Distinct Role of CD80 and CD86 in the Regulation of the Activation of B Cell and B Cell Lymphoma*

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To date, not much has been known regarding the role of CD80 and CD86 molecules in signaling of B cells. The CD28/CTLA4 ligands, CD80 (B7-1) and CD86 (B7-2), are expressed on the surface of freshly isolated splenic B cells, and their expression is up-regulated by lipopolysaccharides. In the present study, we have investigated whether signaling via CD80/CD86 could alter the proliferation and immunoglobulin synthesis of B cells. Splenic B cells were stimulated with lipopolysaccharides in the presence of anti-B7-1 (16-10A1) and anti-B7-2 (GL1) monoclonal antibodies (mAbs). Exciting features observed during the study were that cross-linking of CD86 with GL1 enhanced the proliferation and production of IgG1 and IgG2a isotypes. In contrast, anti-B7-1 (16-10A1) mAb could efficiently block the proliferation and production of IgG1 and IgG2a. Furthermore, GL1 mAb could also induce the secretion of IgG isotypes from B cell lymphomas. Importantly, 16-10A1 could retard the growth of lymphomas and favored the up-regulation of pro-apoptotic molecules caspase-3, caspase-8, Fas, FasL, Bak, and Bax and down-regulation of anti-apoptotic molecule Bcl-x(L). In contrast, GL1 augmented the level of anti-apoptotic molecules Bcl-w and Bcl-x(L) and decreased the levels of pro-apoptotic molecule caspase-8, thereby providing a novel insight into the mechanism whereby triggering through CD80 and CD86 could deliver regulatory signals. Thus, this study is the first demonstration of a distinct signaling event induced by CD80 and CD86 molecules in B cell lymphoma. Finally, the significance of the finding is that CD80 provided negative signal for the proliferation and IgG secretion of normal B cells and B cell lymphomas. In contrast, CD86 encouraged the activity of B cells.

Evidence from a variety of studies has suggested that the B cell contact with T helper cells is important for its optimal activation and responsiveness to cytokines during Ig secretion (1–3). Contact between B and T cells can be mediated by antigen presentation, as well as antigen-independent cell interaction by molecules known as adhesion (LFA-1, LFA-3, ICAM-1, etc.) and costimulatory molecules (CD80, CD86, CD40, etc.) (3–7). Signals from T cells induce two opposite fates in B cells: clonal expansion of B cells that bind specifically to foreign antigens and clonal deletion of equivalent B cells that bind self-antigen. The role of costimulatory molecules is very well established in the activation of T cells (3), but nothing has been determined definitively about how these molecules operate in the activation and differentiation of B cells (8–10).

The best defined co-stimulators to date are two structurally related proteins, CD80 and CD86 (11–14). Both of these play a major role in providing costimulation to T cells, leading to their proliferation, cytokine production, and development of effector functions. CD80 and CD86 could also serve as counter-receptors that transduce distinct signal to the antigen-presenting cells upon engagement by CD28 or CTLA-4. The intracellular domains of CD80 and CD86 are quite distinct and could mediate differential signal transduction. Such signaling could alter the B cells' ability to function as effector cells. The ability of the B cells to deliver the costimulatory signal to T cells by B7 molecules is very well established (15–17). In contrast, whether the engagement of CD80 and CD86 molecules by CD28 and CTLA-4 affect the function of the B cells is very poorly documented (9, 10). Moreover, nothing is known precisely in the case of B cell proliferation and differentiation by the engagement of CD80 and CD86 molecules. However, there is indirect evidence reported earlier that CD28-CTLA4/B7-signaling pathways may affect B cell responses and the regulation of immunoglobulin (Ig) synthesis (18–20). To compare the regulatory role of CD80 (B7-1) and CD86 (B7-2) molecules in humoral immune responses, B7-1$^{-/-}$ and B7-2$^{-/-}$ mice were generated and profound deficits in both antigen-specific IgG1 (Th2-dependent) and IgG2a (Th1-dependent) responses demonstrated that B7 costimulation can influence IgG production *in vivo* (21). However, the basis of this abnormal Ig production was contributed to the absence of Th1- or Th2-specific cytokines in B7-1/2$^{-/-}$ mice that regulate the IgG1 and IgG2a production.

