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Differential Responses to CD40 Ligation Among Burkitt Lymphoma Lines That Are Uniformly Responsive to Epstein-Barr Virus Latent Membrane Protein 1

Nico V. Henriquez,* Eike Floettmann,† Mike Salmon,‡ Martin Rowe,‡ and Alan B. Rickinson2*

Ligation of CD40 on the surface of B cells induces multiple phenotypic effects, many of which are mimicked by the EBV latent membrane protein 1 (LMP1) through its interaction with downstream components of the CD40 signaling pathway. Because the effects of LMP1 have been most closely studied in human Burkitt Lymphoma (BL) cell lines retaining a tumor biopsy-like phenotype in vitro, we have examined the response of a panel of such lines to CD40 ligation. Two distinct patterns of response were observed that were unrelated to the surface level of CD40 or to the EBV genome status of the lines. Following exposure to either CD40-specific mAbs or the soluble trimeric ligand (sCD40L), high responder (HR) lines showed rapid aggregation, activation of NF-κB, up-regulation of cell surface markers ICAM-1/CD54 and Fas/CD95, and growth inhibition. Aggregation was seen at lower doses than those required to elicit the other effects. By contrast, low responder (LR) lines showed no detectable response to CD40 mAbs, while their responses to sCD40L were limited to activation of NF-κB and up-regulation of CD95 only. However, in transfection experiments, LMP1 uniformly induced the full spectrum of phenotypic effects in both HR and LR lines. We conclude that some BL cell lines show a highly restricted response to CD40 ligation but remain fully susceptible to LMP1.


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D40, a cell surface molecule of the TNF receptor/nerve growth factor receptor family expressed on B lymphocytes and on certain other cell types (1–3), can deliver signals following interaction with its counter-receptor, CD40 ligand (CD40L),3 expressed on activated T cells. Like other members of its family, CD40 has been shown to signal through a C-terminal cytoplasmic domain interacting with TNF receptor-associated factor (TRAF) proteins (4–7). Evidence from both mouse and man indicates an essential role for CD40 signaling in B cell differentiation, notably in Ig class switching and in germinal center (GC) formation (3, 8–10).

Multiple effects of CD40 engagement have been described in in vitro systems after ligation with either specific mAb or recombinant CD40L in the soluble or the membrane-bound form. In resting human B cells, CD40 ligation can act to augment the proliferation and Ig secretion induced by anti-Ig (3, 11, 12). On its own, CD40 stimulation of resting B cells induces the formation of homotypic aggregates and up-regulation of several cell surface molecules such as CD23, CD54, and CD95 (13–15). Addition of IL-4 to such cultures leads to rapid B cell proliferation and a pattern of Ig secretion that is skewed toward IgE; other cytokines can cause preferential skewing toward other Ig isotypes in this system (3, 16–18). Parallel studies with cycling GC B cells isolated from tonsillar preparations are complicated by the tendency of these cells to undergo rapid spontaneous apoptosis in vitro. However, it is clear that CD40 ligation and, to a lesser extent, surface Ig cross-linking can provide a short term survival signal to cultured GC cells (3, 19). In combination with specific ILs, such treatments can either promote GC cell transition to a memory B cell phenotype or prolong cell proliferation (12, 20).

In addition to work involving freshly isolated B cells, a number of studies have reproduced several aspects of these responses to CD40 ligation using established human B cell lines, often of Burkitt’s lymphoma (BL) origin. Thus, enhanced cell aggregation, up-regulation of specific cell surface markers such as CD23 and CD54, and activation of the HLA class I Ag processing pathway were observed in a number of different lines (21–24). Furthermore, where cells could be induced into apoptosis by serum deprivation or by exposure to agents such as TGF-β or anti-IgM, then CD40 ligation protected against cell death (21, 25, 26). As in the GC cell system, survival was associated with the up-regulation of anti-apoptotic proteins such as A20 and members of the Bcl-2 family (21, 27, 28). In addition, several (but not all) of the studies with cell lines also reported growth inhibitory effects of CD40 ligation, a finding that was not anticipated from the work with freshly isolated B cells (29–31).

Our interest in these phenomena stem from the parallels that exist between CD40-mediated effects and those induced in B cells by the EBV-encoded latent membrane protein 1 (LMP1). Thus, vectored expression of LMP1 in BL cell lines has been shown to lead to cell aggregation, up-regulation of several cell-surface markers including CD54, activation of HLA class I processing, and protection from apoptosis through up-regulation of A20 and/or Bcl-2 (32–36). Furthermore, a membrane-proximal domain within the cytoplasmic C-terminus of LMP1 (CTAR1) can interact with a
set of TRAF proteins that are also known to associate with CD40. One early effect of TRAF aggregation in both systems is activation of the transcription factor NF-κB (4, 5, 37, 38). Finally, in assays with stably transfected clones, LMP1 has been shown to bring about a slowing of BL cell growth; this cytostatic effect occurs without any induction of cell death (39).

