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INDUCTION OF COSTIMULATORY MOLECULES B7-1 AND B7-2 IN MURINE B CELLS: THE CBA/N MOUSE REVEALS A ROLE FOR BRUTON'S TYROSINE KINASE IN CD40-MEDIATED B7 INDUCTION

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Abstract—The binding of CD40 ligand on activated T cells to CD40 on resting B cells induces the expression of costimulatory molecules B7-1 (CD80) and B7-2 (CD86). The induction of B7 molecules by CD40 ligand-CD40 interaction represents a critical step in rendering B cells competent for antigen presentation. The CBA/N mouse has a defect in CD40 signalling which has been attributed to a mutation in Bruton's tyrosine kinase. We have compared the ability of murine CD40 ligand to induce B7-1 and B7-2 expression on B cells isolated from CBA/N and wild-type CBA/J mice. We find that the CBA/N defect partially impairs both B7-1 and B7-2 induction via CD40. Subsequent experiments investigated the roles of different second messenger systems in B7-1 and B7-2 induction in normal B cells. In M12 B lymphomas either CD40 cross-linking or cAMP treatment can induce B7 molecules. Here we report that treatment with dibutyryl-cAMP also induces B7 molecules in normal B cells provided that they have been preactivated by CD40 cross-linking. We also find that PMA and ionomycin treatment of B cells induces B7-2 but not B7-1 expression. Our data therefore show roles for BTK, cAMP and PMA/ionomycin in B7 induction, as well as providing further evidence for differential regulation of B7-1 and B7-2 induction in B cells. Copyright © 1996 Elsevier Science Ltd.

Key words: B7, CD40, Bruton's tyrosine kinase, xid, cAMP.

INTRODUCTION

To activate T cells, costimulatory signals are required in addition to the antigen-specific signal through the T cell receptor. Occupancy of the T cell antigen receptor alone instead leads to cell death or to the induction of a non-responsive state (Mueller *et al.*, 1989). There is a large body of evidence supporting the role of the CD28 molecule on the T cell as a major costimulatory receptor for T cell activation and prevention of anergy (Jenkins and Johnson, 1993; Linsley and Ledbetter, 1993; Schwartz, 1992). The physiological ligands for CD28, B7-1 (CD80) (Freeman *et al.*, 1989; Linsley *et al.*, 1990) and B7-2 (CD86) (Azuma *et al.*, 1993; Boussiotis *et al.*, 1993; Freeman *et al.*, 1993a, 1993b, 1993c; Hathcock *et al.*, 1993; Lenschow *et al.*, 1993; Razi-Wolf *et al.*, 1993; Wu *et al.*, 1993), are Ig supergene family members expressed constitutively with subsequent upregulation on dendritic cells (Larsen *et al.*, 1992, 1994; Young *et al.*, 1992), inducibly on monocytes by IFN-γ (Freeman *et al.*, 1990), and inducibly on B cells by a variety of stimuli including LPS (Hathcock *et al.*, 1993; Linsley *et al.*, 1990; Razi-Wolf *et al.*, 1993), T cell contact (Koulova *et al.*, 1991; Nabavi *et al.*, 1992; Watts *et al.*, 1993), MHC II cross-linking (Koulova *et al.*, 1991), IL-4 (Stack *et al.*, 1994; Vallé *et al.*, 1991), and CD40 cross-linking (Azuma *et al.*, 1993; Hasbold *et al.*, 1994; Kennedy *et al.*, 1994; Ranheim and Kipps, 1993; Yellin *et al.*, 1994). A major mechanism by which activated T cells induce B7-1 and B7-2 on B cells is through the interaction of CD40, which is constitutively expressed on B cells, and its ligand, gp39 (CD40 ligand) which is induced on T cells upon ligation of the T cell receptor.

CD40 is a member of the nerve growth factor (NGF) receptor gene family, whose members are identified by repeating cysteine-rich extracellular domains (Banchereau *et al.*, 1994). Previous reports describe several roles of CD40 in B cell activation, including enhancing ICAM-1/LFA-1 mediated adhesion (Barrett *et al.*, 1991), preventing apoptosis of germinal centre B cells (Liu *et al.*, 1989), regulating immunoglobulin class switching to IgE (Jabara *et al.*, 1990), and inducing lymphotoxin α gene expression (Worm and Geha, 1994) in addition to inducing B7 molecules. Many studies have investigated mechanisms of CD40 signalling, and suggest that tyrosine kinases including lyn, fyn, syk and Bruton's tyrosine kin-

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Abbreviations: BTK, Bruton's tyrosine kinase; db-cAMP, dibutyryl-cyclic AMP; PH domain, pleckstrin homology domain.