Thus, the aim of our study was to evaluate for the first time the role of CD80- and CD86-mediated signal transduction in the costimulation of B cells. The results show that triggering through CD80 specifically inhibits the proliferation and IgG secretion by LPS[1]-stimulated B cells. By contrast, CD86 chiefly augments the B cell activity. Further, the growth of B cell lymphomas could also be retarded by CD80 cross-linking. Signaling through CD80 inhibited the growth of the B cell lymphoma by up-regulating the expression of pro-apoptotic molecules caspase-3, caspase-8, Fas, FasL, Bak, and Bax and down-regulating the levels of anti-apoptotic molecule Bcl-x(L). In

[1] The abbreviations used are: LPS, lipopolysaccharide; Ab, antibody; SN, supernatant; MFI, mean fluorescence intensity; RPA, ribonuclease protection assay; TNF, tumor necrosis factor; mAb, monoclonal antibody; IL, interleukin; IFN, interferon; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; BCR, B cell receptor; SSC, side scatter; FSC, forward scatter.

contrast, triggering through CD86 augmented the level of anti-apoptotic molecules Bcl-w and Bcl-x(L) and decreased the levels of caspase-8. Thus, the signals delivered by anti-B7-1 and -B7-2 antibodies can differentially regulate the activity of B cell and its lymphoma.

EXPERIMENTAL PROCEDURES

Animals

Female inbred BALB/c mice (8–10 weeks old) were obtained from the National Institute of Immunology (New Delhi, India).

Antigens, Antibodies, Lymphokines, and Reagents

Fetal calf serum was purchased from Sera-Lab (Crawley Down, United Kingdom), RPMI 1640 was from Invitrogen, L-glutamine and streptomycin were from Serva (Heidelberg, Germany), and recombinant IL-4 and IFN-γ were the products of Genzyme (Boston, MA). LPS (from *Salmonella typhosa*), mIgG1, mIgG2a, and rabbit anti-rat FITC-labeled Ab were procured from Sigma and goat anti-rat IgG F(ab)$_2$ from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-B7-1, CD40, Fas-FITC, FasL-biotin mAbs were purchased from PharMingen (San Diego, CA) and anti-B7-2 Ab was a gift from Dr. Vijay Kuchroo (Boston, MA). Rabbit anti-caspase-3 (CPP32) antibody was purchased from Santa Cruz Biotechnologies. The RPA kit was purchased from PharMingen. Enhanced chemiluminescence kit (ECL) was a product of Amersham Biosciences, Inc., and polyvinylidene difluoride membrane was from Bio-Rad.

Cell Lines and Hybridomas

The cell lines and hybridomas used in this study, *i.e.* CRL 1704, TIB 208, HB 188, and HB 170, were procured from American Type Culture Collection (ATCC; Rockville, MD).

Medium

Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (50 μg/ml), streptomycin (50 μg/ml), and 2-mercaptoethanol (0.05 mM).

Cell Preparation

B Cells—B cells were prepared as reported previously (2). Briefly, a single cell suspension of BALB/c mice spleens was prepared in balanced salt solution. The red blood cells were depleted by treatment with hemolytic Gey's solution. The adherent cells were removed by plating on the plastic Petri plates (Nunc, Roskilde, Denmark) for 2 h at 37 °C and 7% CO$_2$. Nonadherent cells were incubated in nylon wool column for 1 h at 37 °C in 7% CO$_2$. The adherent cells eluted from the column were treated sequentially on ice for 45 min each with a mixture of anti-Mac2 and anti-Mac3 Abs and a mixture containing anti-Thy1, L3T4, and CD8 Abs, followed by complement-mediated killing. The purity of cells stained with anti-IgM Ab was over 95%, as analyzed by FACS (Becton Dickinson, Mountain View, CA).

Expression of B7-1 and B7-2 on B Cells and WEHI-279—FACS analysis was done as mentioned previously (2). Briefly, 1,000,000 B cells (stimulated with 20 μg/ml LPS for 48 h) and WEHI-279 cells were incubated with the anti-B7-1, anti-B7-2, and CD40 Abs (1.0 μg) diluted in 100 μl of PBS plus 2% BSA for 1 h at 4 °C. The cells were then washed three times with PBS plus 2% BSA. Anti-rat FITC (1:135 dilution) was then added and the cells were further incubated for 1 h at 4 °C. The cells were washed five times with PBS and fixed in 1% paraformaldehyde and analyzed by FACS for the expression of B7-1 and B7-2 molecules. As a control for anti-B7-1 and anti-B7-2 Abs, the cells were also incubated with RtIg.