Given the extensive studies of LMP1-induced effects in the BL cell system and the availability of a wide panel of well-characterized lines of this type, we elected to screen these lines for their responses to CD40 ligation. Ligation was mediated either through mAb or through purified trimeric CD40L. The work reveals a clear division of BL lines into high responders (HR) showing the full range of responses and low responders (LR) showing only a limited range; in contrast, all lines were fully responsive to vectored expression of LMP1.

Materials and Methods

Cell lines

The EBV-negative BL lines, Ramos, Louckes, DG75, BL2, BL40, BL41, and L3055, and the EBV-positive BL lines, Chep, Mutu-I, Eli, Wan, Rael, and Akata, have been described in previous studies (23, 35, 37, 40). Sal is a recently established, EBV-positive BL line (A.B.R., unpublished observation). All lines had cytogenetic markers characteristic of BL and had retained a tumor biopsy-like phenotype on serial passage. Some assays used lymphoblastoid cell lines (LCLs), generated by EBV transformation of normal human B cells in vitro, as LMP1-positive controls. Cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, gentamicin, 1 mM sodium-pyruvate, α-thioglycerol, and 10% (v/v) FCS. The LMP1-transfected clones of Ramos and Akata were established using a tetracycline-regulated vector system and were maintained in culture medium containing 1.5 mg/ml G418, 0.5 mg/ml hygromycin B, and 1 µg/ml tetracycline. Tetracycline withdrawal induced LMP1 expression as previously described (39). Control and LMP1-transfected clones of Sal were established using the pSG5-Neo and pSG5-Neo/LMP1 vectors, respectively (see below), and transfectedants were selected in culture medium containing 2 mg/ml G418.

Abs and reagents

The murine IgG1 mAbs used as surrogate ligands in this work included two CD40-specific reagents, G28.5 (41) and mAb89 (42), and two control reagents, the α2 integrin-specific mAb 1A3 (ICRF Biotherapeutics and Hybridaoma Development Unit, South Mimms, U.K.) and the CD44-specific mAb BU52 (provided by D. Hardie, Department of Immunology, University of Birmingham, U.K.). All were used as DE-52-purified Abs from hybridoma supernatant, and protein concentrations were determined using the Bio-Rad protein assay (Hercules, CA). An HPLC-purified preparation of trimeric human soluble CD40L (scD40L) was provided by Immunex (Seattle, WA). Bound Ab levels were determined using a phycoerythrin (PE)-labeled goat anti-mouse IgG (Caltag, Burlingame, CA).

CD40 expression was monitored using the G28.5 mAb followed by PE-labeled goat anti-mouse IgG (Caltag). Cell surface changes on cultured cells were detected using CD54- and CD23-specific IgM Abs that were FITC labeled (Serotec, Oxford, U.K.) or unlabeled IgM Abs specific for CD95 (CH11, Upstate Biotechnology, Lake Placid, NY) and CD54 (BU72, Department of Immunology, University of Birmingham), followed by PE-labeled goat anti-mouse IgM (Caltag). Rat CD2 expression of transfected cells was determined using an FITC-labeled rat anti-rCD2-specific mouse mAb, OX34, LMP1 expression was monitored using the C1–4 IgG mAbs (35) followed by Texas Red-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL).

Assays of CD40-mediated effects

Cells were exposed to known concentrations of CD40-specific or control reagents. Cell aggregation was assessed visually by microscopic observation over a 3-day period. Cell surface changes were monitored after 48 h by immunofluorescent staining as described above. NF-κB activation was assessed by bandshift assays using 10 µg of nuclear extract prepared after 3 h. For this, a probe derived from the HIV long terminal repeat was 32P-labeled and used essentially as previously described (43). To assess cell proliferation, microplate wells were seeded at 3 × 105 cells/well to which CD40-specific or control reagents were added at known concentrations. Triplicate cultures of each type were harvested on days 1, 2, and 3, and the viable cell number from a known volume was determined by flow cytometric analysis using a dedicated XL program (Coulter, Hialeah, CA) following addition of propidium iodide to identify dead cells. Evidence of cell death was also obtained by staining Permeafix-treated (Dako, Carpentry, CA) cells with 7-amino-actinomycin D (7-AAD) and determining the proportion of the subdiploid cells by flow cytometric analysis of the resulting cell cycle profile. In some experiments, cells were transiently transfected (as described below) with a CD40 expression vector, pcDNA3-CD40 (44), and levels of CD40, CD54, and CD95 expression on transfected cells were monitored by double staining for these individual markers and rat-CD2 using flow cytometry.

