rejoin DNA DSBs, whereas A-T cell lines rejoin the majority of their DSBs efficiently, although a small increased fraction of breaks remains unrejoined at prolonged times after irradiation (13, 38). For this analysis, we used density-inhibited cells to avoid consideration of the impact of cell cycle checkpoints. The repair of DNA DSBs at varying times after exposure to 30 Gy was examined in IBR3, 87RD102, and 347BR by pulse field gel electrophoresis. Both 87RD102 and 347BR gave a response that was on the lower edge of that found with a large panel of A-T cells lines (Fig. 3). This response is characterized by an elevated rate of repair at early postirradiation incubation times (up to 4 h), followed by an increased fraction of unrejoined breaks at prolonged incubation times. Although small, this difference from control cells is highly reproducible. These results show that both 347BR and 87RD102 display a mild DSB rejoining defect similar to that found in A-T cells.

Analysis of Chromosome Breaks in Interphase Cells Using PCC. To gain further evidence for a repair defect in NBS cells, we examined the repair of damage giving rise to chromosome breaks. PCC is a technique in which interphase cells are induced to condense prematurely by fusion with mitotic cells (39). The presence of chromosome breaks can then be visualized by standard cytogenetic analysis and monitored as the frequency of ECFs. The analysis of ECF frequency was also carried out using nondividing, density-inhibited cells. Although some processing is required to achieve chromosome condensation, the cells neither enter S-phase nor mitosis prior to analysis. The spontaneous and radiation-induced ECF frequency was the same in all of the lines examined. However, 87RD102 and 347BR showed an elevated frequency of ECFs at 24 h after irradiation compared with control cells (Table 1). The elevated frequency of ECFs in NBS cells was slightly less than that observed with A-T cells, consistent with the slightly elevated A-T radiosensitivity (Table 1; Ref. 39). Taken together with the analysis of DSB repair, these results provide direct evidence for aberrant repair in 347BR and 87RD102.

Analysis of Cytogenetic Damage in Mitotic Cells. We also examined the level of chromosome damage detectable in 347BR mitotic cells derived from exponentially growing cells at 4 h after irradiation (Table 2). It has been reported previously that both A-T and NBS cells display an elevated frequency of spontaneous and radiation-induced

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*5 A. M. R. Taylor, personal communication.
6 P-M. Girard, data not shown, and E. Seemanova, personal communication.
Table 2. Spontaneous and radiation-induced chromosomal breakage in 347BR
347BR, AT2BR, an AT cell line, and 1BR3 cells were irradiated 4 h before harvesting in the G2 phase of the cell cycle, n represents the number of metaphases scored; *, induced aberrations — spontaneous aberrations). For the spontaneous breaks, the difference between control and 347 BR was highly significant (χ² = 24.08; 1 df; P < 0.001); the difference between 347BR and AT was not significantly different (χ² = 3.46; 1 df; P > 0.05). For radiation-induced breaks after subtraction of the spontaneous breaks, the difference between control and 347 BR was highly significant (χ² = 14.63; 1 df; P < 0.001). We also observed a slightly greater frequency of aneuploidy in the 347BR cells compared with control cells (60–68% diploid cells for 347BR compared with 84–96% for control cells).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gy</th>
<th>n</th>
<th>% aberrant metaphases</th>
<th>Breaks/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1BR3</td>
<td>0.5</td>
<td>50</td>
<td>20 *(16)</td>
<td>0.26 *(0.20)</td>
</tr>
<tr>
<td>AT2BR</td>
<td>0.5</td>
<td>50</td>
<td>20</td>
<td>0.38</td>
</tr>
<tr>
<td>347BR</td>
<td>0</td>
<td>75</td>
<td>58 *(19)</td>
<td>1.40 *(0.77)</td>
</tr>
<tr>
<td>347BR</td>
<td>1.0</td>
<td>50</td>
<td>74 *(35)</td>
<td>1.96 *(1.33)</td>
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Fig. 4. Micronucleus formation in 347BR. Micronucleus formation was measured at 3 days after irradiation with the doses indicated. The results represent the mean of three experiments each for 347BR and 1BR and a single experiment for AT1BR. Bars, SD of the mean, and where not visible for 1BR, they lie within the symbol. •, 1BR3; ○, 347BR; ▽, AT1BR.

chromatid breaks (20). Strikingly, 347BR showed significantly elevated spontaneous chromosomal instability. Although the spontaneous break frequency was higher than that seen in an A-T control line, the difference was not significant (see legend). In addition to elevated spontaneous chromosome instability, irradiation induced an elevated frequency of aberrant metaphases and breaks/cell that was significantly greater in both 347BR and the A-T line compared with control cells. However, because these cells have traversed a G2-M checkpoint prior to reaching mitosis, we cannot, from this analysis alone, determine whether this feature is the result of a checkpoint or a repair defect.

