Objective. To examine the stress response, including the role of DNA-dependent protein kinase (DNA-PK), in B cells from Sjögren’s syndrome (SS) patients.

Methods. B-cell lines were exposed to gamma radiation and then postincubated to allow inducible stress functions to develop. The magnitude of the DNA damage response was monitored with respect to DNA-PK phosphorylation of a p53 peptide, defence protein levels (Ku, DNA-PK catalytic subunit, ATM, p21 and p53) and flow cytometric determination of cell cycle phases and apoptosis.

Results. B cells from SS patients, compared with healthy controls, displayed enhancement of two stress functions in undamaged cells: DNA-PK kinase activity and apoptosis. In addition, SS showed enhanced cell cycle arrest in gamma-irradiated cells.

Conclusions. Strong kinase activity of DNA-PK, functioning not only in a DNA damage response but also in immunoglobulin gene rearrangement, may be an important component of the heightened stress response displayed by SS cells. In combination with recent reports, our data indicate that constitutional hyper-reactivity to danger signals is a basic pathogenetic factor in SS.

Keywords: Sjögren’s syndrome, DNA-dependent protein kinase, Ku protein, Stress response, Cell cycle arrest.
All cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum, 2mM l-glutamine, 25 mM HEPES and 12 μg/ml gentamicin at 37°C in a humidified 5% CO₂ atmosphere.

The design of the work conformed to the standards currently applied in Sweden at the time of the study and each subject gave informed consent according to the Declaration of Helsinki before entering the study.

Ionizing irradiation
Gamma radiation was delivered by a neutron accelerator (Philips, Germany) at a dose rate of 0.70 Gy/min, at a distance of 50 cm from the cells, which were kept in their plastic culture flask in complete medium at room temperature. All cell cultures in the different assays were handled identically, the only difference being exposure to ionizing radiation. Following irradiation, cells were returned to the incubator and analysed later as indicated.

Extraction of nuclear proteins
Nuclear protein extracts were prepared by a modification of the procedure of Dignam et al. [17]. Cytoplasmic protein was removed by lysis of the cells in a low-salt buffer, and the sedimented cell nuclei were then lysed in a high-salt buffer, as described [10].

DNA-dependent protein kinase activity
The phosphorylating function of DNA-PK was measured using a synthetic p53 peptide (Promega, Madison, WI, USA) containing a DNA-PK specific phosphorylation site (serine 15) as substrate, as described previously [18]. Briefly, nuclear protein extract was mixed with [γ-³²P]ATP and the synthetic p53 peptide, with or without sonicated DNA. The DNA-PK activity was calculated as counts per minute (c.p.m.) in the presence of DNA minus the c.p.m. in the absence of DNA. HeLa cells were included in each analysis and used as a calibrator in order to reduce the interassay variation. All nuclear extracts were analysed in triplicate, with an intra-assay coefficient of variation (CV) of 8.7%. The interassay CV, estimated using HeLa cells and determinations made on seven separate dates, was 19.4%.

Western blotting
A standard immunoblotting was performed as described [18]. Membranes were probed with the following antibodies: anti-Ku86 (p80, Ab-3) (clone S10B1) and anti-p53 (Ab-2) (clone PAb1801) from Oncogene (Cambridge, MA, USA); anti-DNA-PKcs (C-19) (SC-1552) and anti-p21 (F-5) (SC-6246) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-ATM (AHP397) from Serotec (Serotec, UK). The peroxidase-conjugated secondary antibodies were from Dako (DAKO A/S, Denmark). The blots were developed using the enhanced chemiluminescence method (ECL; Amersham-Pharmacia, UK), together with X-ray film or a Bio-Rad Personal Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA).

Cell cycle analysis
For cell cycle analysis a Coulter Epics XL-MCL flow cytometer (Coulter, Miami, FL, USA) was used. According to a standard total DNA labelling protocol the cells were incubated on ice for 10–60 min in a hypotonic solution containing 0.01 M NaCl, 0.1% NP-40, 50 μg/ml propidium iodide and 10 μg/ml RNase A. To calculate the fractions of cells located in the different cell cycle phases MacCycle II software (Phoenix Flow, Phoenix, AZ, USA) was used and at least 8000 events were scored. For each individual three to six determinations were made at 24 h and one or two determinations 48 and 72 h after exposure to 15 Gy of irradiation.

