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Epstein–Barr virus efficiently immortalizes human B cells without neutralizing the function of p53

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Epstein–Barr virus (EBV) efficiently converts resting human B cells into actively cycling, immortal, lymphoblastoid cell lines (LCLs). Here we show that LCLs expressing the full complement of latent viral genes are very sensitive to DNA-damaging agents such as cisplatin. The response includes a rapid accumulation of the tumour suppressor protein p53 and induction of the cellular genes *mdm2* and *WAF1/p21*. Although the levels of Bcl2 protein and Bax mRNA appear unaltered by the activation of p53, within 24 h the majority of cells undergo apoptosis. Over-expression of wild-type p53 in an LCL also resulted in apoptosis; this was preceded by the dephosphorylation of the retinoblastoma gene product, pRb. Primary resting B cells showed no response to cisplatin and even after drug treatment, p53 remained undetectable. However, after infection with EBV, p53 gene expression was induced to a similar level to that found in mitogen-activated B cells. When the physiologically activated primary B cells were exposed to cisplatin, although p53 accumulated as in LCLs, the outcome was growth-arrest rather than gross cell death. We conclude that, in contrast to the transformation of fibroblasts by adenovirus, SV40 or HPV, when B cells become activated and immortalized by EBV they are sensitized to the p53-mediated damage response. When the resulting LCLs are treated with genotoxic agents such as cisplatin, they are unable to arrest like normal cells because they are driven to proliferate by EBV and consequently undergo apoptosis.

Key words: apoptosis/DNA damage/EBV/p53

Introduction

In vitro, EBV can induce the continuous proliferation of normal resting human B cells. The resulting lymphoblastoid cell lines (LCLs), which resemble normally activated B lymphoblasts, express nine latent viral proteins (reviewed, Liebowitz and Kieff, 1993). It is generally assumed that together these proteins are responsible for the activation of the resting B cells, induction of their continuous proliferation and maintenance of the EBV genome in its episomal latent form. It is not known, however, whether any of these proteins function by overcoming the action of cell cycle checkpoint factors such as the tumour suppressor proteins p53 and retinoblastoma gene product, pRb.

Apoptosis or programmed cell death is an active process resulting in cell suicide. It probably evolved as an essential component of developmental and morphogenetic programmes (reviewed by Raff, 1992; Williams and Smith, 1993; Wyllie, 1994). In many, if not all, multicellular organisms apoptosis is also an important cellular response to a damaged genome (Lowe *et al.*, 1993a), inappropriate or conflicting growth signals (Evan *et al.*, 1992; Hermeking and Eick, 1994; Wu and Levine, 1994) and possibly even viral infection (Vaux *et al.*, 1994). The product of the cellular *bcl2* gene is a survival factor and inhibitor of apoptosis (Hockenbery *et al.*, 1990). In some cells Bcl2 is induced by the expression of the EBV latent membrane protein 1 (LMP1) (Henderson *et al.*, 1991; Martin *et al.*, 1993) and the nuclear protein, EBNA2 (Finke *et al.*, 1992). Thus it is thought that EBV, through the expression of its latent cycle genes, can protect infected human B cells from programmed cell death induced when contradictory growth signals are caused by factor withdrawal (Gregory *et al.*, 1991; Henderson *et al.*, 1991). Bax is a Bcl2-related cell protein which was shown to heterodimerize with Bcl2 and accelerate cell death (Oltavi *et al.*, 1993). It has been suggested that the stoichiometry of Bcl2–Bax interactions determines whether cells survive or die following an apoptotic stimulus (Oltavi *et al.*, 1993; reviewed by Wyllie, 1994).

DNA tumour viruses such as SV40, adenovirus and human papillomavirus encode oncoproteins which interact with the tumour suppressor protein p53 (reviewed, Ludlow, 1993; Moran, 1993; Vousden, 1993; Vaux *et al.*, 1994). The function of p53 is suppressed by all three viral oncoproteins: p53 is inactivated by the SV40 T antigen (Mietz *et al.*, 1992; McCarthy *et al.*, 1994) and by the adenovirus E1B p55 protein (Debbas and White, 1993) and is degraded in response to papillomavirus E6 (Scheffner *et al.*, 1990; Mietz *et al.*, 1992). p53 can be an important mediator of apoptosis: normally it is activated and rapidly accumulates in cells responding to DNA damage. In some cell types it induces G_1-arrest but in others it activates the apoptosis pathway (Kastan *et al.*, 1991; Clarke *et al.*, 1993; Lowe *et al.*, 1993a,b). Expression of the adenovirus E1A protein (in the absence of E1B) can sensitize fibroblasts to the p53-mediated apoptosis induced by DNA damage (Lowe and Ruley, 1993; Lowe *et al.*, 1993a). Activation of p53 results in its specific binding to DNA and the transcriptional activation of cellular genes such as *WAF1/p21* (El-diery *et al.*, 1993) and *mdm2* (Chen *et al.*, 1994). WAF1 inhibits cyclin-Cdk function which leads to the dephosphorylation of pRb and

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is consequently responsible for the p53-mediated G_1-arrest seen in fibroblasts (reviewed by Hunter, 1993; Dulic *et al.*, 1994). Mdm2 and its human equivalent bind to and inactivate p53 (Chen *et al.*, 1994). The roles of WAF1, pRb and Mdm2 in apoptosis are unknown. There are reports that Bcl2 expression can induce resistance to p53-mediated apoptosis (Sentman *et al.*, 1991; Wang *et al.*, 1993; Chiou *et al.*, 1994). In myeloid leukaemia cells, p53 differentially regulates Bcl2 and Bax, it represses the former and upregulates the latter (Miyashita *et al.*, 1994).

Various viral proteins interfere with the p53-mediated cellular growth and death programmes and the ability of these viruses to immortalize cells *in vitro* is dependent on these p53-abrogating functions. EBV can very efficiently immortalize resting B cells, can sustain high levels of Bcl2 and also encodes a latent protein, EBNA5 or Leader Protein (LP) which has been reported to bind p53 *in vitro* (Szekely *et al.*, 1993). Following on from these observations we tested the response of B cells, latently infected with EBV, to DNA damage induced by chemotherapeutic agents. The results of this study show that, although EBV protects B cells from apoptosis which would have been activated by the absence of growth or survival factors in serum, it does not appear to block the p53-dependent activation pathway initiated by damage to DNA. Furthermore, during infection, viral gene expression activates transcription of the p53 gene and therefore primes, but does not trigger, the p53-dependent route to cell death. A comparison of LCLs with mitogen-activated primary B cells suggests that, rather than protecting B cells from DNA damage, EBV is actually responsible for the apoptotic response. Finally, because it has recently been shown that the response of proliferating lymphocytes to DNA damage is not solely mediated by p53 (Strasser *et al.*, 1994) we contrived a system where the level of p53 protein could be increased in an LCL without causing significant damage to cellular genomes. In this assay, as in their response to DNA-damaging drugs, the cells showed no evidence of impaired p53 function and rapidly underwent apoptosis when wild-type p53 expression was increased.