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ase (BTK) (Faris *et al.*, 1994; Hasbold and Klaus, 1994; Kansas and Tedder, 1991; Knox and Gordon, 1993; Ren *et al.*, 1994; Uckun *et al.*, 1991), serine/threonine kinases (Uckun *et al.*, 1991), PLCγ2 (Ren *et al.*, 1994), and cAMP pathways (Kato *et al.*, 1994; Knox *et al.*, 1993) are all activated by CD40 ligation. In addition, nuclear transcription factors that have been identified as activated by CD40 signalling include NF-AT, NF-κB, and AP-1 (Berberich *et al.*, 1994; Choi *et al.*, 1994; Francis *et al.*, 1995). Recently, three groups have identified a TRAF-1 like molecule (called CD40bp, LAP1 or CRAF-1) which associates with the cytoplasmic domain of CD40 (Cheng *et al.*, 1995; Hu *et al.*, 1994; Mosialos *et al.*, 1995). Although various signalling mechanisms and outcomes of CD40 ligation have been identified, it is unclear which signalling pathways play roles in which outcomes. Therefore, we have begun to examine the signalling pathways involved in B7-1 and B7-2 induction upon CD40 ligation.

The CBA/N mouse strain has a point mutation in the pleckstrin homology (PH) domain of BTK, which has been identified as the cause of the *xid* phenotype observed in these mice (Rawlings *et al.*, 1993; Thomas *et al.*, 1993). Similarly, mutations in human BTK cause the disorder X-linked agammaglobulinemia (XLA) (Tsukada *et al.*, 1993; Vetrie *et al.*, 1993). Previous work has shown that B cells from CBA/N mice fail to proliferate upon ligation of CD40, implicating BTK as a key factor in CD40 mediated signalling (Hasbold and Klaus, 1994). In this study we show that there is a quantitative impairment in the induction of both B7-1 and B7-2 upon signalling through CD40 in B cells from CBA/N mice with a more reproducible effect on B7-2 induction, suggesting that BTK plays a role in B7-1 and B7-2 induction upon CD40 signalling.

We also examined the roles of different second messenger systems in B7 induction. Previous studies showed both that cAMP induces B7 molecules in an M12 B lymphoma line (Freeman *et al.*, 1993a; Nabavi *et al.*, 1992; Watts *et al.*, 1993), and that CD40 ligation causes an increase in intracellular cAMP (Kato *et al.*, 1994; Knox *et al.*, 1993). In this study we examined the ability of cAMP to induce B7 molecules in primary murine B cells. Although treatment of primary B cells with db-cAMP proved to be lethal, we showed that once CD40 ligation occurs, db-cAMP treatment greatly enhances B7-1 and B7-2 induction. Finally, we observed that upon treatment of normal murine B cells with PMA and ionomycin, B7-2 but not B7-1 is induced—yet another example of the differential induction of B7-1 and B7-2.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

Cells were maintained in RPMI-1640 containing 10% FCS (P.A. Biologicals, Sydney, Australia), 50 μM 2-mercaptoethanol, MEM non-essential amino acids (Gibco-BRL), antibiotics, pyruvate and glutamine, as previously described (Watts *et al.*, 1993). Dibutyryl-cAMP (db-cAMP), PMA, and ionomycin were purchased from Sigma (St Louis, MO). LPS was purchased from Calbiochem (San Diego, CA) or Difco Laboratories (Detroit, MI).

The hybridoma secreting the B7-1 specific antibody 16.10A1 (Razi-Wolf *et al.*, 1992) was kindly provided by Dr Hans Reiser, Dana Farber Cancer Institute. The α-B7-2 secreting hybridoma GL-1 (Hathcock *et al.*, 1993) and the α-CD8 secreting hybridoma 53-6.72 were obtained from the American Type Culture Collection. The hybridoma, MR1, secreting the α-gp39 antibody (Noelle *et al.*, 1992) was kindly provided by Dr Randy Noelle, Dartmouth Medical School. Antibodies were purified using protein A or protein G sepharose (Pharmacia, Uppsala, Sweden). For flow cytometry, antibodies were labelled with biotin according to Goding (Goding, 1983), and used at a concentration which gives maximal binding on db-cAMP-treated M12 cells (1–3 μg per 1–2 $\times$ 10^6 cells).