The cells from each suspension were acquired on CellQuest software for FACScan (Becton Dickinson). Debris in the cell suspension was excluded from the analysis by suitable gating that allowed the collection of data only from those light-scattering events (*i.e.* cells) of a size consistent with B cells. The analysis for the mean fluorescence intensity (MFI) was done on histograms, where abscissa and ordinate denote log FITC fluorescence and relative cell count, respectively.

B Cell Proliferation—B cells activated with 20 μg/ml lipopolysaccharide (5 × 10^3 to 1 × 10^5 cells/well) were incubated with anti-B7-1 and -B7-2 mAbs at a concentration of 0.01–1.0 μg/ml in a total volume of 200 μl of RPMI, 10% FCS at different (24–96 h) time intervals. The LPS-activated B cells (1 × 10^5/well) were also cultured with optimum dose of IFN-γ (10 units/ml) and IL-4 (100 units/ml) in the presence of different concentration of anti-B7 mAbs. The suitable controls containing B cells, LPS-activated B cells incubated with control RtIg, or B cells without antibodies were also kept. The cells were incubated at 37 °C in a

humidified atmosphere containing 7% CO$_2$. After the specified time period, the cultures were pulsed with 0.5 μCi of [^{3}H]thymidine/well. After 16 h, the plates were harvested and incorporation of thymidine was determined by using an automatic cell harvester (Skatron, Tranby, Norway) and liquid scintillation counting.

IgG1 and IgG2a Isotypes

The cultures were set as mentioned in B cell proliferation assays. The SNs were collected on day 6 from the experimental as well as control wells and were analyzed for IgG1 and IgG2a by enzyme-linked immunosorbent assay (2). Briefly, triplicate wells were coated overnight at 4 °C with 5 μg/ml goat anti-mouse IgG1 and IgG2a Abs in carbonate-bicarbonate buffer, pH 9.6. The unbound sites were blocked with 2% BSA, and then log 6 dilutions of culture SNs were added for 2 h at 37 °C. The plates were then incubated with rabbit anti-mouse IgG horseradish peroxidase-labeled antibody at 37 °C for 2 h. The usual steps of washings with PBS-Tween 20 were carried at each step. The color developed because of the substrate OPD was visualized at 492 nm. The concentration of Abs is represented as nanograms/ml, as computed by using standard mouse IgG1 and IgG2a isotypes.

Flow Cytometric Analysis of Intracellular IgG1 and IgG2a Synthesis

The B cells (5 × 10^5/ml) stimulated with LPS (20 μg/ml) were cultured with anti-B7-1 and -B7-2 mAbs (1.0 μg/ml) for 72 h. Four hours before harvesting the cells, brefeldin A (10 μg/ml) was added. The cells were harvested and resuspended in PBS containing brefeldin A and fixed by adding equal volume of 4% paraformaldehyde for 20 min. The cells were incubated for 10 min in PBS/BSA/saponin to permeabilize the cells. The cells were blocked with 0.5% rabbit sera and saponin and stained immediately for intracellular expression of IgG1 and IgG2a using a modified method described by Murphy *et al.* (22). The cells were incubated with anti-IgG1-biotin and anti-IgG2a-biotin (2 μg/ml) for 30 min and then with avidin-FITC (1:1000 dilution). The usual steps of washings were followed at each step using 1% BSA and 0.5% saponin. The last washing was done in PBS/BSA without saponin to allow membrane closure. The samples were analyzed by FACScan flow aptometer (Becton Dickinson). Results were analyzed by CellQuest software.

The Proliferation of WEHI-279 Lymphoma and the Secretion of IgG1 and IgG2a Isotypes

Different concentrations of WEHI-279 cells (5 × 10^2 to 5 × 10^3/well) were cultured with anti-B7-1, B7-2, and CD40 mAbs (0.01–1.0 μg/ml) in a total volume of 200 μl of RPMI, 10% FCS. The suitable controls containing WEHI-279 cells cultured in the presence or absence of LPS, control RtIg, or without antibodies were also kept. The cells were incubated for 72 h, pulsed with 0.5 μCi of [^{3}H]thymidine for another 4 h, and then processed for β-scintillation counting. Similar types of cultures were also kept for 4–5 days, and SNs were harvested and analyzed for IgG1 and IgG2a secretion as mentioned above.

The Expression of Fas and FasL Demonstrated by FACScan on the Surface of B Cells and WEHI-279 Lymphoma Stimulated with Anti-B7-1, B7-2, and CD40 Abs

B cells (LPS-stimulated) and WEHI-279 cells were cultured for 48 h with 1.0 μg/ml anti-B7-1, B7-2, and CD40 Abs and control RtIg in 200 μl of RPMI 1640 and 10% FCS. The cells were washed and labeled with anti-Fas-FITC and anti-FasL-biotin/avidin-FITC. As mentioned above, the expression was demonstrated by FACScan.