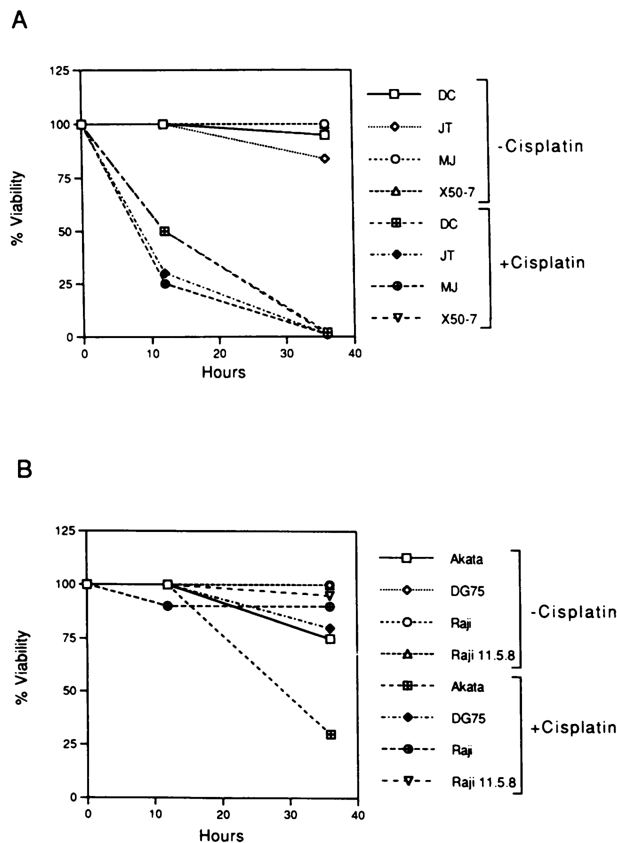

Fig. 1. Viability of LCLs and BL cell lines after treatment with cisplatin. (**A**) Four LCLs were incubated at 37°C in either complete RPMI medium or medium supplemented with cisplatin (10 μg/ml). Viability was assessed by trypan blue exclusion after 12 and 36 h. All the LCLs were immortalized by B95-8 EBV (our unpublished data and Allday *et al.*, 1993). (**B**) Similar samples of four BL cell lines were treated and analysed in the same way as the LCLs. All the BL lines have mutations in their p53 gene: Akata is EBV-positive, expresses only EBNA1 ('group 1 phenotype') and has a 190 frameshift mutation and deleted allele (Farrell *et al.*, 1991). DG75 is EBV-negative and has a 283 Arg-His mutation (K.Wiman, Stockholm, personal communication); Raji is EBV-positive, expresses all the EBV latent genes except EBNA3C and has a 213 Arg-Gln mutation and on the other allele, a Tyr-His at 234 (Farrell *et al.*, 1991; Duthu *et al.*, 1992); Raji 11.5.8 is a subclone of Raji constitutively expressing a transfected EBNA3C gene (Allday et al., 1993).

Results

A consistent finding in preliminary experiments was that LCLs immortalized by EBV, and thus expressing the viral genes essential for growth transformation, were very sensitive to chemotherapeutic agents, such as cisplatin, actinomycin D and adriamycin, all of which damage DNA (Fritsche *et al.*, 1993).

Apoptosis in LCLs

Although all the drugs tested had similar effects, cisplatin was chosen for a detailed study because of its specificity of action as an agent which cross-links DNA, produces DNA-adducts and induces strand breaks (Eastman, 1990; Fritsche *et al.*, 1993). A typical assay for cell viability after treatment with cisplatin at 10 μg/ml is shown in Figure 1A. By 12 h up to 75% of the cells were dead and at 36 h less than a few percent of viable cells could be found in any LCL tested. In parallel experiments, Burkitt's lymphoma (BL) cell lines carrying mutant p53 genes (see legend to Figure 1) appeared relatively resistant to similar doses of the drug (Figure 1B). This observation is consistent with reports that thymocytes from p53-null, transgenic mice and T cells expressing SV40 T antigen are resistant to DNA damage (Clarke *et al.*, 1993; Lowe *et al.*, 1993b; McCarthy *et al.*, 1994).

In order to determine more precisely the fate of the lymphoblastoid cells, they were analysed by flow cytometry and fluorescence microscopy after 24 h of drug treatment. Representative cell cycle profiles of cells stained with propidium iodide are shown in Figure 2A and B. The profile of the untreated proliferating cells (Figure 2A) has the expected distribution of cells in all phases of the cell cycle. The cells incubated with cisplatin for 24 h (Figure 2B) mostly have a DNA content of less than the diploid, G_0/G_1 peak; this is consistent with nuclear fragmentation. Untreated cells, stained with acridine orange and viewed under a fluorescence microscope, appeared as smooth, intact spheres (Figure 2C) whereas the cisplatin-treated cells showed the chromatin condensa-

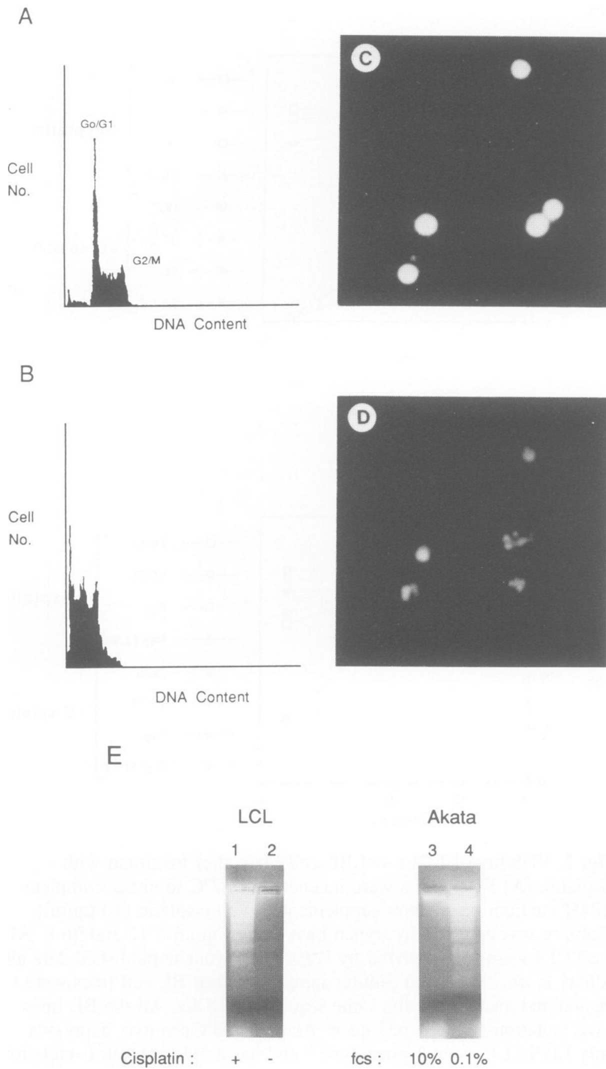

Fig. 2. Apoptosis in a representative LCL. (**A**) Cell cycle profile from flow cytometric analysis of actively proliferating DC-LCL cells. (**B**) Similar analysis of the LCL after 24 h treatment with cisplatin (10 μg/ml). (**C**) Morphology of acridine orange stained LCL cells from an actively proliferating culture. (**D**) Acridine orange stained DC-LCL cells after a 24 h treatment with cisplatin (10 μg/ml) showing cells with chromatin condensation and nuclear fragmentation. (**E**) Agarose gel analysis of DNA fragmentation in apoptotic cells. The soluble DNA fraction from 10^7 cells was separated on 1.2% agarose; lane 1, DNA from DC-LCL treated with cisplatin (10 μg/ml) for 24 h and lane 2, from untreated cells. As a positive control for oligonucleosomal fragmentation the 'group 1' BL line Akata was induced to undergo apoptosis by reducing the serum concentration in the growth medium from 10% (lane 3) to 0.1% (lane 4).