As an alternative procedure to monitor unjoined chromosome breaks, we also examined the formation of micronuclei that arise when acentric fragments are generated (40). A striking feature of both 347BR and A-T cells was a significantly increased frequency of spontaneous micronuclei indicative of an elevated level of spontaneous chromosome breakage (Fig. 4). Both A-T and 347BR cells also showed an elevated level of micronuclei after radiation exposure. However, when the spontaneous level of micronuclei was subtracted, the difference with control cells was only significant after 2 Gy. These results are consistent with those obtained by measuring chromosome breaks as described above and show that 347BR cells have a highly elevated frequency of spontaneous chromosome breakage. We were unable to carry out either of these analyses with 851129W or 87RD102 because of their low mitotic indices.

347BR Cells Show Nearly Normal S-Phase Checkpoint Arrest.
A characteristic phenotype of NBS cells is RDS, which represents a failure to arrest DNA synthesis in response to IR (41, 42). To determine whether failed S-phase arrest could contribute to the radiosensitive phenotype of NBS cells, we next analyzed the NBS lines using the RDS assay. We examined 347BR, 851129W, and 87RD102 together with an A-T cell line and control cell lines for their ability to arrest DNA synthesis after irradiation with varying doses of γ-rays and labeling with [3H]thymidine for 4 h. 87RD102 and 851129W showed an RDS phenotype that was similar to the A-T line used in the same experiments and to other A-T lines examined in our laboratory (Fig. 5). In contrast, 347BR showed a significant arrest that was close to that observed with the control line used in the same experiment. The results with 347BR were, however, slightly impaired compared with three control lines that we have examined in our laboratory (Fig. 5).

Analysis of p53 Induction and G1-S Arrest. NBS cell lines have also been reported to show a reduced level of induction of p53 and p21 after irradiation compared with control cell lines (17–19). The nearly normal RDS response of 347BR suggested that failed S-phase arrest was not responsible for its γ-ray sensitivity. We therefore examined 347BR further for other checkpoint responses. We consistently observed a high level of p53 in untreated 347BR, although after irradiation, the induced levels of p53 were similar to the levels seen with control cells (Fig. 6A). This meant that the fold induction of p53 was lower than that found in control cells (Fig. 6B). p21 levels in untreated 347BR and 1BR cells were similar, however, resulting in a similar magnitude of p21 induction (Fig. 6C). This response was distinct from that found in several A-T lines examined by us that also had a higher untreated level of p53 but showed very little subsequent induction. Our analysis of 87RD102 and 851129W for p53 and p21 induction has been limited by their early senescence. However, based on the limited analysis undertaken, we have observed reduced induction consistent with previous findings with NBS cells (17–19).

Induction of p53 activates a signal transduction pathway that in
untransformed skin fibroblasts results in arrest at the G₁-S boundary. The significantly delayed and reduced ability to induce p53 in A-T cell lines is accompanied by an inability to arrest at the G₁-S checkpoint (43). We therefore investigated the operation of the G₁-S checkpoint in 347BR. To investigate the G₁-S checkpoint without being influenced by the impact of the G₂-M checkpoint, we used a procedure in which S-phase cells are labeled by a 30-min exposure to bromodeoxyuridine immediately prior to irradiation (32). After analysis by FACS at various postirradiation incubation times, the progression of unlabeled cells into S-phase provides an estimation of cells that have traversed the G₁-S phase boundary. A dramatic decrease in the unlabeled S-phase population was observed in a normal fibroblast cell line and in 347BR in marked contrast to the A-T line, AT5BI (Fig. 6D). Taken together, these results show that after irradiation, 347BR cells activate p53 and p21 and effect G₁-S arrest as efficiently as controls.