Aptoptosis
The same Coulter Epics XL-MCL flow cytometer was used, with filters detecting emission at 515–545 nm for fluorescein isothiocyanate (FITC) and 565–595 nm for propidium iodide. Background autofluorescence was monitored for each sample by analysing unlabelled cells. Apoptosis was measured as described [19], and at least 3000 events were scored. For each individual, two or three determinations were performed in unirradiated and 24 h post-irradiation cells. FITC-labelled Annexin V was from Alexis (Switzerland) and PharMingen.

Statistical analysis
The Mann–Whitney U-test was used for statistical analysis of the DNA-PK activity data, which included results from three controls and four SS patients. The lack of overlap between these two groups yielded a P value of less than 0.05 (0.028), considered statistically significant.

Results
High level of DNA-dependent protein kinase activity in SS
EBV-transformed B-cell lines were exposed to gamma radiation, post-incubated for 24 h under optimal growth conditions, and then subjected to nuclear protein extraction. DNA-PK kinase activity was determined by the incorporation of radiolabelled ATP into a synthetic p53 peptide containing a DNA-PK phosphorylation site. DNA-PK activity was calculated by subtracting the amount of radioactivity incorporated in the absence of sheared DNA from that incorporated in the presence of DNA. The assay takes advantage of the observation that DNA-PKcs appears to be the major DNA-activated kinase in mammalian cells [20]. The DNA-PK activity was higher in all four SS patients than in all three controls; i.e. there was no overlap between the groups (P = 0.028) (Fig. 1). The increased activity in SS was significant at all radiation doses, including unirradiated cells. There was no sign of induction of DNA-PK activity due to radiation exposure (except for the SS3 patient at 2 Gy); instead, a dose-dependent reduction was seen.

Normal Ku86 and DNA-PKcs protein content in SS
The nuclear protein extracts were also used for Western blotting semiquantitation of Ku86 and DNA-PKcs protein content. SS and control samples, at each radiation dose, were electrophoresed on the same gel. The resulting bands suggest that there is no apparent difference between SS and controls, as shown by representative experiments in Fig. 2. These data do not permit evaluation of the influence of radiation on the protein level, since the extracts representing different radiation doses were analysed on separate immunoblotting gels. However, it has been reported that DNA-PK shows no inducibility in response to DNA damage [21].

Enhanced cell cycle arrest due to gamma radiation in SS
At 24, 48 and 72 h after 15 Gy of irradiation, samples were drawn from the B-cell cultures and the DNA content was analysed by flow cytometry in order to determine cell cycle progression.
Normal p53, p21 and ATM protein levels in SS
p53 is known to activate transcription of p21, leading to inhibition of cell cycle progression from the G1 phase. Western blotting of nuclear protein extracts revealed induction of p53 and p21 upon gamma-irradiation in all cells examined; this is in accordance with the observation of G1 cell cycle arrest. ATM expression was unaffected by radiation, in agreement with the reported lack of inducibility of ATM [21]. There was no apparent difference between SS and healthy controls in the protein levels, as shown by a representative experiment in Fig. 5. However, it should be emphasized that this result does not exclude a difference in biological activity of these DNA damage response modifiers; compare our DNA-PK results in Figs 1 and 2 showing no effect on protein level but higher kinase activity in SS cells.

Enhanced apoptosis in SS B cell lines
Early apoptosis was analysed by flow cytometry of the same radiation-exposed B-cell cultures as in the cell cycle experiments, and defined by the binding of annexin V to cells impermeable to propidium iodide. Results from representative experiments are visualized by the annexin V-positive but propidium iodide-negative events of the dot plot in the lower right quadrant (Fig. 6). The controls display a result similar to that reported in the literature for normal individuals, with a pre-irradiation value of approximately 10% apoptotic cells and becoming significantly increased by gamma radiation [23]. Two of the three SS lines differed markedly from this normal pattern even before irradiation, by having high median values for apoptotic cells (24 and 36% respectively) (Table 1). At 24 h after irradiation, the higher apoptosis frequencies were found in the SS cases (medians of 34–71%) than in the controls (33–35%). There was no apparent difference between SS and healthy controls in radiation-induced apoptosis; however, such a comparison is complicated by the very high apoptosis frequency (up to 71%) in irradiated SS cells (Table 1).

Multiple facets of the stress response are enhanced in SS
An overview of the results of the present study is attempted in Table 1, by assigning a ranking number to each normal and SS individual, number 1 denoting the strongest defence reaction. The SS patients hold all the top positions in the pre-irradiation defence functions (DNA-PK kinase activity and apoptosis) as well as in the radiation-induced cell cycle arrest response (Table 1).