tion and nuclear fragmentation typical of apoptotic death. To confirm that the LCL cells were dying by apoptosis, soluble DNA was extracted from normal and treated cells and analysed by agarose gel electrophoresis; the characteristic oligonucleosomal fragmentation is seen only in the extract from drug-treated LCLs (e.g. see Figure 2E, lane 1). BL-derived cells analysed in the same way as the LCLs showed little or no evidence of apoptosis (data not included) or even significant growth-arrest (Figure 3A–D). However, the BL-derived line Akata (Takada and Ono, 1989), which has the 'group I' phenotype characteristic of BL biopsy specimens (Rowe *et al.*, 1987; Farrell *et al.*, 1991) was shown to undergo apoptosis upon reduction of fetal calf serum in the growth medium (Figure 2E, lane 4). This is consistent with previously published results (Gregory *et al.*, 1991; Henderson *et al.*, 1991).

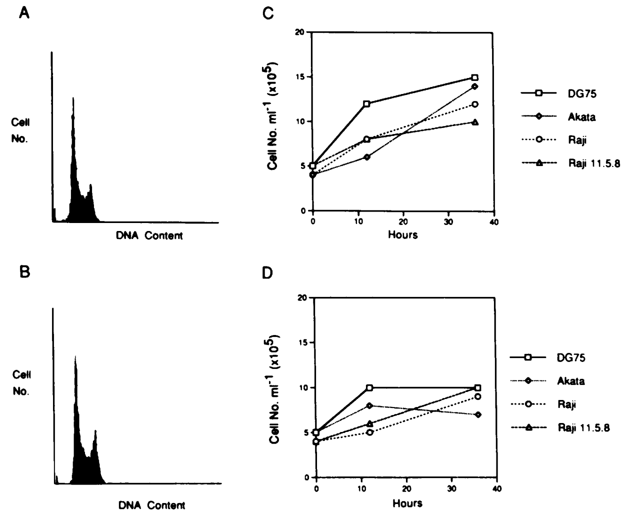

Fig. 3. The effect of cisplatin on BL cell lines. Cell cycle profiles from flow cytometric analysis of the DG75 BL cell line (**A**) before and (**B**) after 24 h treatment with cisplatin (10 μg/ml). Growth kinetics of the four BL cell lines (described in the legend to Figure 1) over 36 h in the absence (**C**) and presence (**D**) of cisplatin.

Induction of transcriptionally active p53 in LCL by DNA-damaging drugs

Functional p53 is necessary in mouse thymocytes for apoptosis induced by DNA damage. The BL-derived cell lines (which carry p53 lesions) failed to respond to DNA-damaging drugs in the same way as LCLs. This variation in response to DNA damage led us to examine the steady-state levels of p53 protein in all the lines investigated. Consistent with the findings in thymocytes and other cell types, cisplatin caused a dramatic accumulation of p53 protein in the LCLs (Figure 4, lanes 1–8). This was presumably due to post-translational modification resulting in increased protein stability (reviewed by Lane, 1994). Experiments using other chemotherapeutic drugs, for example actinomycin D and adriamycin (doxorubicin), resulted in the accumulation of p53 with similar kinetics (our unpublished data). These results are similar to published reports describing the action of these drugs on other cell types and consistent with the response in B cells being initiated by DNA damage (Fritsche *et al.*, 1993; Lowe *et al.*, 1993a; Strasser *et al.*, 1994). The BL cells either expressed high constitutive levels (Figure 4, lanes 11–16) or were negative for p53 protein (see Akata in lanes 9 and 10 and Farrell *et al.*, 1991).

Time course studies revealed that in the LCLs, an increase in p53 can be detected within an hour of adding the drug and it continues to accumulate for at least 8 h. Figure 5A shows a representative Western blot of such an experiment. It also shows that during the 8 h period the levels of detectable Bcl2 and the EBV LMP1 protein remain constant. Human Mdm2 protein is induced concurrently with the stabilization of p53; this induction indicates that the p53 is transcriptionally active.The observation that Mdm2 was not induced in the p53-null cell line Akata

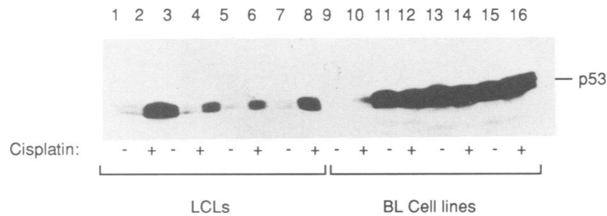

Fig. 4. Induction of p53 by cisplatin. Western blot analysis of protein extracts from LCL and BL cells treated with cisplatin (10 μg/ml) showing relative level of p53. Lanes 1 and 2 contain DC-LCL; lanes 3 and 4, JT-LCL; lanes 5 and 6, MJ-LCL; lanes 7 and 8, X50-7; lanes 9 and 10, Akata; lanes 11 and 12, DG75; lanes 13 and 14, Raji; lanes 15 and 16, Raji 11.5.8.

(data not shown) is consistent with the induction in the LCLs specifically requiring p53 activation. The induction of *WAF1* mRNA during a similar 8 h period of treatment (Figure 5B) provides further evidence for p53 being transcriptionally active. During various time-course experiments samples were taken and examined under the microscope—these revealed small numbers of cells with the morphological characteristics of apoptosis (as described in Figure 2D) appearing in <4h.

The balance between Bcl2 and Bax appears unaltered by DNA damage

The rapid onset of apoptosis in the (EBV-immortalized) LCLs and the constant level of Bcl2 found in these cells prompted us to determine whether the level of Bax, its partner in heterodimeric complexes and its functional antagonist, was affected by p53 activation. Since it has been suggested that in some cells p53 can induce the expression of *bax* mRNA (Miyashita *et al.*, 1994) a Northern blot of total RNA from various LCL and BL cells was probed with a Bax-specific cDNA (Figure 5C). There was no increase in the steady-state level of Bax mRNA species after cisplatin treatment. We assume the two RNA species to which the purified probe hybridizes correspond to the two differentially spliced messages of 1.0 and 1.5 kb which are found in mouse cells (Oltavi *et al.*, 1993)

p53 expression in primary B cells

To compare the kinetics of p53 accumulation in EBV-immortalized cells with normal, resting B cells, the usual target of EBV infection, a purified population of human, peripheral blood, B lymphocytes was incubated with and without cisplatin. Western blot analysis showed that although protein extracts from 2×10^6 cells were used, no p53 could be detected, even after 8 h treatment with cisplatin (Figure 6A, upper panel). Prolonged exposure of the filter to X-ray film failed to elicit a signal corresponding to p53 (Figure 6A, lower panel). From reconstruction experiments (not shown) we estimate that the steady-state level of p53 in primary B cells is at least 40-fold less than in LCL cells. The accumulation seen in the LCLs after 8 h of exposure to cisplatin is at least 10- to 20-fold greater than basal expression. Bcl2 was readily detectable in the primary cells, which is consistent with previous reports (see Martin *et al.*, 1993). Similar results were obtained when total peripheral lymphocytes were analysed for their expression of Bcl2 and p53 (data not shown).