Demonstration of Apoptosis on B Cells and WEHI-279 Cells Induced by Anti-B7-1 and -B7-2 Abs

Apoptosis was detected by utilizing the method developed by Nicoletti (23). Briefly, B cells and WEHI-279 cells were stimulated with anti-B7-1 and -B7-2 Abs (1.0 μg/ml) for 48 h. The cells were further incubated with anti-Fas Ab (1.0 μg/ml) for a period of 24 h. The cells harvested from the cultures were centrifuged; the cell pellet was fixed in 0.5 ml of cold 70% ethanol at 4 °C for 1 h. The cells were centrifuged again and resuspended in 0.5 ml of RNase (1.0 mg/ml) and incubated at room temperature for 20 min. Finally, 30 μl of propidium iodide (0.1 mg/ml) was added into each sample and incubated for another 10 min in the dark at room temperature before cell cycle was analyzed by flow cytometry. Apoptosis in the cells was analyzed by a FACScan with cytomation, data acquisition, and software (lysis II) for red fluorescence. Initial identification of the cells was made with the help of FSC/SSC plots. The apoptotic cells were selected on the basis of the expression of higher SSC because of the condensation of the nuclear

chromatin. The debris was excluded on the basis of its very low SSC and FSC signals, and normal cells were gated and analyzed.

The Induction of the Expression of Pro-apoptotic and Anti-apoptotic Molecules after Cross-linking CD80 and CD86 Molecules on WEHI-279 Cells

Multi-probe Ribonuclease Protection Assay—The WEHI-279 cells were cultured with anti-CD80 Ab, anti-CD86 Ab, and RtIg Ab and cross-linked with anti-RtIg Ab as mentioned for the proliferation assay. Total RNA was extracted from WEHI-279 cells by the guanidinium isothiocyanate-phenol-chloroform method using Trizol reagent (Invitrogen). The expression was determined using the RiboQuant multiprobe RNase protection assay system (PharMingen) following the manufacturer's instruction (24). Briefly, 5 μg of total RNA was hybridized to ^{32}P-labeled RNA probes overnight at 56 °C, followed by treatment with RNase for 45 min at 30 °C. In each set we have included L32 (which encodes a ribosomal protein) and glyceraldehyde-3-phosphate dehydrogenase genes to serve as housekeeping gene controls. The samples were submitted to electrophoresis through a 8 M urea, 5% polyacrylamide gel. The probe lengths are greater than the "protected" fragment lengths; this is because of the presence of flanking sequences. The resulting resolved bands were imaged using a phosphorimager (Bio-Rad Molecular Imager FX). The normalized quantity for each band was obtained by dividing with L32 housekeeping gene control. The -fold activation (increase or decrease) was calculated by dividing the value of normalized quantity of the experimental samples with that of WEHI-279 cells cultured with medium alone.

Western Blotting—WEHI-279 cells were incubated with anti-B7-1 Ab or RtIg and cross-linked with anti-RtIg as mentioned for the proliferation assay. The experiment was done as mentioned previously (25, 26). Briefly, after incubation, cells were washed twice with ice-cold PBS and re-suspended in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml aprotinin, 1.0 μg/ml leupeptin, 2 mM dithiothreitol, 10 mM Tris-HCl, pH 8) for 5 min at 4 °C followed by protein determination with BCA method (Pierce). The protein (50 μg) was resolved on 12% polyacrylamide gel and was assayed by Western blotting using anti-caspase-3 Ab. The expression levels were analyzed using NIH Image software.

RESULTS

Expression of CD80 and CD86 on B Cells and WEHI-279 Cells

The FACScan revealed the presence of CD80 (MFI of 106.49) and CD86 (MFI of 155.80) molecules on B cells incubated with LPS for 48 h (Fig. 1*a*). We also evaluated the expression of CD80, CD86, and CD40 molecules on the B cell lymphoma, WEHI-279. WEHI-279 expressed CD80 (MFI of 190.09), CD86 (MFI of 111.51), and CD40 (MFI of 125.73) molecules (Fig. 1*b*). The RtIg used as control for anti-B7-1 and -B7-2 mAbs failed to show any significant shift in MFI.