Assays of LMP1-induced effects

In transient transfection assays cells were transfected at 2 × 106/ml in 0.5 ml of culture medium by electroporation using a Bio-Rad apparatus at 960 µF and a predetermined optimal voltage (270–310 V depending on the cell line). The electroporation cuvette contained 2 µg of pSG5-LMP1 or pSG5 control DNA (37), 2 µg of pSG5-rat-CD2 (45), and 2 µg DNA of the NF-κB reporter construct 3Enh.xB.Con A-luc; the latter construct contains three tandem NF-κB binding sites from the Igk promoter linked to a minimal conalbumin promoter and to the luciferase gene (46). Cells were harvested after 48 h, and transfection efficiency was monitored by immunofluorescent staining for rat-CD2. Cell surface changes induced by LMP1 were monitored by double staining with CD40- and CD95-specific IgM Abs, which were followed by anti-mouse IgM-PE, then analyzing the rat-CD2-positive population for expression of the second marker. NF-κB activation was determined using a standard luciferase assay, and results were normalized for transfection efficiency. Levels of LMP1 expression were determined by double staining of fixed cell smears with LMP1-specific IgG mAbs CS1–4 plus CD45-specific IgM mAb Bu72 followed by Texas Red-labeled anti-IgG plus FITC-labeled anti-IgM; then staining was quantitated using a Bio-Rad MR500–600 confocal microscope.

Stable LMP1 transfecteds were established using either the tetracycline-regulated vector system as previously described (39) or a G418-selectable pSG5 derivative. For this, the G418 expression cassette was removed from pSV2neo as a BamHI fragment, blunt ended, and cloned into the blunt-ended XbaI site of pSG5 to yield pSG5-Neo. For the construction of the pSG5-LMP1, the LMP1 gene was removed from pSG5-LMP1 (37) as an EcoRI fragment and cloned into the EcoRI site of pSG5-Neo. Cell proliferation of LMP1-positive and -negative clones was assessed as described above. Levels of LMP1 expression were monitored by Western blotting using the CS1–4 mAbs (35).

Results

BL cell lines differ in their aggregation response to CD40 ligation

The initial sets of experiments used a panel of 14 BL cell lines (seven EBV positive and seven EBV negative), selected for their retention of a biopsy-like phenotype in vitro, i.e., growth as single cells and generally low to undetectable expression of surface markers such as CD23, CD54, and CD95. Note that in EBV-positive BL lines of this kind, the virus exhibits a form of latency like that of the original tumor where LMP1 is not expressed (40). Each of the lines was first assessed for cell aggregation following CD40 ligation over a period of 3 days. For this, we used three CD40-specific reagents, namely two murine IgG1 mAbs (G28.5 and mAb 89) and an HPLC-purified preparation of soluble trimeric CD40 ligand (scCD40L). Each agonist was tested over a 100-fold concentration range (0.1–10 µg/ml), and all three gave an essentially similar pattern of results.

The BL lines clearly fell into one of two categories based on their aggregation response. Typical results are illustrated in Fig. 1 from cultures exposed to mAb G28.5. Several lines, here represented by Mutu-I, showed aggregation across the full range of concentrations of CD40 mAbs and CD40L tested; the aggregation occurred within 8 h and was maintained over the 3-day assay period. Other lines, here represented by Rael, showed no detectable aggregation at any dose tested. As a control, IgG1 mAbs directed against other cell surface markers (for example, mAb 1A3 against α2 integrin; Fig. 1) were used, and these did not induce cell aggregation in any of the cell lines tested at any concentration. This
control the possibility of Fc-Fc receptor interactions as mediators of the aggregation induced by CD40-specific mAbs.

Table I summarizes the aggregation response of all 14 BL lines to all three CD40-specific stimuli and highlights the consistency of the response patterns observed. Note that differences in response were not related to the EBV status of the lines. Furthermore, Table I also records the level of CD40 cell surface expression on each line, as determined by indirect immunofluorescent staining and expressed as the mean fluorescence intensity (MFI) ± SD of three independent assays. Clearly, all lines are CD40 positive, and levels of surface expression are not related to CD40 responder status. Based on these initial experiments, we grouped the BL lines into CD40 high responder (HR; aggregating) and CD40 low responder (LR; nonaggregating) categories. This nomenclature is used throughout the rest of the manuscript.