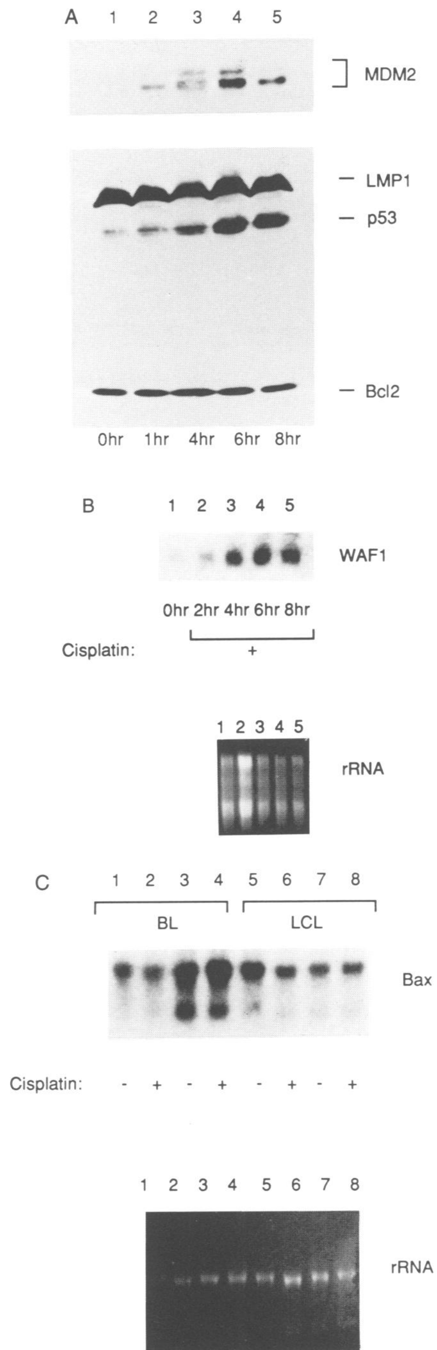

Fig. 5. (**A**) Induction of MDM2 while the Bcl2 and LMP1 levels remain unaltered. Western blot analysis of the X50-7 LCL treated with cisplatin (10 μg/ml) and sampled over an 8 h time course.The blot was probed with a cocktail of mAbs recognizing Bcl2, p53, EBV LMP1 and MDM2. A film was exposed to the filter for about six times longer to produce the MDM2 autoradiograph. (**B**) Induction of WAF1/p21 mRNA. A Northern blot was performed on total RNA extracted from X50-7 LCL cells 0, 2, 4, 6 and 8 h after the addition of cisplatin (10 μg/ml): the filter was probed with a cDNA clone corresponding to human *WAF1*. The ethidium bromide stained gel, with rRNA bands, is also shown. (**C**) The level of Bax mRNA is unaffected by p53 activation. A Northern blot was performed on total RNA extracted from various LCL and BL cells after they had been incubated with or without cisplatin (10 μg/ml) for 8 h: the filter was probed with a cloned cDNA corresponding to human *Bax* . Lanes 1 and 2, DG75; lanes 3 and 4, Raji; lanes 5 and 6, X50–7 LCL; lanes 7 and 8, DC-LCL. The ethidium bromide stained gel is also shown.

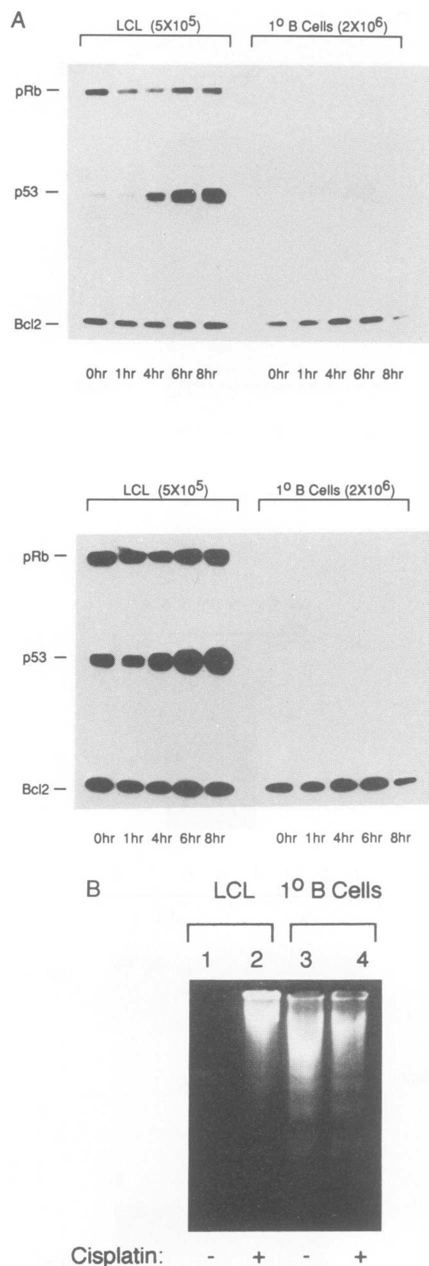

Fig. 6. p53 is not detected in primary resting B cells. (**A**) Upper panel: Western blot of total protein extracts from X50–7 LCL (~5×10^5 cells) and primary resting B cells (~2×10^6 cells). Samples were taken 0, 1, 4, 6 and 8 h after treatment with cisplatin; the blot was probed with a cocktail of mAbs recognizing Bcl2, p53 and pRb. After development using an ECL system (Amersham) the filter was exposed to X-ray film for 30 s. Lower panel: after exposing the filter to film for more than 12 h still no signal corresponding to p53 was detected in the extracts from primary cells. (**B**) Agarose gel analysis of DNA fragmentation in cultured primary B cells. An LCL and primary cells purified as described in Materials and methods were incubated with or without cisplatin at 10 μg/ml for ~26 h. The soluble DNA fraction from 10^7 cells was separated on 1.2% agarose: lane 1, untreated X50-7, LCL; lane 2, cisplatin-treated X50-7; lane 3, untreated primary B cells; lane 4, cisplatin-treated primary B cells.