B cell isolation

Male or female CBA/J and CBA/N mice were obtained from Jackson Laboratories (Bar Harbor, ME) and used at 6–20 weeks of age. In each experiment, mice from both strains were age and sex matched. Six- to 8-week old Balb/c female mice were obtained from Charles River Laboratory (St Constant, Quebec). Spleens were obtained aseptically, teased into a single cell suspension and treated with 0.15 M NH_4Cl solution to remove erythrocytes. T cells were depleted (Hathcock, 1991) using α-CD4 (RL172), α-CD8 (3.168) and α-Thy-1.2 (HO-13-4) plus rabbit complement (Cedarlane, Hornby, Canada). Antibodies used for T cell depletion were provided by Dr Michael Julius, University of Toronto. Resting B cells were further purified by sedimentation through Percoll (Mond and Brunswick, 1991) (Pharmacia). The small high-density resting cells were collected from the 60–70% Percoll interface. Total B cells were collected from both the 50–60% and 60–70% interfaces. Cells were >95% μ+ from CBA/J and Balb/c, and >80% μ+ from CBA/N mice.

Induction of B7 and analysis by flow cytometry

Normal splenic B cells were cultured at a final concentration of 1–2 $\times$ 10^6 cells/ml for all experiments. PMA and ionomycin were added to final concentrations of 10 ng/ml and 1 μM respectively. LPS was added to a final concentration of 50 μg/ml. A soluble form of the murine CD40 ligand, murine gp39-CD8 was produced in the same way as described for the human CD40 ligand fusion protein (Hollenbaugh *et al.*, 1992). It was used as a COS cell supernatant that contained less than 0.1 endotoxin units/ml as determined by the Limulus amebocytes lysate kit (BioWhittaker, Inc., Walkersville, MD). To signal via CD40, normal B cells were cultured with dilutions of the murine gp39-CD8 fusion protein varying from 1 in 2 to 1 in 64. The fusion protein was cross-linked either by precoating plates overnight at 4°C with supernatant from the α-CD8-producing hybridoma 3.168 (Table 1, Fig. 4), or by adding soluble α-CD8 antibody 53-6.72 to a final

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Table 1. Induction of B7-1 and B7-2 in CBA/J and CBA/N B cells

B cells[a]			gp39[b]		LPS[c]	
			ΔB7-1[d]	ΔB7-2	ΔB7-1	ΔB7-2
Resting	Expt. 1	CBA/J	4.5	41.2	27.2	204.4
		CBA/N	2.0	17.5	18.3	664.7
Resting	Expt. 2	CBA/J	7.3	29.4	27.4	104.9
		CBA/N	4.7	15.7	42.3	269.4
Total	Expt. 3	CBA/J	23.6	148.2	67.9	248.9
		CBA/N	3.1	119.9	61.9	378.6
Total	Expt. 4	CBA/J	37.1	159.8	84.7	360.8
		CBA/N	6.1	80.9	128.1	575.7

[a]Resting (high density) or total B cells were isolated from CBA/N or CBA/J spleen as described in the Materials and Methods section.
[b]B cells were stimulated with plastic immobilized anti-CD8 plus a 1 in 2 dilution (expt. 2) or 1 in 5 dilution (expts. 1, 3, 4) of COS cell supernatant containing murine gp39-CD8.
[c]B cells were stimulated with LPS at 50 μg/ml.
[d]After 61–64 hr, B cells were stained for B7-1 and B7-2 expression as described in the Materials and Methods section. Results are expressed as the difference in mean fluorescence intensity between stimulated and unstimulated B cells.

concentration of 10 μg/ml (Figs 1–3). After 18–92 hr, analysis of B7-1 and B7-2 expression was determined by flow cytometry.

After washing once in PBS, up to 2×10^6 B cells in 200 μl of PBS, 1% FCS, 2 mM NaN_3 were incubated with irrelevant antibody to block Fc receptors prior to the addition of biotinylated antibody. Following incubation and washing, samples were then incubated with 200 μl of a 3 μg/ml solution of PE-streptavidin (Molecular Probes, Eugene, OR or Biomeda Corp., Foster City, CA). To control for increased autofluorescence upon activation of the B cells, each experiment included an unstained sample and a sample stained with a negative control antibody for each treatment. Samples were analysed on a Becton Dickinson FACSCAN (Mountain View, CA), or in one case an EPICS Profile analyser (Coulter Electronics, Inc., Hialeah, FL). Alignment was checked using Immuno-check beads (Coulter Electronics) and the instrument was calibrated using Calibrite beads (Becton Dickinson) and autocomp software. Propidium iodide staining and forward and side scatter were used to gate on live cells. Data were analysed and plotted using Becton Dickinson Lysis II software.