**CD40 ligation selectively inhibits the growth of HR lines**

We next examined the effects of CD40 ligation on cell proliferation. Cells were seeded at 3 × 10^5/ml and exposed to a 0.1–10 μg/ml concentration range of sCD40L, mAb G28.5, and an isotype-matched control mAb 1A3. Proliferation was assessed by counting viable cell numbers over the next 3 days. As illustrated in Fig. 2 for three representative HR lines (Akata, Mutu-I, and Ramos), 10 μg/ml concentrations of sCD40L or mAb G28.5 caused a substantial inhibition of cell growth compared with that seen in untreated cultures or cultures containing 10 μg/ml control mAb 1A3. In contrast, none of the LR lines showed any detectable effect on cell growth by CD40 ligation; this is illustrated in Fig. 2 for the representative LR lines, L3055, Rael, and Sal. Note that in the above growth-inhibited HR cell cultures, the cells remained viable. No evidence of apoptotic cell death could be observed either microscopically or by flow cytometric analysis of the DNA content quantified by 7-amino-actinomycin D staining (data not shown).

In subsequent experiments we determined the dose dependence of the growth-inhibiting effects of CD40 ligation using several HR lines. Typical results are illustrated in Fig. 3 using mAb G28.5. In every case the inhibitory effect was already maximal at a mAb concentration of 1 μg/ml, whereas at 0.1 μg/ml there was either no effect on cell growth (Akata and Ramos), or suboptimal inhibition occurred (Mutu-I). In the same assays sCD40L gave a similar pattern of dose dependence, as did the other CD40-specific mAb 89 (data not shown). These experiments therefore showed that the growth inhibitory effect of CD40 ligation was restricted to HR lines, i.e., those lines showing an aggregation response. Interestingly, induction of growth inhibition required a higher concentration of the CD40 stimulus than was required to induce aggregation.

**CD40 ligation-induced up-regulation of CD54 and CD95**

In parallel experiments all HR and LR lines were examined for two reported downstream effects of CD40 signaling in B cells, namely increases in cell surface expression of CD54 (ICAM-1) and CD95 (Fas). Where responses were observed, pilot studies using different concentrations of mAb G28.5 or sCD40L again showed that doses of 1 and 10 μg/ml were indistinguishable in their effects. Furthermore, the lower dose of 0.1 μg/ml produced either no detectable change or a suboptimal response. Note that isotype-matched control mAbs never produced any detectable changes in CD54 and CD95 expression compared with untreated cultures. The illustrative data in Fig. 4 were obtained in experiments using 1 μg/ml doses of mAbs or sCD40L.

Fig. 4A shows the MFI ± SD from three independent experiments in which cell surface levels of CD54 were determined after

### Table I. Aggregation responses of BL cell lines to CD40 ligation

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EBV Status</th>
<th>Cell Aggregation Following:</th>
<th>CD40 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mAbG28.5</td>
<td>mAb89</td>
</tr>
<tr>
<td>BL2</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BL41</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DG75</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Louckes</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ramos</td>
<td>−</td>
<td>+</td>
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</tr>
<tr>
<td>Akata</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Chep</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Eli</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mutu-I</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>BL40</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>L3055</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sal</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Wan</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Rael</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

α Cell aggregation was assessed microscopically following treatment with the CD40 mAbs G28.5 or 89, or with sCD40L; in each case consistent results were observed over a 0.1-10 μg/ml concentration range (see Fig. 1). Untreated cultures and cultures exposed to control mAbs 1A3 (anti-α, integrin) or BU52 (anti-CD44) showed no aggregation.

β Cell surface levels of CD40 on the various BL lines were determined by fluorescence staining. Data are expressed as MFI ± SD from three independent experiments. Background levels of staining in the same assay never exceeded an MFI of 0.2; parallel staining of an EBV-transformed lymphoblastoid cell line gave an MFI of 27 ± 3.
48 h in the presence of the CD40-specific stimuli or of control mAb 1A3. With the single exception of Akata, a line that has previously been reported to be deficient in CD54 up-regulation in a variety of circumstances (40), all HR lines showed a clear CD40-induced response to both mAb G28.5 and sCD40L. The level of CD54 induction was 2- to 3-fold in lines with appreciable baseline expression (e.g., Ramos and Chep) and up to 10-fold in lines with very low baseline levels (e.g., Mutu-I). In contrast, the four LR lines illustrated showed no significant response to either mAb G28.5 or sCD40L regardless of their baseline levels of CD54 expression; the fifth LR line, BL40, showed no response to mAb G28.5, but there was a weak (<2-fold) increase in CD54 staining in response to sCD40L (data not shown).

The corresponding findings for CD95 are shown in Fig. 4B. Here all HR lines including Akata show clear up-regulation of CD95 from low baseline levels in response to either mAb G28.5 or sCD40L. In the same experiments, LR lines did not show any detectable change in their low baseline surface expression of CD95 in response to mAb G28.5. However, with the exception of Rael, all LR lines did show a small but significant increase (Student’s t test, p < 0.05) in CD95 expression following exposure to sCD40L.