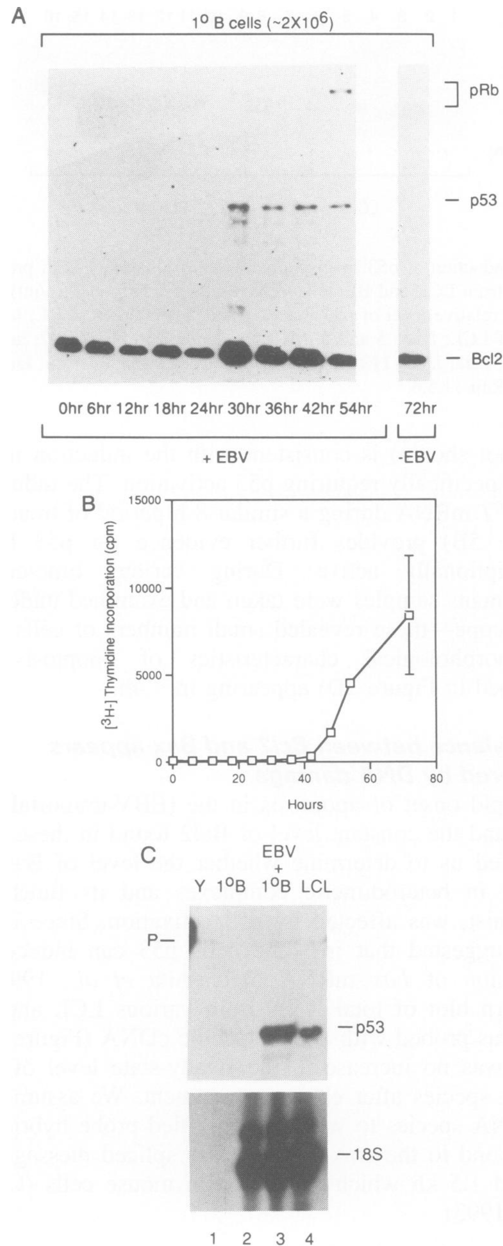

Fig. 7. (**A**) Induction of p53 after the infection of primary B cells with EBV. Western blot of extracts from ~2×10^6 resting peripheral blood B cells made at the times after infection with B95.8 EBV as indicated. The filter was probed with the Bcl2/p53/pRb cocktail of mAbs. An extract from uninfected cells after 72 h in culture is also shown. Due to a sampling error the 30 h lane contains two to three times as much total protein. (**B**) Cumulative [^{3}H]thymidine incorporation at the times shown after infection of primary resting B cells with B95-8 EBV. The experiment was performed in triplicate and the standard error is indicated where it is large enough to register on the scale used. (**C**) RNase-protection analysis shows that EBV infection of resting primary B cells induces p53 gene expression. Lane 1, yeast RNA; lane 2, uninfected primary B cells; lane 3, primary B cells infected with the B95-8 strain of EBV; lane 4, an LCL established using B95-8 virus. The upper panel shows the bands corresponding to the protected p53-specific fragments (260 bp) and the lower panel shows 18S RNA (80 bp protected fragments) The position of the undigested p53-specific probe (P) is indicated.

Apoptosis in primary B cells

As primary B cells contain little or no p53, we determined whether this largely resting (G_0) population would respond to cisplatin as LCLs do and undergo apoptosis. Experiments were performed on purified, peripheral B cells to determine whether DNA fragmentation occurred in response to cisplatin. However, the results were uninformative in this respect because the oligonucleosomal laddering

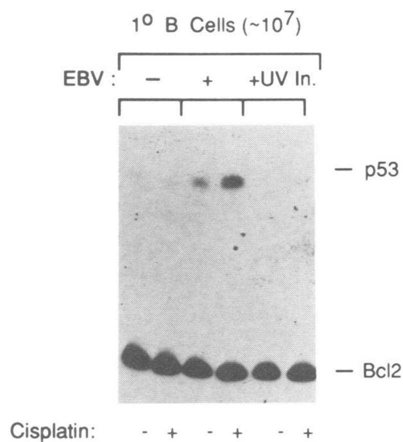

Fig. 8. Induction of p53 and sensitization to DNA damage after the infection of resting B cells with B95-8 EBV. Approximately 10^7 primary B cells were infected with EBV, UV-inactivated EBV (UV in.) or left untreated. After 24 h incubation cisplatin (10 μg/ml) was added and following a further 8 h incubation protein extracts were made and Western blotted. The filter was probed with a mixture of the Bcl2 and p53-specific mAbs.

was evident whether or not cisplatin was added (Figure 6B, lanes 3 and 4). Apoptosis clearly occurs in a subpopulation of these cultured primary cells irrespective of whether DNA damage was sustained. As p53 is undetectable in these cells, we assume that here apoptosis is p53-independent and induced by the lack of survival factors and/or cell–cell contact in the culture; it is not augmented by cisplatin treatment. As expected, an LCL released soluble, fragmented DNA only when cisplatin was added (Figure 6B, lane 2 and also Figure 2E, lane 1).

p53 expression during the infection of primary B cells with EBV

LCLs express a low but readily detectable basal level of p53, whereas primary resting B cells do not. Purified peripheral B cells were therefore infected with the immortalizing B95-8 strain of EBV and protein extracts were made from 2×10^6 cells at intervals post-infection. Western blotting and probing for p53 showed that p53 protein becomes detectable ~30 h after infection, and thereafter is maintained at a constant level (Figure 7A). The level of Bcl2 also increases at this time. The first EBV antigens to be expressed, EBNA-LP and EBNA2, were detected in the extracts taken 18–24 h after infection (data not shown and Sinclair *et al.*, 1994). This confirms that infection by EBV has occurred and is consistent with the report that EBNA2 can upregulate Bcl2 (Finke *et al.*, 1992). p53 remains undetectable in uninfected cells cultured for 72 h, showing that the increased expression was specific for EBV infection. S-phase of the cell proliferation cycle, operationally defined by a significant increase in the incorporation of thymidine, did not start in EBV-infected primary B cells until at least 40 h after infection (Figure 7B; Alfieri *et al.*, 1991; Sinclair *et al.*, 1994). The induction of p53 expression associated with EBV must therefore take place in the middle of the G_1-phase while the cells are still being activated and undergoing blast-transformation.

RNase-protection analysis performed on total RNA extracted from primary B cells, 24 h after infection with

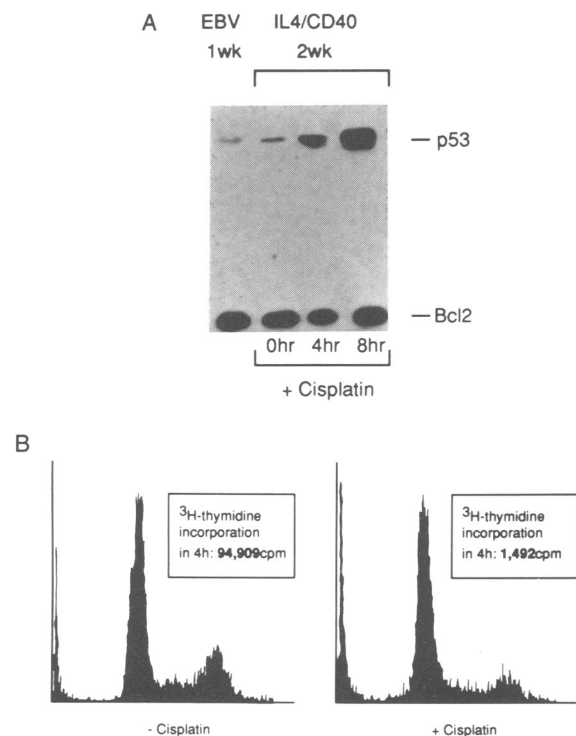

Fig. 9. p53 and the DNA damage response in mitogen-activated primary B cells. (**A**) p53 expression in EBV-infected cells is similar to that in mitogen-activated B cells. Primary B cells were activated and induced to proliferate using recombinant IL4 and a monoclonal antibody to CD40 (Banchereau *et al.*, 1991). After 2 weeks in culture the cells were treated with cisplatin (10 μg/ml), samples were taken at the times shown and protein extracts were analysed by Western blotting. The filter was probed with a mixture of mAbs to p53 and Bcl2. An extract from primary B cells infected with B95–8 EBV and harvested 1 week post-infection is shown for comparison. Each lane contains protein extract from $\sim5\times10^5$ cells. (**B**) Mitogen-activated primary B cells undergo growth-arrest in response to cisplatin. Cells from a second donor were cultured for 1 week in IL4 and anti-CD40 monoclonal antibody, divided into two flasks and cisplatin (10 μg/ml) was added to one. After 26 h a sample of each was taken and pulsed with [^{3}H]thymidine for 4 h — the results of liquid scintillation counting the precipitable DNA are shown. After 30 h the cultures were harvested and cell cycle analysis was performed by flow cytometry. The profiles show cells distributed in all phases of the cell cycle regardless of whether cisplatin was present.