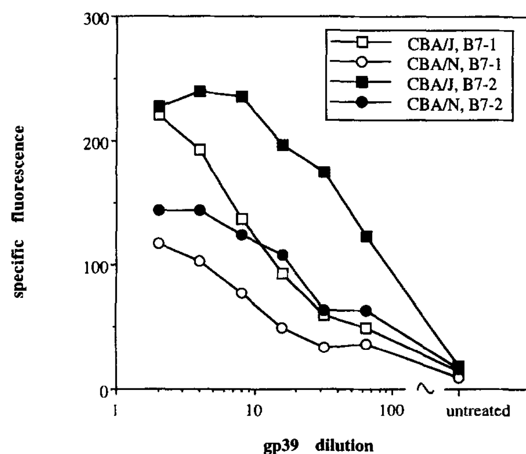

Fig. 1. B cells from CBA/N mice have impaired induction of B7-1 and B7-2 upon treatment with CD40 ligand. Resting B cells from CBA/J (squares) and CBA/N (circles) mice were incubated for 65 hr at 10^6/ml with various dilutions of COS cell supernatant containing the murine gp39-CD8 fusion protein, cross-linked by the soluble α-CD8 antibody 53-6.72 at a final concentration of 10 μg/ml. Expression of B7-1 (open symbols) and B7-2 (closed symbols) was determined by flow cytometry as described in the Materials and Methods section. Specific fluorescence is defined as the difference in mean fluorescence intensity between cells stained with the B7 specific antibody and an irrelevant antibody.

Proliferation assay

Proliferation of normal B cells (10^5 cells in 100 μl) was determined by incorporation of 1 μCi/well of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) during the final 6 hr of a 69 hr culture. Cultures were harvested onto glass fibre and radioactivity determined by liquid scintillation counting.

RESULTS

B7 induction in B cells from CBA/N mice

Previous studies show that B cells from CBA/N mice fail to proliferate when signalled through CD40 (Hasbold and Klaus, 1994), suggesting that signalling through CD40 may proceed through BTK. As CD40 is known to signal in many different ways, we chose to study B cells from CBA/N mice to see if BTK is required for induction of B7-1 and/or B7-2 upon CD40 signalling. Figure 1 indicates that there is a quantitative defect in both B7-1

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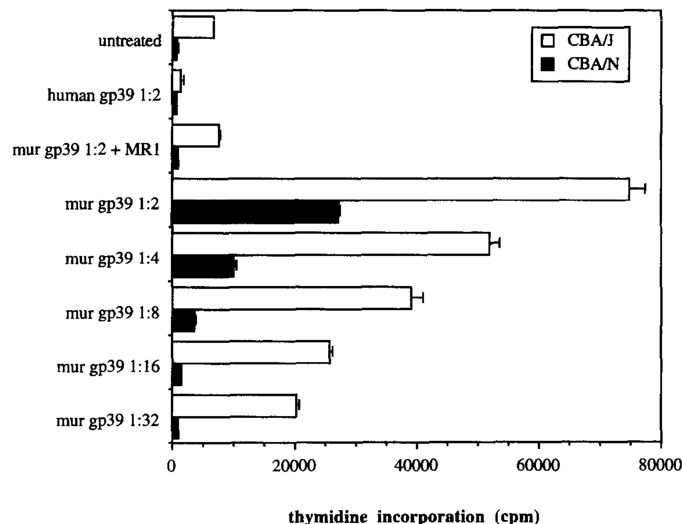

Fig. 2. B cells from CBA/N have a defect in proliferation upon treatment with CD40 ligand. Resting B cells from CBA/J (open bars) and CBA/N (shaded bars) mice were cultured at 10^5/well in 100 μl final volume. Cells were untreated, or cultured with varying dilutions of COS cell supernatants containing either murine or human gp39-CD8 fusion protein cross-linked by the soluble α-CD8 antibody 53-6.72 at a final concentration of 10 μg/ml. In addition, cells were treated with COS cell supernatant containing murine gp39-CD8 at a 1 in 2 dilution that had been preincubated for 30 min at 4°C with the 40 μg/ml of the α-gp39 antibody MR1. Proliferation was measured during the final 6 hr of a 69 hr incubation as described in the Materials and Methods section. Results are presented as the mean $\pm$ S.E. of duplicate cultures. Comparable results were obtained in three separate experiments.

and B7-2 induction in B cells from CBA/N mice. However, it should be noted that in some experiments with the gp39 fusion protein cross-linked by soluble α-CD8 (data not shown) we observed defects in B7-2 but not in B7-1 induction. Overall, a defect in B7-1 induction was observed in seven of the 10 total induction experiments performed. The reason for this irreproducibility is not clear. Figure 1 also shows that the optimum dose of gp39 required for induction of B7-1 and B7-2 is approximately a 1 in 4 dilution of supernatant. Thus, subsequent experiments were performed using doses of gp39-CD8 supernatant ranging from 1 in 2 to 1 in 5.