CD40 ligation-induced activation of NF-κB

We next examined one of the more proximal reported effects of CD40 ligation, namely activation of NF-κB. For this purpose, we measured NF-κB activity in nuclear extracts prepared from CD40-stimulated cells in a bandshift assay using a radiolabeled oligonucleotide probe containing a defined NF-κB binding sequence from the HIV long terminal repeat (43). Specific retardation of the probe was visualized by autoradiography. Pilot studies again showed that 1 μg/ml of mAb G28.5 or sCD40L was sufficient to induce an optimal response in this assay. The optimal stimulation time was approximately 3 h, with detectable NF-κB levels beginning to fall by 12 h poststimulation.

All lines were tested in this way, and Fig. 5 shows representative data from a single experiment; similar results were obtained in two additional independent experiments. Note that nuclear extracts of all the BL lines tested produced two retarded bands on the gel, both of which appeared to be specific in that they were competed out only by the unlabeled cognate oligonucleotide probe and not by irrelevant competitor sequences (data not shown). All HR lines showed increased NF-κB binding activity in response to both mAb
the transfected cells formed only a small proportion of the whole activation. In this case bandshift analysis was not feasible because of 10
g or 10
m 3 independent experiments as the mean luciferase counts.
crease in NF-
(illustrated by L3055 and Sal), we did observe a significant in-
 stimulation. However, in four of the five LR lines tested
Akata, and Eli) and two LR lines (Sal and Rael) were sufficiently
transfectable (i.e., producing 8–20% rat-CD2-positive cells) to al-
ways be found in this way. The results from their independent experiments for surface expression are expressed as the MFI ± SD of
CD95 (Fig. 7A) and CD54 (Fig. 7B), in each case analyzing only
the rat-CD2-positive population. We observed consistent up-regulation of CD95 in all four HR lines and in both LR lines (Fig. 7A).
CD54 was up-regulated in three of four HR lines (the exception again being Akata-BL) and again in both LR lines (Fig. 7B). In
these experiments, transfection per se did not affect the baseline levels of either cell surface marker. Thus, in control cotransfec-
tions of rat-CD2 and pSG5 vector, the rat-CD2-positive cells showed the same CD95 and CD54 expression levels as the rat-
CD2-negative cells in the same culture. Fig. 8 shows the results of confocal microscopic analysis following double staining for LMP1 and CD54 in one of the LR lines, Rael, transiently transfected with either the pSG5 or the pSG5-LMP1 vector; EBV-transformed LCL cells were simultaneously analyzed as a positive control. The data show firstly that CD54 up-regulation is restricted to LMP1-trans-
secreting cells, and secondly that LMP1 levels produced by transient transfection with the pSG5-LMP1 construct do not exceed normal LCL-like levels. In an extension of these experiments, we tran-
siently transfected Rael cells with the rat-CD2 vector and a vector,
pcDNA3-CD40, that is known to express a functional CD40 mol-
ecule (44). Subsequent exposure of the cells to CD40L did not result in any detectable CD54 or CD95 up-regulation in the rat-
CD2-positive population (data not shown).
Finally, to examine the effects of LMP1 on cell growth in HR
and LR cell backgrounds, it was necessary to establish stable LMP1-positive and control transfectants using drug selection. We
noted that previous reports of an inhibitory effect of LMP1 on BL
cell growth had only involved the use of HR target lines. For the present work we chose previously established transfectants on two
HR cell backgrounds, namely Ramos and Akata; in both cases the
cells carried a tetracycline-regulatable LMP1 expression vector.
Thus, removal of tetracycline from the medium induced expression of
LMP1, and uninduced cultures served as the control (39). To extend
the analysis we established new sets of control and LMP1-
positive transfectants on the background of a representative LR

FIGURE 3. Dose response of CD40 ligation-induced growth inhibition in representative HR lines (Akata, Mutu-I, and Ramos). Proliferation was assessed, as described in Fig. 2, in cultures containing either 10 μg/ml of control mAb 1A3 (○) or CD40 mAb G28.5 at 0.1 μg/ml (▲), 1.0 μg/ml (●), or 10 μg/ml (◆). The data represent the mean of three experiments; SDs were within 15% of the means in every case.

Both HR lines and LR lines are fully responsive to LMP1
In light of these clear differences in BL cell responsiveness to
CD40 ligation, we used a similar approach to compare HR and LR
lines in their responsiveness to EBV-encoded LMP1 expressed in
the cell lines by transient transfection. In these experiments cells were electroporated with the pSG5-LMP1 expression vector or with the parental pSG5 vector as a control, in each case alongside a marker vector expressing rat-CD2. Immunofluorescent staining of the cells for surface expression of rat-CD2 thus provided both an indicator of transfection efficiency and a means of identifying the transfected subpopulation. Four HR lines (Ramos, DG75, Akata, and Elii) and two LR lines (Sal and Rael) were sufficiently transfectable (i.e., producing 8–20% rat-CD2-positive cells) to allow their study in this way.