EBV and from an LCL showed that the increase in p53 seen after infection (Figure 7A) is (largely) due to transcription of the p53 gene followed by *de novo* protein synthesis (Figure 7C, cf. lanes 2 and 3).

Superinduction of p53 in cells activated by EBV

EBV caused p53 expression ~30 h after infection of primary B cells. We tested whether the p53 which was induced by EBV was responsive to cisplatin and whether UV-inactivated virus (i.e. viral DNA alone) could also initiate p53 synthesis in resting cells. The results of Western blotting are illustrated in Figure 8; they show that, even when protein extracts from 10^7 cells are loaded, p53 is still undetectable in primary cells which have not been infected with EBV. Similarly, it is undetectable after cells were infected for 32 h with UV-inactivated virus. However, p53 is clearly detectable ~32 h after infection of cells with B95–8 EBV and when an equivalent sample of this culture was treated between 24 and 32 h with

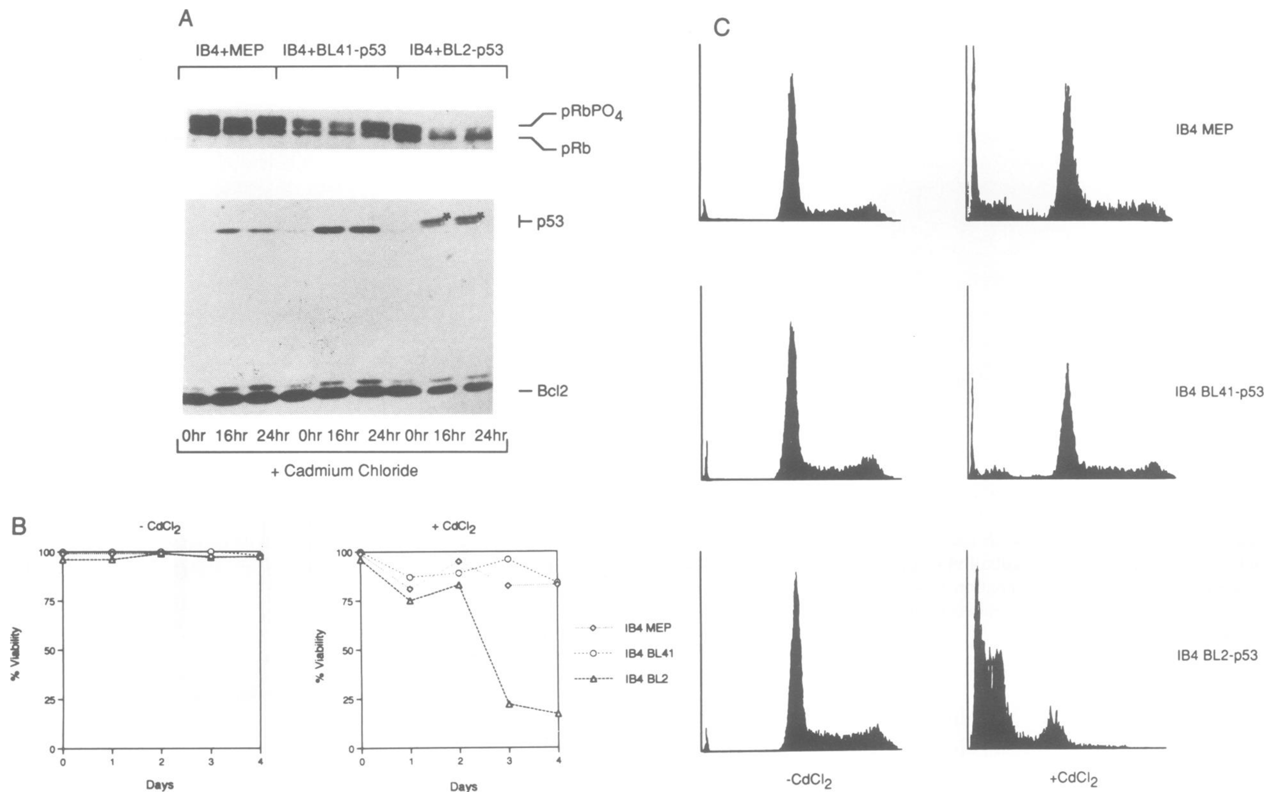

Fig. 10. Induction of apoptosis in an LCL by the overexpression of wild-type p53. (**A**) The lower panel shows a Western blot of protein samples from three lines of stably transfected IB4 cells taken at the times indicated after treatment with $CdCl_2$ (5 μM). The filter was probed with a mixture of mAbs to p53 and Bcl2. The BL2-derived p53 is indicated (*); this molecule has a slightly slower mobility relative to the endogenous species due to the polymorphism at amino acid 72 (see Farrell *et al.*, 1991). The slight reduction in the level of Bcl2 after 16 and 24 h reflects a reduction in the total protein loaded. This also results in an under-representation of the accumulated p53. The upper panel shows a similar western blot of samples from the same cells run on a lower percentage polyacrylamide gel (7.5%) in order to resolve the differentially phosphorylated forms of pRb. The filter was probed with an anti-Rb monoclonal antibody. (**B**) The viability of the various IB4 cells was determined each day after induction with $CdCl_2$ (5 μM). The percentage viability was estimated by trypan blue exclusion — it therefore merely reflects the integrity of the cytoplasmic membrane. (**C**) Cell cycle profiles from flow cytometric analysis of samples of the IB4 cells (described above) taken 48 h after induction with $CdCl_2$. The p53 gene derived from BL2 has a wild-type DNA sequence; the gene derived from BL41 has an Arg(248)-Gln mutation (Farrell *et al.*, 1991).

cisplatin, the level of p53 protein is further induced, presumably as a result of modification and stabilization.