A series of further experiments using a single dose of the gp39 fusion protein (Table 1) also indicates that there are defects in both B7-1 and B7-2 induction both in resting B cells and in total B cells. B cells treated with plate bound α-MHC I showed no induction of either B7-1 or B7-2 (data not shown), verifying that the effect observed was not caused by the CD8 portion of the fusion protein. Furthermore, use of a human gp39-CD8 fusion protein as a COS cell supernatant did not lead to increases in B7-1 or B7-2 expression (data not shown), arguing that the effect is due to the interaction of gp39 with CD40 and not due to other effects of the COS cell supernatant.

Induction of B7-1 and B7-2 was also measured upon treatment with LPS. As shown in Table 1, there is no defect in B7-1 or B7-2 induction upon LPS treatment; in fact, B7-2 induction is enhanced in CBA/N B cells. There does not therefore appear to be an intrinsic inability of CBA/N B cells to express B7 molecules on their surface.

In Table 1, it can be seen that when total B cells (activated plus resting) are analysed, higher B7-1 and B7-2 induction is observed with gp39 and with LPS than when resting B cells are used. This is true for both the CBA/N and CBA/J strains. Thus activated B cells appear more competent to upregulate costimulatory molecules both in response to LPS and to CD40 signalling, consistent with the improved antigen presentation ability of activated B cells. However, the partial defect in B7-1 and B7-2 induction observed upon gp39 treatment of resting B cells is also seen with total B cells from CBA/N mice.

We also examined proliferation of B cells from CBA/J and CBA/N mice upon treatment with the gp39 fusion protein. B cells from both mouse strains proliferate to this treatment; however, similar to the effects observed with B7-1 and B7-2 induction, B cells from CBA/N mice have a quantitative but not absolute defect in proliferation upon CD40 signalling (Fig. 2). Figure 2 also shows that proliferation is completely inhibited in both mouse strains by the addition of the α-gp39 antibody MR1 and that treatment with a COS cell supernatant containing the human gp39-CD8 fusion protein has no proliferative effect.

The kinetics of B7 induction in the two mouse strains were compared in order to verify that the impaired B7 induction observed could not be explained by differences in the kinetics of B7 induction in CBA/N and CBA/J mice. As shown in Fig. 3, the kinetics of B7-1 and B7-2 induction are virtually identical in the CBA/N and CBA/J mouse strains. These experiments also show impaired induction of both B7-1 and B7-2 in the CBA/N mouse strain, and thus further support the data shown in Fig. 1

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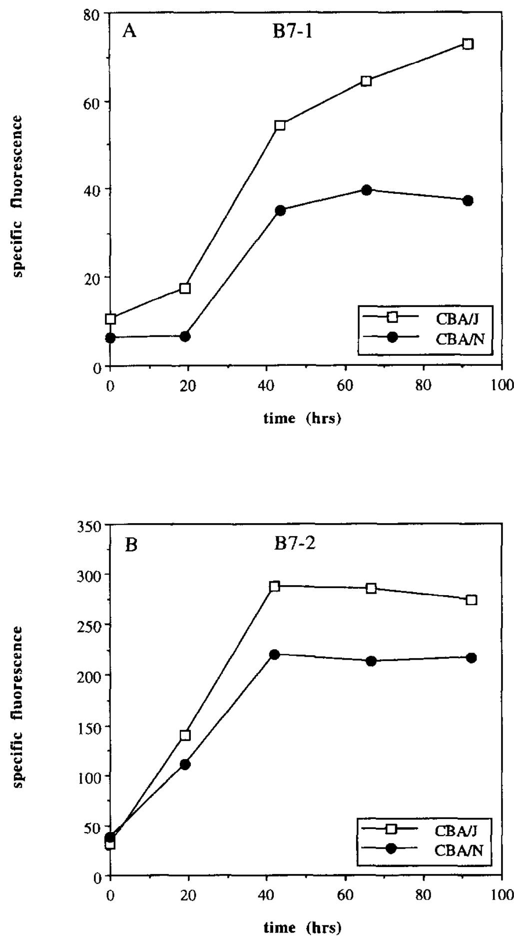