We first examined the effects of LMP1 expression on NF-κB activation. In this case bandshift analysis was not feasible because the transfected cells formed only a small proportion of the whole population. Instead, we assayed NF-κB activity using a cotransfected reporter construct expressing luciferase under the control of a NF-κB-responsive promoter (46). Fig. 6 presents the results of three independent experiments as the mean luciferase counts ± SD following normalization for transfection efficiency. We observed significant activation of NF-κB in all four HR lines and in both LR lines tested; the effects were reproducible and independent of the baseline NF-κB levels in each line.

In the same experiments, the effects of LMP1 on cell surface expression of CD95 and CD54 markers were determined by double staining for rat-CD2 (i.e., for successfully transfected cells) and for the marker in question. The results from three independent experiments for surface expression are expressed as the MFI ± SD of CD95 (Fig. 7A) and CD54 (Fig. 7B), in each case analyzing only the rat-CD2-positive population. We observed consistent up-regulation of CD95 in all four HR lines and in both LR lines (Fig. 7A). CD54 was up-regulated in three of four HR lines (the exception again being Akata-BL) and again in both LR lines (Fig. 7B). In these experiments, transfection per se did not affect the baseline levels of either cell surface marker. Thus, in control cotransfections of rat-CD2 and pSG5 vector, the rat-CD2-positive cells showed the same CD95 and CD54 expression levels as the rat-
CD2-negative cells in the same culture. Fig. 8 shows the results of confocal microscopic analysis following double staining for LMP1 and CD54 in one of the LR lines, Rael, transiently transfected with either the pSG5 or the pSG5-LMP1 vector; EBV-transformed LCL cells were simultaneously analyzed as a positive control. The data show firstly that CD54 up-regulation is restricted to LMP1-trans-
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the analysis we established new sets of control and LMP1-
positive transfectants on the background of a representative LR
In all cases, LMP1 expression in the appropriate cultures was monitored by immunoblotting and was shown to be no greater than that typically seen in EBV-transformed LCL cells (data not shown). Fig. 9 presents the cell proliferation data from one representative set of experiments comparing LMP1-expressing vs control cell populations. LMP1 expression was associated with a slowing of cell growth not only in the HR cell backgrounds Ramos and Akata, but also in the LR line Sal. We consistently observed this result in assays comparing a total of four control and four LMP1-positive clones established on the Sal background.

FIGURE 4. Effect of CD40 ligation on cell surface levels of CD54 (A) and CD95 (B) in representative HR lines (Ramos, Mutu-I, Akata, and Chep) and LR lines (L3055, Sal, Rael, and Wan). Cells were exposed to 1 μg/ml concentrations of control mAb 1A3 (open bars), CD40 mAb G28.5 (hatched bars), or sCD40L (black bars) and assayed 48 h later by immunofluorescence. Results are expressed as the MFI ± SD and are shown for three assays.

FIGURE 5. Effect of CD40 ligation on NF-κB activity in representative HR lines (Ramos, Mutu-I, and Akata) and LR lines (L3055, Sal, and Rael). NF-κB was assayed by gel shift of a radiolabeled NF-κB-specific probe using nuclear extracts made 3 h post-treatment with 1 μg/ml doses of control mAb 1A3 (Neg. control), CD40 mAb G28.5, or sCD40L. The results shown are from a single experiment that is representative of three independent experiments.
Discussion

Studies of CD40 ligation in B cell systems have revealed multiple effects, but it is still not clear what the relationship between these different effects might be. The picture is complicated by the variety of B cell preparations tested and, in work with established B cell lines, by the tendency to use only a very limited number of lines in each study (14, 27, 29, 30). Here we have screened a large number of lines all derived from BL, a human B cell tumor that is well characterized by cytogenetic and phenotypic criteria (47). Furthermore, the BL cell background has been extensively used to document the phenotypic effects of EBV-LMP1, a molecule that interacts with components of the CD40 signaling pathway (4, 38).

Our experiments revealed two patterns of responsiveness to CD40 ligation among BL lines, and these patterns were consistently observed using either CD40 mAbs or a purified preparation of soluble CD40L as the agonist.

The HR group of lines was easily identified by virtue of the homotypic aggregation invariably induced by a wide range of agonist concentrations. Every one of these lines also showed other well-documented features of the CD40-induced response, namely

<table>
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<th>Aggregation</th>
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<th>CD54 Up-Regulation</th>
<th>CD95 Up-Regulation</th>
<th>NFκB Activation</th>
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* A + * indicates weak responses where the up-regulation of CD54 or CD95 was less than twofold; */-* indicates weak responses that were seen on some but not all occasions of testing. NT, not tested.