The response to cisplatin of primary B cells cultured on IL4 and anti-CD40

The steady-state level of p53 protein in primary B cells 1 week after infection with EBV was compared with that in mitogen-activated primary B cells. Figure 9A shows a Western blot of protein extracts from human, peripheral B cells activated and induced to proliferate for 2 weeks by IL4 and anti-CD40 monoclonal antibody (Banchereau *et al.*, 1991). The amount of p53 is essentially similar irrespective of the method of activation and consistent with EBV infection mimicking the physiological activation of B cells. When the cells maintained on IL4/anti-CD40 were treated with cisplatin (10 μg/ml) they accumulated p53 with similar kinetics to an LCL (compare Figure 9A with Figures 5A and 6). In further experiments, cell cycle analysis was performed on primary B cells driven to proliferate by IL4/anti-CD40 after they had been treated with cisplatin for 30 h. Surprisingly, unlike the LCLs treated in a similar way, although p53 induction was as expected (data not shown), relatively little apoptosis was seen (compare Figure 9B with Figure 2B). Viability was verified by microscopical examination. Most of the cells (>75%) are distributed throughout the cell cycle. [^{3}H]thymidine incorporation was determined between 26 and 30 h after the addition of cisplatin. The presence of the drug resulted in a >60-fold reduction in thymidine incorporation compared with the control culture; very few, if any, cells entered S-phase of the cell cycle. The response to cisplatin is thus growth-arrest rather than apoptosis.

Apoptosis induced by overexpression of wild-type p53

It has recently been reported that damage to DNA can induce apoptosis in proliferating lymphoid cells by both p53-dependent and independent mechanisms (Strasser *et al.* , 1994). Therefore, to examine more precisely p53-mediated apoptosis in B cells latently infected with EBV, plasmids expressing p53 genes under the transcriptional regulation of the metallothionein promoter (Vousden *et al.*, 1993) were introduced into, and stably maintained in, the LCL, IB4. Three representative lines of transfected cells, one expressing wild-type p53 (derived from BL2), one expressing a mutant (derived from BL41) and one carrying the empty MEP4 vector, were treated with $CdCl_2$ (at 5 μM). Consistently, within 8 h there was a detectable

increase in the expression of exogenous p53 (not shown). The level of protein then remained constant for at least 24 h (Figure 10A, lower panel). In addition, it can be seen that the $CdCl_2$ treatment induced a modest but significant increase in the endogenous wild-type p53 (see for example IB4-MEP, and the lower p53 band in IB4-BL2). When the viability was determined daily for 4 days after induction with $CdCl_2$, the MEP4- and mutant-p53 (BL41)-carrying lines showed only a slight drop in the number of viable cells (to ~80%), however, those expressing the wild-type BL2-p53 rapidly lost viability and by 3 days only ~20% of cells were able to exclude trypan blue (Figure 10B). These results were reflected in the flow cytometric cell cycle analysis of samples taken at 48 h after induction (Figure 10C). DNA from the BL2-p53-transfected cells consistently produced profiles consisting of largely sub-diploid fragments characteristic of apoptosis (compare with the cisplatin-treated LCL in Figure 2B). Microscopical examination and also agarose gel electrophoresis of 'Hirt'-extracted DNA both revealed very marked nuclear and oligonucleosomal fragmentation (data not shown). Athough there was evidence of some cell death in the two control lines of IB4 (MEP4 and BL41), most of the cells can be seen distributed in all phases of the cell cycle.

Further Western blot analysis of the protein extracts which showed p53 induction by $CdCl_2$ (Figure 9A, lower panel) revealed that in the BL2-p53-expressing cells, the pRb protein is dephosphorylated prior to, or simultaneously with, activation of the apoptotic pathway (Figure 9A, upper panel).

Discussion

This investigation has shown that LCLs immortalized by EBV are very sensitive to DNA-damaging chemotherapeutic agents.They respond with a rapid commitment to apoptosis. Prior to cell death there is a substantial accumulation of p53 tumour suppressor protein. Increases in the expression of the p53-responsive genes MDM2 and WAF1/p21 are consistent with the p53 being translationally modified in response to drug-induced DNA damage, becoming stabilized and transcriptionally activating these two cell genes. LCLs express no EBV function which significantly represses p53 accumulation or this trans-activating capacity. Although EBNA-LP has been shown to bind to p53 (Szekely *et al.*, 1993) this study provides no evidence that it (or any other EBV latent protein) interferes with or alters p53 function in cycling B cells.

We have also shown by sensitive assays for both protein and mRNA that resting primary B cells are essentially p53-negative. Some of these cells spontaneously undergo apoptosis when they are placed in culture, presumably due to a lack of growth/survival factors. This appears to be a p53-independent process. When EBV infects resting B cells it rescues them from this spontaneous cell death but it also stimulates p53 synthesis by activation of the p53 gene. This action seems to mimic the normal activation of B cells into the proliferation cycle and does not result in an abnormal accumulation of p53 protein. The induction requires viral gene expression and is therefore unlikely to be a response to viral DNA. In this respect the p53 gene resembles a number of cellular genes including *CD23*, c-*fgr*, c-*myc* and cyclin D2 (Alfieri *et al.*, 1991; Sinclair *et al.*, 1994). The expression of these diverse genes presumably reflects the transition of cells from G_0 into the cell cycle. Linear EBV DNA which was introduced from virions [and exists in cells prior to the ends linking to form covalently closed circles (Hurley and Thorley-Lawson, 1989)] is not interpreted by cells as a damaged genome and does not provide an adequate stimulus for the induction of p53.

Perhaps the most surprising result from this study is that 'normal' peripheral B cells cultured in IL4 and anti-CD40 do not respond in the same way as LCLs to a similar degree of DNA damage. Although p53 accumulates with similar kinetics they clearly do not apoptose to any significant degree, they do, however, cease DNA synthesis. In this respect they more closely resemble normal diploid fibroblasts than LCLs.

Cells expressing EBV genes show some resistance to apoptosis induced by factor withdrawal or calcium ionophores (Gregory *et al.*, 1991; Henderson *et al.*, 1991) but they are very sensitive to DNA-damaging drugs. It is widely accepted that this latter pathway is largely mediated by p53 (reviewed in Lane, 1994). The accumulation of p53 in LCLs treated with cisplatin is apparently normal, however, since DNA damage can initiate apoptosis in proliferating lymphoid cells in the absence of p53 (Strasser *et al.*, 1994) we have shown that over-expression of wild-type p53 from extrachromosomal plasmids can also induce apoptosis in an LCL. In addition these experiments showed that the pRb protein becomes dephosphorylated in LCL cells which are proceeding towards programmed cell death. This is consistent with transcriptional activation of *WAF1* by p53 (which is also seen in the cisplatin-treated cells in Figure 5B) which causes the inhibition of cyclin/Cdk-mediated phosphorylation of pRb. In normal fibroblasts this results in G_1-arrest because cells in which pRb is largely hypophosphorylated cannot proceed into S-phase (reviewed, Hunter, 1993; Dulic *et al.*, 1994). If the E1A protein (Lowe and Ruley, 1993; Lowe *et al.*, 1993a), cMyc (Hermeking and Eick, 1994) or the transcription factor E2F (Wu and Levine, 1994) are constitutively expressed in fibroblasts and a conflict of signals is created, for instance, if those cells sense they should growth-arrest, then the outcome is apoptosis. The data presented here, therefore, suggest that EBV may deregulate c-*myc* or possibly encode an E1A-like function which generates high levels of transcriptionally active E2F.