Cross-linking of CD80 Inhibits but CD86 Increases the Proliferation of LPS-activated B Cells

We next determined whether triggering of CD80 and CD86 molecules by their respective mAbs will affect the proliferation and differentiation of LPS-activated B cells. The experiments were performed with different concentrations of responder cells and readout at different time points after stimulation. Surprisingly, cross-linking of CD80 with anti-B7-1 mAb (16-10A1) significantly inhibited the proliferation of different doses (5 × 10^3 to 1 × 10^5 cells/well) of LPS stimulated B cells from as early as 24 h of the cultures. The maximum decline (61.56%) in the proliferation was observed when the B cells were cultured at the concentration of 5 × 10^4 with 16-10A1 (1.0 μg/ml) for 48 h (Fig. 2*A*). It is worth to mention here that 16-10A1 was found to be highly potent for a range of 5 × 10^3 to 1 × 10^5 cells at a concentration of 1.0 μg/ml in significantly down-regulating the proliferation of B cells at any time period (24–96 h) of the study. The response was observed in a dose-dependent manner.

Unlike anti-B7-1 mAb, signaling by anti-B7-2 mAb (GL1) significantly enhanced the proliferation of LPS stimulated B cells (Fig. 2*B*). There was gradual increase in the proliferation
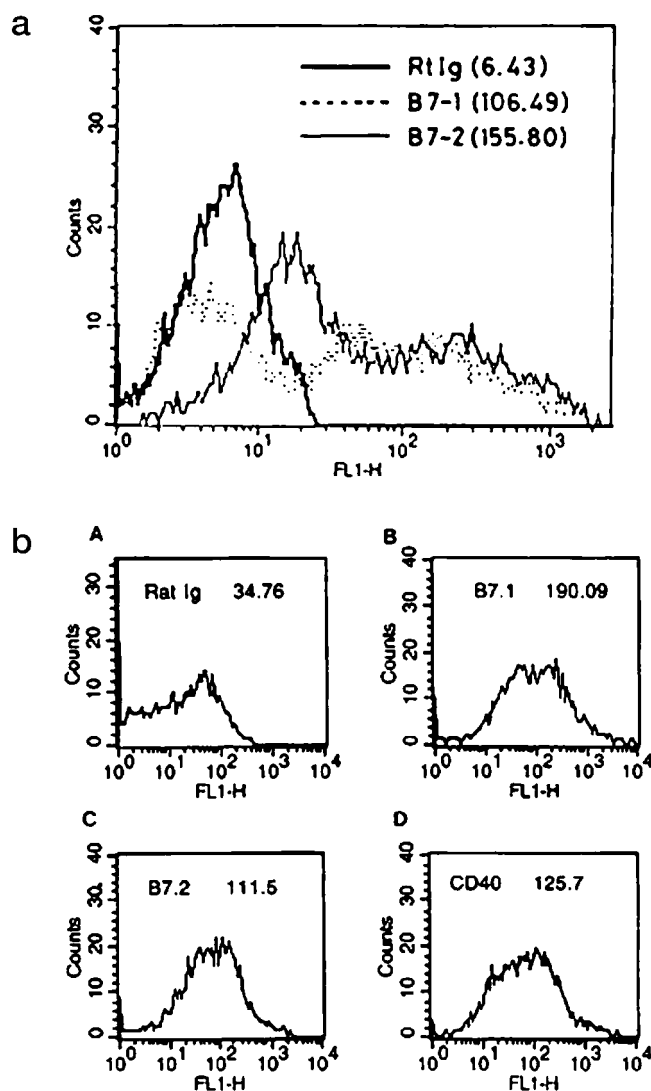

FIG. 1. **B cells and WEHI-279 express B7-1 and B7-2 molecules.** The B cells (stimulated with LPS) (*a*) and WEHI-279 (*b*) were stained with anti-B7 Abs and secondary anti-RtIg-FITC. In the case of WEHI-279, the data depict probing of the cells with RtIg (*A*), anti-B7-1 Ab (*B*), anti-B7-2 Ab (*C*), and anti-CD40 Ab (*D*). The expression of the molecules on the cells was analyzed by FACS. The data shown in the *parentheses* depict MFI. The figure is representative of at least three experiments.

of B cells, irrespective of cell concentration (5 × 10^3, 1 × 10^4, 5 × 10^4, and 1 × 10^5/well). The maximum and significantly high proliferation (97.86%) was observed when 1 × 10^5 cells/well were cultured with GL1 (1.0 μg/ml) for 48 h. In contrast, only 20.70% enhancement in the growth of B cells was observed when the B cells were cultured for 96 h. On account of over-proliferation and the death of the cells, the magnitude of proliferation declined by the end of 96 h.