FIGURE 6. Effect of LMP1 expression on NF-κB activity in representative HR lines (Ramos, DG75, Akata, and Eli) and LR lines (Sal and Rael). Cells were transfected with pSG5-control vector (open bars) or pSG5-LMP1 (black bars) in conjunction with an NF-κB-responsive luciferase expression vector and a rat-CD2 vector as a marker of transfection efficiency. Luciferase counts were determined in lysates made 48 h after transfection and normalized for transfection efficiency using the percentage of rat-CD2 expressing cells as determined by immunofluorescence. The data show the mean normalized count ± SD of three experiments.

FIGURE 7. Effect of LMP1 expression on cell surface levels of CD95 (A) and CD54 (B) in representative HR lines (Ramos, DG75, Akata, and Eli) and LR lines (Sal and Rael). Control transfected cells (open bars) and LMP1-transfected cells (closed bars), transfected as described in Fig. 6, were harvested after 48 h for immunofluorescent staining. Results from three independent experiments are expressed as MFI values ± SD obtained by gating on rat-CD2-expressing cells.
activation of NF-κB and specific changes in cell surface phenotype such as up-regulation of CD95 and (except for Akata) of CD54. Finally, all the HR lines displayed clear evidence of growth inhibition following CD40 ligation. This is the first systematic analysis of such growth effects in BL lines and adds to earlier reports of CD40-mediated growth inhibition in particular B cell lines of murine and human origin (29, 31). In the present study a marked growth inhibition of HR lines was consistently observed in the absence of detectable cell death by apoptosis. In this respect the results mirrored the growth inhibition induced by CD40 ligation in human epithelial cell lines (43) and were unlike those observed in another study following high dose CD40L treatment of human/mouse heterohybridomas in vitro (30).

It is significant that in our experiments the lowest dose of either CD40 mAb or sCD40L (0.1 μg/ml) was sufficient to induce optimal aggregation in all HR lines, whereas other aspects of the response required at least 1 μg/ml for optimal induction. Hence, aggregation per se was distinguishable from other effects, in particular from the up-regulation of ICAM-1/CD54, an adhesion molecule that in CD40-stimulated normal B cells can mediate homotypic aggregation through binding to its counter-receptor, LFA-1 (13, 23). This distinction is further emphasized by results from the HR line Akata, which is universally negative for detectable CD54 expression under any condition of CD40 ligation and yet showed a clear aggregation response. Interactions between LFA-1 and alternative ligands such as ICAM-2 and ICAM-3 can also mediate adhesion (48). However, LFA-1/ICAM interactions do not appear to be important in the present context, since attempts to block CD40-induced aggregation in the HR lines using mAbs to either CD18 (the β subunit of LFA-1) or CD54 produced only a weak transient effect, whereas in parallel experiments they achieved complete disaggregation of EBV-transformed lymphoblastoid lines (N.V.H., unpublished observations). This is reminiscent of other reports that LFA-1 blocking Abs led to only partial inhibition of CD40-mediated cell aggregation in B cell systems (30). Together the data imply that other receptor-ligand interactions must be responsible for the bulk of the CD40-induced homotypic aggregation in the BL system. Another candidate pathway, the CD23-CD21 interaction (45), is also unlikely to be involved, because CD23 was virtually undetectable on most lines, and CD40 ligation up-regulated CD23 expression on only one of the 14 BL lines tested, Ramos (data not shown).

It is also important to note that aggregation and growth inhibition were differentially dependent on the CD40 agonist dose, with only aggregation observable at the lowest concentration tested. Hence, the observed effects on cell growth at higher agonist doses are not nonspecific consequences of cell aggregation per se. This is further supported by the fact that one can induce aggregation in cells treated with the control mAbs (such as 1A3) by adding anti-mouse IgG as a secondary cross-linking Ab, yet this never impaired the growth of any of the HR lines tested (N.V.H., unpublished observations). Finally, although cell surface phenotypic changes occurred at similar agonist doses as did growth inhibition, the data from the HR line Akata clearly show that growth inhibition can occur in the complete absence of at least one feature of surface phenotypic change, i.e., CD54 up-regulation.

The LR group of lines, initially identified by a complete absence of CD40-induced aggregation, showed a much more limited range of CD40-induced responses when tested in the full range of assays. Thus, neither CD40 mAb nor sCD40L had any detectable effect on cell growth or on cell surface expression of CD54. However, most (but not all) LR lines did show positive responses in the CD95 and NF-κB induction assays, but usually only following exposure to sCD40L. The limited nature of CD40-induced effects in LR lines was observed despite relatively strong CD40 surface expression (Table I). Furthermore, addition of secondary cross-linking anti-mouse IgG to CD40 mAb-treated LR lines did cause aggregation (as did cross-linking of anti-α3 mAb-treated cells; see above), but, again, this did not induce any of the other parameters of the CD40-induced response in these lines (N.V.H., unpublished observations). Such data imply that the different responses observed between LR and HR lines cannot be explained by quantitative
differences in CD40 expression and/or in CD40 cross-linking, but, rather, reflect qualitative differences in engagement of downstream signaling pathways.