In summary, by activating resting B cells EBV stimulates the synthesis of p53; this is not, however, triggered by viral DNA alone. No EBV-encoded protein(s) appear to interfere with the activation of p53 in response to DNA damage or its function as a mediator of apoptosis. The presence of EBV in these cells may actually be providing the proliferative drive which produces apoptosis rather than cell cycle-arrest. In this respect, EBV differs fundamentally from the adeno- papilloma- and SV40 tumour viruses which encode proteins that directly inhibit p53 function and thereby block growth-arrest and/or apoptotic responses which normally occur in response to DNA damage (Rao *et al.*, 1992; Debbas and White, 1993; Kessis *et al.*, 1993; McCarthy *et al.*, 1994). These viruses appear to depend absolutely on the abrogation of p53 function in order both to immortalize cells in culture and produce tumours *in vivo*

(reviewed by Ludlow, 1993; Moran, 1993; Vousden, 1993). EBV, on the other hand, may have evolved a unique strategy to induce the activation of resting B cells into the proliferation cycle without disrupting the function of this major regulator of cell cycle progression and mediator of cell death. There is no evidence that EBV in a latent infection encodes an equivalent of the E1B p55 protein, E6 or T antigen; there is no reason, therefore, to expect the type of genomic instability associated with these oncoproteins (Lane, 1994; McCarthy *et al.*, 1994) in cells immortalized by EBV.

Materials and methods

Cell culture, DNA-damaging agents and viability assays

Cells were maintained at 37°C in RPMI medium with 10% fetal calf serum. Cisplatin (David Bull Laboratories, Warwick, UK), actinomycin D (Merck, Sharp and Dohme Ltd, Hoddesdon, UK) and adriamycin (Sigma, Poole, UK) were used at concentrations of 10 μg/ml, 2 μg/ml and 0.5 μg/ml respectively (Fritsche *et al.*, 1993; Lowe *et al.*, 1993a; Strasser *et al.*, 1994). Cell viability was assessed by trypan blue exclusion.

Flow cytometry and fluorescence microscopy

Cell cycle analysis by flow cytometry was performed as previously described (Allday and Farrell, 1994). Actively proliferating cells or cells treated with DNA-damaging drugs were also stained with acridine orange (5 μg/ml Sigma) for ~30 min and visualized by fluorescence microscopy to assess their morphology.

DNA fragmentation assays

The DNA fragmentation assay was essentially a modified 'Hirt' extraction: briefly ~10^7 cells were harvested, washed in PBS and lysed in 5 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 0.5% Triton X-100 and 1 mM PMSF at room temperature for 15 min. The lysate was then spun for 20 min at 16 000 *g* in a microfuge. The supernatant containing the soluble DNA was removed and, after extraction with phenol/chloroform, ethanol precipitated. The precipitated DNA was resuspended in TE pH 8.0 containing RNase A (100 μg/ml) and separated by electrophoresis through a 1.2% agarose gel.

Antibodies and Western blotting

Total protein extracts from the number of cells indicated in each experiment were solubilized in SDS-sample buffer, boiled and separated in 7.5 or 10% SDS–polyacrylamide gels and transferred to nitrocellulose as described previously (Allday *et al.*, 1993) They were probed with the following monoclonal antibodies (mAbs) an anti-p53 mAb DO1 (Vojtesek *et al.*, 1992), an anti-LMP1 mAb S12 (Mann *et al.*, 1985), an anti-Bcl2 (Dako UK Ltd, High Wycombe, UK), an anti-MDM2 mAb SMP14 (Picksley *et al.*, 1994) and an anti-Rb mAb (Pharmingen, San Diego, CA, USA). The visualization process using an antibody sandwich and ECL (Amersham International, UK), was performed essentially as described previously (Allday *et al.*, 1993).

Northern blotting

Total RNA was extracted from cells by the phenol/guanidinium thiocyanate method (RNAzol™ Biogenesis Ltd, Bournemouth, UK). Approximately 20 μg of each RNA sample were loaded and separated in formaldehyde–1% agarose gels and transferred to nitrocellulose. The filters were hybridized under standard conditions to gel purified cDNA probes labelled with ^{32}P by 'random priming' (Amersham)

Cell culture and purification of primary B lymphocytes

Primary B lymphocytes were purified from human peripheral blood purchased from North London Blood Transfusion Service. Buffy coats were subjected to centrifugation over Ficoll paque gradients (Pharmacia LKB, Uppsala, Sweden) and the CD19-positive lymphocytes were immunoselected with pan-B Dynal M450 beads (Funderud *et al.*, 1990). After release from the beads with Detachabead (Dynal, New Ferry, UK), the cells were cultured in RPMI 1640 supplemented with penicillin, streptomycin and 15% heat inactivated fetal calf serum at a density of 1×10^6 cells/ml for 16 h prior to use.

Purification of B95-8 EBV virus

EBV was purified from B95-8 cells which had been treated with 30 ng/ml TPA for 6 days. Following centrifugation at 16 000 *g* for 5 min and filtration of the medium through a 0.45 mm filter (Nalgene, Europe Ltd, Rothewas, UK), the virus was pelleted by centrifugation at 141 000 *g* for 60 min. The pellet was washed carefully to remove any traces of TPA, re-pelleted, resuspended in 1/50th the original volume of medium and stored at −80°C. Where indicated, virus stocks were inactivated by pre-treatment with 9 mJ of UV radiation with a Stratalinker (Stratagene Cloning Systems, La Jolla, CA, USA).

Thymidine incorporation assays

Samples of 10^6 cells were incubated in medium containing 1 mCi of [^{3}H]thymidine and harvested at various times after infection by EBV. The cells were lysed in 100 ml of water, precipitated with 10% TCA and the precipitated material collected on a GF/C filter (Whatman). Assays were performed in triplicate and the incorporation of [^{3}H]thymidine was quantitated by liquid scintillation counting. For the analysis of the IL4/anti-CD40 cultures, 3× 100 μl aliquots were removed from each culture 26 h after the addition of cisplatin and placed in round bottom wells. 1 μCi of [^{3}H]thymidine was added to each well. After 4 h incubation at 37°C cells were harvested and analysed as described above.

RNase-protection assays

The vector pGEM4Zp53 (Farrell *et al.*, 1991) was linearized with the restriction enzyme *Ssp*I and T7 polymerase was used to synthesize a 325 bp transcript. The 18S vector (Ambion Inc., Austin, TX) was used in a similar manner to generate a 109 bp transcript. The protection assay was performed as described previously (Sinclair *et al.*, 1994) using 10 μg of total RNA in each reaction.

IL4/anti-CD40 cell culture

E-rosette-negative, peripheral B cells were resuspended at 2×10^6 cells/ml in RPMI-1640 containing 10% fetal calf serum and antibiotics.The cells were cultured in 25 cm flasks on a feeder layer of X-irradiated (5000 rads) Lmtk$^-$CDw32 cells (kindly provided by Dr K.Moore, DNAX, Palo Alto, CA) with recombinant IL4 (R&D Systems Inc., Abingdon, UK) and anti-CD40 monoclonal antibody (1 μg/ml, Dako UK Ltd). Cells were harvested for analysis after shaking free from the adherent irradiated feeder cells.

Establishment of IB4/p53 transfectants

The p53 cDNAs cloned into pMEP4 have been described (Vousden *et al.*, 1993). Plasmid DNAs were electroporated and hygromycin-resistant lines selected as described (Lau *et al.*, 1992). The cells were maintained in RPMI-1640 containing 10% fetal calf serum and 0.3 mg/ml hygromycin B. p53 expression was induced with cadmium chloride at a final concentration of 5 μM.

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