Discussion
Human cells express a number of complex and interacting mechanisms that constitute the stress response to DNA damage, including cell cycle arrest, allowing time for enzymatic DNA repair, and apoptosis of severely damaged cells. Some molecular components of these defence functions are expressed constitutively, whereas others become induced upon DNA damage formation. Double-stranded DNA breaks are thought to arise continuously in all cells, due to, for example, environmental radiation and endogenous radical oxygen species. Even a low frequency of this potent damage type, perhaps a single lesion per cell, is capable of inhibiting DNA replication and may threaten cellular homeostasis unless a stress response is evoked [5]. For the present work on the stress response in SS, we decided to focus on B cells and on gamma radiation-induced damage. Double-stranded DNA breaks occur physiologically in B cells during the rearrangement of immunoglobulin genes, and the DNA-PK complex participates in this recombination, not only in the bone marrow but also in mature B cells responding to antigen
Moreover, DNA-PK is essential for the repair of double-stranded DNA breaks by the mechanism of non-homologous end-joining, occurring mainly in G1, and also for the activation of the G1 arrest function mediated by p53 accumulation and p21 gene expression [5].

Accordingly, if a disease (such as SS) is found to have abnormalities in both immune regulation and stress response, we reasoned that the molecular basis may be shared between these two processes, DNA-PK being a likely candidate. EBV-transformed, continuously growing B-cell lines were used, since sufficient peripheral blood B cells could not be obtained from patients. In a previous study, Zeher et al. [26] found that the increased susceptibility to apoptosis of peripheral blood CD4^+ T cells from SS patients correlates with lymphocyte activation. We find it unlikely that our EBV lines, cultivated for several weeks and for more than ten generations in vitro at the time of these experiments, should retain an imprint of in vivo activation status. Therefore, we considered it wise to avoid primary B cells, acknowledging that SS-related in vivo activation of B cells might influence any result in a stress response study.

To what extent is the cellular stress response affected by the status of EBV transformation and its accompanying growth alterations? Infection of human B lymphocytes with EBV in vitro induces a G0 to G1 transition followed by DNA synthesis and cell division. This cell cycle activation closely mimics the antigen-dependent normal B-cell activation pathway and is mediated by the virus-encoded latent membrane protein 1 (LMP1), which is a functional homologue of CD40, and LMP2, which mimics the
survival signal that is usually provided by the B-cell antigen receptor [27]. Significant interaction between EBV and a stress response factor such as p53 or DNA-PK, to the best of our knowledge, has not been demonstrated. Furthermore, G1 arrest function with p53 stabilization and p21 production has been reported in gamma-irradiated EBV lines [23]. Therefore, we consider B-cell lines to be an approximation of normal antigen-stimulated B cells, with no difference to be anticipated between SS patients and controls in activation level. Even though the number of cell lines studied is small, we regard the results as representative of SS since the patients fulfill appropriate criteria for inclusion, and the observed differences between patients and controls were distinct, with no overlapping.

The present study demonstrates an enhanced stress response capacity in SS B-cell lines. Two of the defence functions we assayed were found to be enhanced in undamaged cells: (i) the capacity of DNA-PKcs to become activated in vitro by broken DNA and phosphorylate a p53 peptide; and (ii) apoptosis. The third stress function assayed, G1 cell cycle arrest, depends on the accumulation of p53 being induced by DNA damage. Therefore, our observation of an elevated G1 arrest function illustrates an enhanced capacity in SS cells also in a stress-induced defence function. The evaluation of cell cycle data in proliferating cell lines is complicated by the induction of DNA damage, and possibly also arrest mechanisms, in all cycle phases. For example, a deficient S/G2 arrest may simulate enhanced G1 arrest by the exportation of cells into the next-coming G1, and thereby increase the percentage of G1 cells. However, our interpretation of enhanced G1 arrest in the SS lines is supported by reports of AT cells lacking the ATM stress regulatory protein, leading to deficient cell cycle arrest in response to gamma radiation [28]. EBV lines from AT patients display features which are strikingly opposite to those found by us in SS [22–24]. Thus, radiation causes the percentage of AT cells in G1 to decrease more than in controls, and apoptosis is reduced. Also, in AT blood lymphocytes the frequency of chromosome translocations mediated by V(D)J recombinase is increased [29], whereas we have reported a lack of translocations in SS patients [9]. This comparison with AT supports the notion that the present SS findings reflect hyperactive G1 arrest.