The results with LR lines were also interesting in that they provided an example in which the CD40L-induced response was distinct from that induced by CD40 mAb. Indeed, the results observed with sCD40L highlighted an apparent correlation between the induction of CD95 expression and the activation of NF-κB; both aspects of the CD40L-induced response were seen in four of five LR lines tested, but both were absent in the fifth line, Rael. It is possible that NF-κB plays a role in CD95 induction, and this would be consistent with the reported presence of NF-κB response elements in the CD95 promoter region (49). A corollary of these results is that NF-κB activation (at least as measured by the bandshift assay used here) and also CD95 induction can occur independently of any effect on the CD54 expression level or on cell growth. In this context there were no obvious quantitative differences in the levels of NF-κB induced by sCD40L in HR and LR lines. Certainly, the NF-κB activation seen in L3055 (Fig. 5) and in BL40 (data not shown) was at least as marked as that observed in several HR lines (e.g., Ramos; Fig. 5). It will be interesting to determine how another facet of the CD40 response in BL cells, namely protection from apoptosis, compares in HR and LR lines. The reported ability of sCD40L to rescue the line L3055 from ionomycin- and anti-IgM-induced apoptotic cell death suggests that such rescue may be part of the limited LR cell response (25, 27). However, the point needs to be examined in a proper comparative study, and this will require standardization of the various apoptosis-inducing protocols to one that is similarly effective across a range of lines (21, 27).

Given the differences in response between HR and LR lines to CD40 ligation, we were interested to determine whether a similar dichotomy could be observed in the response to LMP1. This was particularly important because perusal of the literature showed that almost all reports of LMP1-induced phenotypic effects in BL cells had examined lines that we identified as HR in the present study (32–34, 37, 39); studies on representative LR lines, therefore, became particularly significant. The final sets of experiments clearly show that in transient transfection assays LMP1 induces NF-κB, CD95, and CD54 equally well in HR and LR cell backgrounds. The capacity of LMP1 to induce CD95 in BL cell lines has not been shown previously and provides yet another parallel between LMP1- and CD40-mediated effects. Note that one of the LR lines used in this part of the work, Rael, was uniformly unresponsive to any form of CD40 ligation and yet (along with a second LR line, Sal) showed the full spectrum of changes following LMP1 transfection. We were concerned that the use of transient transfection to study LMP1 responsiveness might be a source of artifact, especially if the LMP1 levels thus produced were atypically high. However, this was not the case; LMP1 expression in transiently transfected Rael cells was below LCL-like levels (Fig. 8). Moreover, in a converse experiment, Rael cells were transiently transfected with a functional CD40 expression vector but did not acquire responsiveness to CD40L (data not shown). Furthermore, in stable LMP1 transfectants on the LR cell background, Sal, where there was a significant slowing of growth compared with that in controls, LMP1 was again expressed at or below LCL-like levels. These observations make it clear that LMP1 is capable of inducing the full range of phenotypic effects in cells that show grossly impaired responses to CD40 ligation. This may reflect the fact that CD40 signaling is ligand dependent, whereas LMP1 functions constitutively. In addition, however, it is likely that the two molecules signal through overlapping but nonidentical combinations of TRAFs. Firstly, even in the immediate vicinity of their PxxT TRAF-binding motif, CD40 and LMP1 show little sequence homology (37, 38), implying that the two domains may be functionally distinct. Secondly, each molecule appears to have other unique TRAF-interacting domains that, again, may induce differential effects. Thus, CD40 contains a domain interacting with TRAF6, whereas the CTAR2 domain of LMP1 can use TRADD (TNFR-associated death domain protein) as an adapter molecule that provides an additional link to TRAF pathways (50, 51).

The present findings imply that in at least a proportion of BL tumors, CD40 is expressed at the tumor cell surface, but CD40-mediated signaling may be impaired. It will be important to understand the molecular basis of the differential responsiveness to CD40 ligation in HR and LR lines and to determine whether such differences are apparent between freshly isolated tumor cell preparations as well as between tumor-derived lines. The possibility remains that since reception of a CD40-mediated signal by B cells in the absence of cognate Ag can lead to cell elimination in vivo (9), then the selection of cells with a lesion in the CD40 pathway may in some cases be a contributory step in BL pathogenesis.

Acknowledgments

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References