No spectacular difference in the activity of LPS-activated B cell was noticed in the control cultures incubated with RtIg. B cells incubated with medium and anti-B7-1 and -B7-2 mAbs did not register any proliferation.

Regulation of the Secretion of IgG1 and IgG2a Isotypes by B Cells Stimulated with Anti-B7-1 and -B7-2 mAbs

Anti-B7-1 mAb Inhibits whereas Anti-B7-2 mAb Augments the Production of IgG Isotypes—We also evaluated the impact of signaling through CD80 and CD86 on the secretion of IgG1 and IgG2a isotypes. We have chosen to measure IgG1 and
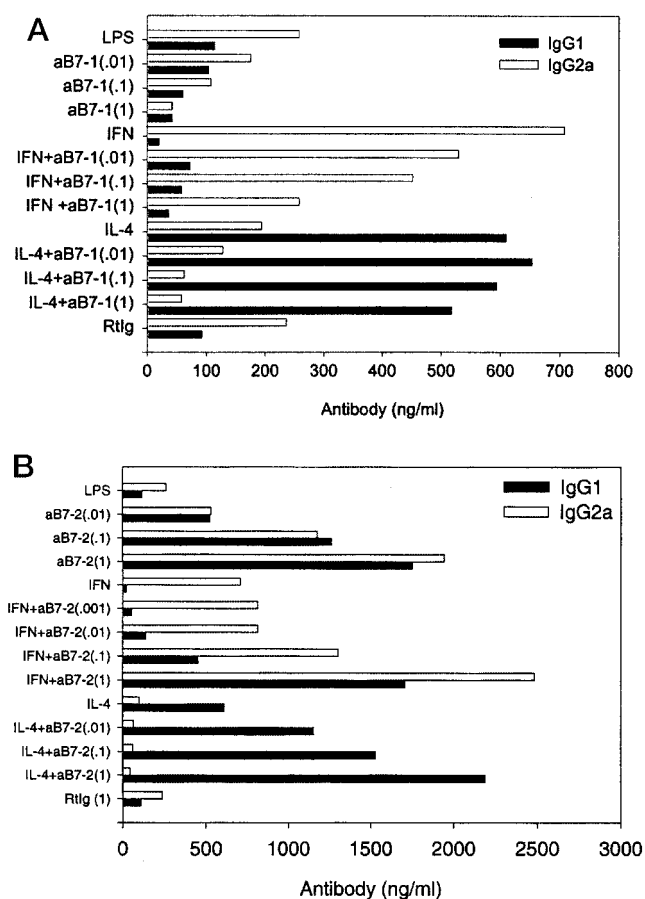
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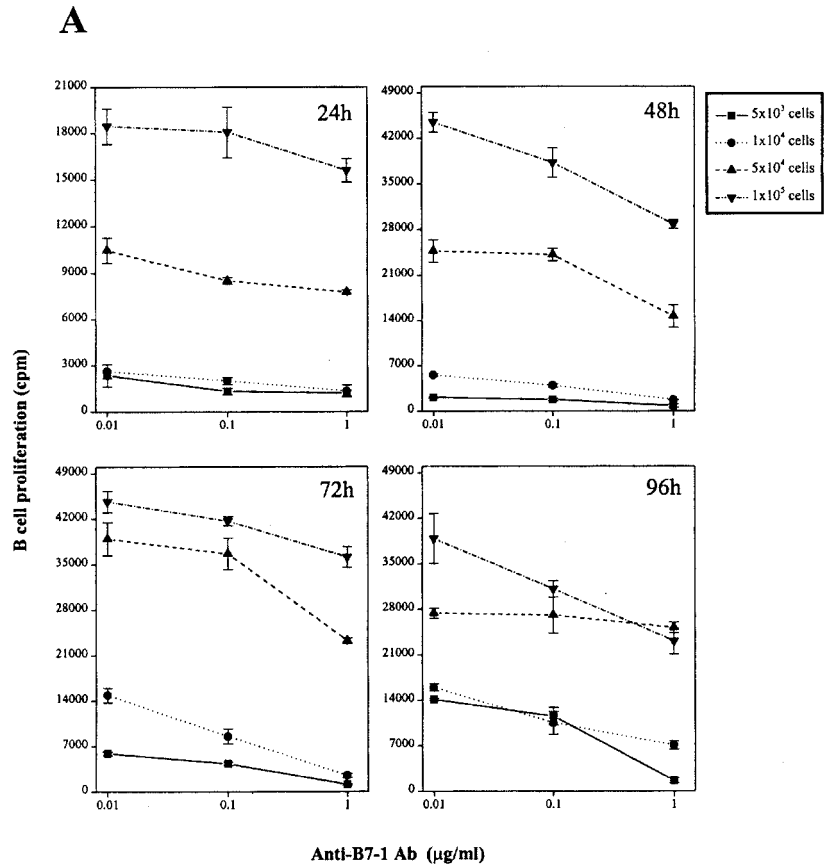
FIG. 2. **Anti-B7-1 mAb down-regulate but anti-B7-2 mAb enhances the proliferation of LPS-activated B cells.** Different concentration of B cells was incubated with anti-B7-1 (*A*) and anti-B7-2 Abs (*B*) and cross-linked with anti-Rt Ab (2 μg/ml). LPS (20 μg/ml) was also added, and the cultures were kept for various time periods. After completion of incubation, [^{3}H]thymidine (0.5 μCi/well) was added, the cells were harvested, and radioactivity incorporated was counted 16 h later. RtIg was used as a control for anti-B7-1 and -B7-2 Abs. B cells incubated either with medium or anti-B7-1 Ab or anti-B7-2 Ab or RtIg alone could not generate more than 2000 cpm. The data expressed as mean ± S.D. are the representative of at least three experiments.