Fig. 3. B cells from CBA/J and CBA/N mice have indistinguishable kinetics of B7-1 and B7-2 induction. Total B cells from CBA/J (open squares) and CBA/N (closed circles) mice were cultured for 19–92 hr at 2×10^6/ml with a 1 in 4 dilution of COS cell supernatant containing the murine gp39-CD8 fusion protein and the α-CD8 antibody 53-6.72 at a final concentration of 10 μg/ml. Expression of B7-1 (A) and B7-2 (B) was determined by flow cytometry as described in the Materials and Methods section. Specific fluorescence is defined as the difference in mean fluorescence intensity between cells stained with the B7-specific antibody and an irrelevant antibody. Results shown are representative of two such experiments.

and Table 1. It therefore appears that the immunodeficiency in CBA/N mice, attributed to a point mutation in BTK, partially affects both B7-1 and B7-2 induction, with a more reproducible effect on B7-2.

The role of cAMP in B7-1 and B7-2 induction in normal mouse B cells

Previous studies have shown that the ability of a B lymphoma M12 to activate T cells is dependent on treatment of the B cells with db-cAMP (St Pierre *et al.*, 1989), a treatment which leads to the induction of B7-1 and B7-2 (Freeman *et al.*, 1993a; Nabavi *et al.*, 1992; Watts *et al.*, 1993). Treatment with db-cAMP is specific for cAMP as sodium butyrate alone has no effect on B7 induction (Watts *et al.*, 1993). It was therefore of interest to determine if cAMP would also induce B7 molecules in primary B cells. Treatment of primary B cells with db-cAMP proved to be lethal (data not shown). As shown in Fig. 4 however, treatment with the gp39 fusion protein followed by db-cAMP induces both B7-1 and B7-2 well on primary B cells from CBA/J mice. Primary B cells treated with the gp39 fusion protein cross-linked by plate bound α-CD8 for 64 hr showed moderate B7-1 and B7-2 induction (Fig. 4C, D). However, if db-cAMP was added for the final 38 hr of culture, dramatic induction of both B7-1 and B7-2 was observed (Fig. 4E, F). cAMP therefore plays a role in B7 induction in primary B cells as well as in M12 B lymphomas. However, it appears that normal B cells must first be primed by a signal through CD40 before they survive cAMP treatment. We were therefore interested to know whether B cells from CBA/N mice would be capable of responding to cAMP after priming by gp39. In fact, treating B cells from CBA/N mice with gp39 + db-cAMP is lethal, just as treatment with db-cAMP alone is lethal (data not shown). Thus it appears that B cells from CBA/N mice are not appropriately primed by a signal through CD40 to allow them subsequently to respond to cAMP treatment.

Treatment of primary B cells with PMA and ionomycin induces B7-2 but not B7-1

In experiments to investigate further mechanisms of B7 induction, primary B cells were treated with a combination of PMA and ionomycin. In contrast to results obtained with gp39, LPS or cAMP, treatment with PMA and ionomycin induced B7-2, but not B7-1 (Fig. 5A). The data shown are after 60 hr of treatment, at which time we observe optimal B7-1 induction with gp39 or LPS treatment. However, qualitatively similar results were obtained after 18, 24 or 40 hr of incubation (data not shown). The observation that PMA and ionomycin induced similar levels of B7-2 as were obtained with LPS, whereas LPS treatment also induced significant levels of B7-1, argues that the failure to detect B7-1 induction by PMA and ionomycin was not due to insensitivity of the detection method. The effect of adding PMA and ionomycin separately to the B cells was also tested. Figure 5B shows that PMA alone induces little or no B7-2 (MFI = 13.8), whereas ionomycin alone induces significant levels of B7-2 (MFI = 138). The combination of PMA and ionomycin, however, is synergistic for B7-2 induction (MFI = 352).

It has previously been shown that Ig cross-linking on murine B cells induces B7-2 but not B7-1 (Lenschow *et al.*, 1994). Because PMA and ionomycin mimic the effects of Ig signalling, our results are consistent with the above studies but differ from results with human B cells, where Ig cross-linking has been shown to induce both B7-1 and B7-2 (Boussiotis *et al.*, 1993).

DISCUSSION

In this report we have shown that there is a quantitative defect in both B7-1 and B7-2 induction in B cells from

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