A number of reports on both salivary glands and blood lymphocytes, when combined, may be taken to suggest a constitutional predisposition in SS to over-react when confronted with stress stimuli. These reports include DNA repair alterations [30, 31], apoptosis dysregulation [32, 33], elevated cellular DNA damage response [8], up-regulation of cell cycle inhibition [10], and protein kinase C signalling abnormalities [34]. A rationale for the salivary gland to be a locus minoris resistentiae to stress signals has recently been outlined by Konttinen et al. [35], based on a unique tissue architecture and on continuous exposure to ascending microorganisms and other foreign materials. In support of this view—the salivary gland being a vulnerable organ—experimental evidence of a constitutively high level of stress has been provided by demonstration of DNA strand breaks and DNA repair Ku protein in ductal cells [36], as well as by extremely high radiosensitivity of salivary glands (C. Barlow, personal communication). Consequently, in the salivary gland reactivity to stress and DNA damage seems to be of great significance. Interestingly, our observations of a tendency in SS patients to display a heightened genotoxic response are in agreement with previous reports on the overexpression of wild-type p53 and its transcription factor p21 in labial salivary glands [37, 38].

In summary, our present results suggest that DNA-PK activity is part of a heightened stress response in SS B cells involving cell cycle arrest and apoptosis. Because of a role of DNA-PK also for

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**Table 1. Enhanced stress response functions in SS, illustrated by ranking**

<table>
<thead>
<tr>
<th>Individual</th>
<th>DNA-PK activity</th>
<th>G1 arrest</th>
<th>Apoptosis</th>
<th>DNA-PK activity</th>
<th>G1 arrest</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>5 (57)</td>
<td>n.a.*</td>
<td>4 (5)</td>
<td>n.a.*</td>
<td>4 (24)</td>
<td>3 (31)</td>
</tr>
<tr>
<td>C2</td>
<td>6 (33)</td>
<td>n.a.*</td>
<td>4 (5)</td>
<td>n.a.*</td>
<td>5 (32)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>C3</td>
<td>7 (28)</td>
<td></td>
<td></td>
<td>n.a.*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS1</td>
<td>2 (66)</td>
<td>n.a.*</td>
<td>3 (7)</td>
<td>n.a.*</td>
<td>1 (2)</td>
<td>5 (26)</td>
</tr>
<tr>
<td>SS2</td>
<td>3 (65)</td>
<td>n.a.*</td>
<td>2 (24)</td>
<td>n.a.*</td>
<td>3 (15)</td>
<td>1 (42)</td>
</tr>
<tr>
<td>SS3</td>
<td>4 (58)</td>
<td></td>
<td></td>
<td>n.a.*</td>
<td>2 (12)</td>
<td>2 (35)</td>
</tr>
<tr>
<td>SS4</td>
<td>8 (88)</td>
<td>n.a.*</td>
<td>1 (36)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ranking of all SS patients and control subjects according to the results for stress response functions. The rank number is given in bold type. Figures in parentheses are median values: for DNA-PK activity, the c.p.m. x 1000 for p53 phosphorylation; for cell cycle arrest, the reduction of % cells in G1 phase caused by radiation at 48 h post-incubation; for apoptosis, the percentage of annexin V-positive, propidium iodide-negative cells 24 h after irradiation.

*n.a., not analysed. Cell cycle arrest, by definition, cannot be determined in unexposed cells.

**No induction of DNA-PK activity due to gamma radiation was seen.**

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**Fig. 6. Apoptosis in gamma-irradiated (15 Gy) B cells, analysed by flow cytometry.** Dot plots from representative experiments using unirradiated and 24 h post-incubated cells are shown, each dot representing one cell. Annexin V-positive, propidium iodide-negative (located in the lower right quadrant) are considered to be in apoptosis, whereas double-positive cells (upper right quadrant) are regarded as necrotic (or late apoptotic).
immunoglobulin rearrangement, our findings raise the question of whether the DNA-PK alteration influences B-cell maturation and, thus, the development of autoimmunity features. B-cell activating factor, secreted by macrophages and dendritic cells, has recently become strongly linked to the development of SS in both man and mice [39]. It is therefore intriguing to consider whether an elevated stress response is a primary aetiopathological factor in SS [11], leading to hyper-alert innate immunity, and also to derangement of adaptive immunity, as predicted by the Danger model for lymphocyte activation [40].

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The authors have declared no conflicts of interest.

### References