B
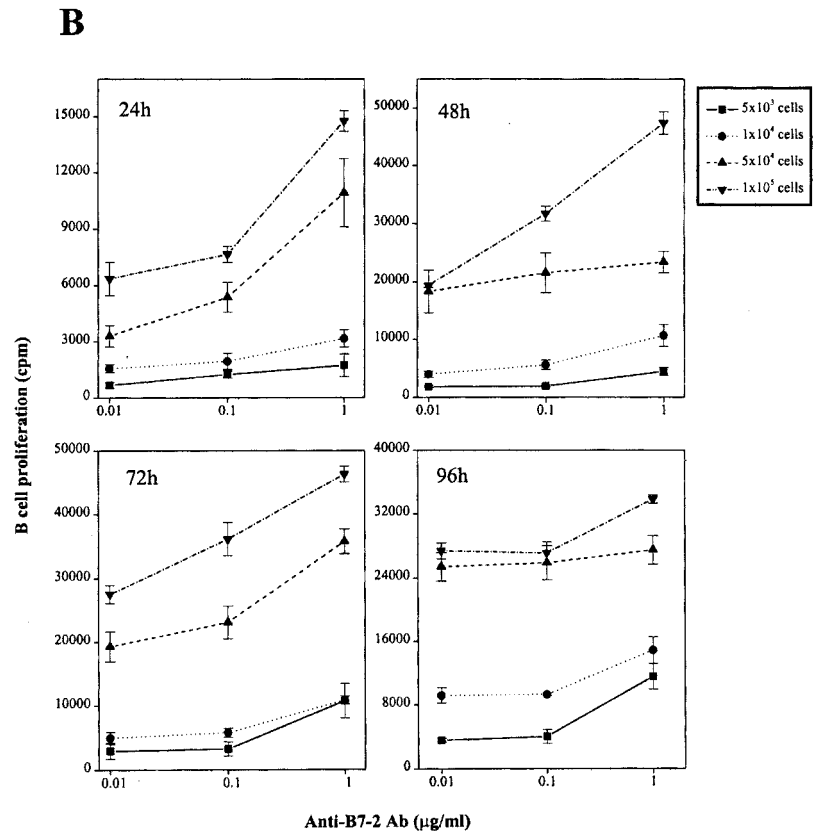
IgG2a isotypes because of their production precisely by IL-4 and IFN-γ, respectively (17). Interestingly, anti-B7-1 mAb down-regulated the production of both IgG1 and IgG2a (Fig. 3*A*). The concentration measured of IgG1 Ab was 114 ng/ml,