Analysis of the G₂-M Checkpoint Arrest. It has been reported previously that A-T cells in G₂ at the time of irradiation show reduced arrest at a G₂-M checkpoint (6, 7). To analyze the operation of this checkpoint in 347BR, the accumulation of mitotic cells was observed. Colcemid, was scored at varying postirradiation incubation times. Fig. 7A shows the accumulation of mitotic cells for A-T, control, and 347 BR. Our results here, however, show that after irradiation 347BR cells showed a significant delay in accumulation of mitotic cells similar to that found in control cells, whereas there was only a very short delay in A-T cells.

Phosphorylation of Chk2 Occurs in 347BR. Previously, it has been reported that after irradiation, Chk2 is rapidly phosphorylated in an ATM-dependent manner (2). Here, we show that after 20 Gy irradiation, there are enhanced levels of a form of Chk2 with reduced mobility likely representing a phosphorylated product in 1BR and 347BR but absent in AT5BI (Fig. 7B). We therefore conclude that 347BR can phosphorylate Chk2 efficiently after radiation, providing further evidence for effective checkpoint responses in 347BR cells. This response is currently being analyzed in the NBS lines that show a more marked RDS phenotype.

DISCUSSION

Our aim here was to determine whether the radiosensitivity of NBS cells could be attributed to an inability to effect cell cycle checkpoint arrest or whether it is the result of a repair defect. Using pulse field gel electrophoresis, we found a modest but reproducible defect in DNA DSB rejoining in 347BR and 87RD102 cells. The kinetics and magnitude of this response are similar to that reported previously in A-T cells (13, 44). This is not a feature common to all radiosensitive cell lines and to date has only been observed in A-T and the NBS lines reported here (for an example, see Ref. 45), which describes a radi-
An inability to undergo S-phase arrest, represented by the RDS phenotype, is a consistently reported phenotype of NBS cells and is used below to compare the checkpoint versus repair defect in NBS cells (47). 851129W and 87RD102 displayed an RDS response similar to that shown by other NBS lines and by A-T cells. 347BR, however, displayed an S-phase arrest that was only slightly impaired compared with normal cells. From studies emerging, NBS cells appear to display a range of severity of RDS responses, suggesting that 347BR may not represent a distinct NBS phenotype but rather the lower end of a spectrum (for an example, see Ref. 24). Notwithstanding this, 347BR and 851129W represent two lines with differing RDS phenotypes but similar γ-ray sensitivity, suggesting that the γ-ray sensitivity of NBS cannot be attributed to impaired S-phase arrest because a causal relationship should be reflected in similarly decreased levels of both phenotypes. Additionally, 347BR and 87RD102 have different RDS phenotypes, yet similar repair defects. Taken together, these data suggest that the γ-ray sensitivity of 347BR correlates with the repair defect rather than the S-phase checkpoint defect.

To assess further whether other aberrant checkpoint responses could contribute to the γ-ray sensitivity of 347BR, we examined it for additional checkpoint responses. A reduced ability to induce or stabilize p53 and p21 has been reported in other NBS cells (17–20); G1-S arrest has been reported to be normal in one study but abnormal in another (17, 19). Most of this analysis has been carried out with lymphoblastoid lines, however. Thus far, our analysis of other NBS fibroblast lines is consistent with these previous observations (data not shown). However, the majority of our primary NBS fibroblast lines grow extremely poorly, which has restricted a detailed assessment of the response of such cells. Here, however our focus has been on 347BR cells, because they showed a nearly normal S-phase arrest. 347BR showed G1-S and G2-M checkpoint responses that were identical to normal cells. The level of p53 after radiation treatment appeared similar to control cells, but the fold induction was lower because of a higher level of p53 in untreated 347BR cells, a feature that may reflect the elevated level of spontaneous damage in 347BR cells. A-T cells also show elevated spontaneous p53 levels, which has been suggested to be attributable to elevated oxidative damage. The raised p53 levels appear insufficient to trigger p21 induction in unirradiated A-T or the 347BR cells, because increased levels of p21 are not observed. However, the ability to induce p21 normally after radiation treatment and to effect G2-M arrest suggests that the p53 response to radiation is normal. Additionally, Chk2 is phosphorylated normally. Taken together, our findings show that the γ-ray sensitivity of 347BR cells cannot be attributed to defects in either the G1-S or G2-M checkpoints. Because the RDS phenotype of 347BR was slightly abnormal and because of limitations of the RDS assay, we cannot rule out some contribution of failed S-phase arrest to the radiosensitivity of 347BR cells. However, the experiments to monitor the repair defect used noncycling cells and therefore cannot be attributed to failed S-phase arrest. It is worth noting that irradiation-induced, Mre11-dependent foci formation is dramatically decreased or absent in 347BR cells. Thus, such foci formation does not appear to be a prerequisite for the operation of these checkpoints.

Despite the differences in ability to effect checkpoint arrest, 347BR, 87RD102, and 851129W proved to have the same homozygous mutation in NBS1, a mutation commonly found in NBS patients of Eastern European origin. Normally the severity of cellular phenotype of cell lines with known defects is related to the nature of the
DNA REPAIR DEFECT IN NIJMEGEN BREAKAGE SYNDROME

mutational change. For example, A-T variant cell lines display milder defects compared with classical A-T lines because of the presence of a missense rather than a null mutation (48). The difference between 347BR and 87RD102/851129W is therefore surprising and suggests that the phenotype of cells bearing this mutation must be affected by differences in the genetic background. Because hMre11 and hRad50 knock-out mice are embryonic lethal, the viability of NBS patients is surprising, raising the possibility that they may not represent a null phenotype. To date there is no direct evidence for residual Nbs1 function nor how such activity may arise. However, although the 5-bp deletion in the NBS cells studied here results in premature truncation of the protein in the NH2 terminus, it lies downstream of the FHA domain, leaving the possibility of residual activity. Differences in the stability of such a fragment could provide an explanation for the variation between lines. In this context, it should be noted that our findings do not necessitate different functions for Nbs1 in repair versus checkpoint control but may merely reflect quantitative differences in the operation of the pathway between the cells. Finally, notwithstanding the explanation for the lack of RDS phenotype in 347BR cells, our results raise a note of caution in relying solely on the RDS phenotype for NBS diagnosis.

Recent evidence for the overlap between A-T and NBS is the finding that a class of A-T variant patients are defective in hMre11, a protein that interacts with Nbs1 (24). Our results demonstrate that A-T and NBS cell lines share a similar repair phenotype as well as shared checkpoint defects and suggest that ATM and Nbs1 either control or function in the same repair mechanism. What is the nature of this aberrant repair phenotype? The marked phenotype of A-T and NBS cells is a modest defect in DSB rejoining coupled with a more marked defect in the repair of chromosome breaks. The modest DSB rejoining defect is distinct from that observed in NHEJ defective cells, but our results, however, do not eliminate a role of Nbs1 in some aspect of NHEJ, such as maintenance of the fidelity of the process or the repair of a critical subset of breaks. It is also possible that Nbs1 is required for HR in mammalian cells, consistent with findings in yeast (28). However, A-T cells have been reported to display a hyper-re. phenotype rather than be recombination defective (49). Additionally, 347BR cells are not sensitive to MMC, a characteristic phenotype of HR defective XRC2- and XRC3-deficient cells (50). One possibility is that ATM/Nbs1 control the utilization of NHEJ versus HR and that elevated radiosensitivity is the end result of the inappropriate usage of the two repair mechanisms. The more pronounced defect in repair of chromosome breaks raises a second possibility that NBS cells are not defective in the rejoining of DNA ends per se but rather that this phenotype is a manifestation of some other repair defect, of which one interesting possibility is a failure to reassemble higher order DNA structure.

A further distinctive feature of 347BR is an elevated level of spontaneous instability. Elevated aberrations have been reported in lymphocytes from A-T and NBS patients (4) and in A-T and NBS lymphoblastoid cells. Previous work has been compatible with this being attributed to impaired G2-M checkpoint arrest (7, 20, 51, 52). Because 347BR arrests normally at the G2-M checkpoint, our results show that the spontaneous instability cannot be the result of such a defect and suggests that in the absence of Nbs1, either elevated breaks arise during replication or breaks arise at a normal frequency but fail to be repaired efficiently.

In conclusion, we show here that NBS cells have a modest defect in DNA DSB rejoining as well as a more pronounced impaired ability to rejoin chromosome breaks. Our results provide direct evidence for a repair defect in NBS cells that, we suggest, underlies their γ-ray sensitivity. Furthermore, analysis of an unusual NBS line with only a very mild checkpoint defect is inconsistent with the explanation that impaired checkpoint arrest is responsible for their γ-ray sensitivity. Additionally, 347BR like other NBS cells displays elevated spontaneous damage that cannot be attributed to impaired G2-M arrest. Taken together, our results suggest that Nbs1 functions in concert with ATM in a damage response mechanism that impacts upon a repair process as well as cell cycle checkpoint control.

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