and that of IgG2a was 258 ng/ml, when the B cells were cultured with LPS. There was significant decline in IgG1 from 114 to 44 ng/ml and in IgG2a from 258 to 44 ng/ml, when anti-B7-1 Ab was added into the cultures (Fig. 3*A*). More interestingly,
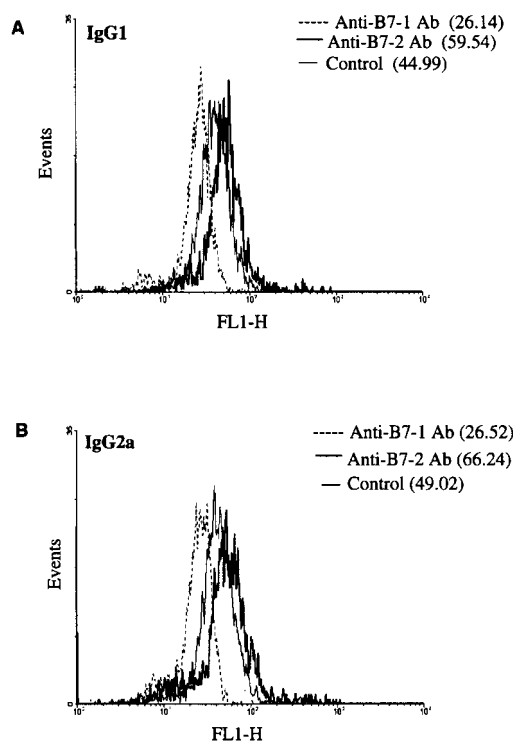
FIG. 4. **Anti-B7-1 declines whereas anti-B7-2 enhances the intracellular expression of IgG1 and IgG2a isotypes.** The culture conditions were same as those mentioned in Fig. 3. After cross-linking B cells with antibodies to B7-1 and B7-2 and culturing with LPS, intracellular staining was done using biotinylated anti-IgG1 and IgG2a Abs and avidin-FITC (for details, see "Experimental Procedures"). The analysis was done by FACScan. The figure is representative of at least three experiments.

FIG. 3. **Anti-B7-1 Ab inhibits but anti-B7-2 Ab augments the secretion of IgG1 and IgG2a isotypes by LPS stimulated B cells.** B cells (1×10^5/well) were incubated at 4 °C for 1 h with different concentrations of anti-B7-1 Ab (*A*) and anti-B7-2 Ab (*B*) and LPS (20 μg/ml), IL-4 (100 units/ml), and IFN-γ (10 units/ml). Anti-B7-1 Ab and anti-B7-2 Ab were also present throughout the culture period. The cells were incubated for 5–6 days, the supernatants were collected from triplicate wells of control and experimental cultures, and IgG1 and IgG2a were monitored by enzyme-linked immunosorbent assay. B cells cultured with anti-B7-1 Ab in the absence of LPS could not generate Ab response. The data (shown as nanograms/ml) are representative of at least three similar experiments.

anti-B7-2 Ab critically increased the production of IgG1 (from 116 to 1750 ng/ml) and IgG2a (from 260 to 1944 ng/ml) isotypes, when cocultured with LPS-stimulated B cells (Fig. 3*B*).

The inhibitory signals delivered by anti-B7-1 mAb (1.0 μg/ml) were powerful enough to down-regulate the secretion of IgG1 and IgG2a induced by IL-4 (IgG1; from 610 to 518 ng/ml) and IFN-γ (IgG2a; from 708 to 258 ng/ml), respectively (Fig. 3*A*). However, in the case of anti-B7-2 Ab (1.0 mg/ml) stimulation, IL-4 (100 units/ml) and IFN-γ (10 units/ml) worked in a synergistic fashion with the Ab and augmented the yield of IgG1 (from 610 to 2188 ng/ml) and IgG2a (from 710 to 2482 ng/ml), respectively (Fig. 3*B*). Anti-IL-4 and IFN-γ Abs could not block the production of IgG1 and IgG2a isotypes, respectively, induced by anti-B7-2 mAb (data not shown). The mAbs worked in a dose-dependent manner, and the maximum activity was attained at a concentration of 1.0 μg/ml. The control RtIg used in the cultures could not influence any change in B cell activity.

Cross-linking of CD80 Down-regulates but Signaling through CD86 Increases the Intracellular Expression of IgG1 and IgG2a Isotypes

We also observed the distinct effect on the intracellular expression of IgG1 and IgG2a isotypes upon cross-linking CD80

and CD86 molecules with their respective mAbs. Similar results were seen as observed in the case of IgG secretion. Anti-B7-1 mAb diminished, whereas anti-B7-2 mAb enhanced, the intracellular exhibition of IgG1 and IgG2a-isotypes (Fig. 4).

CD80 Cross-linking Fails to Alter the Viability of B Cells

It was of concern for us whether the decrease in the proliferation in B cells is a direct result of the death of the cells induced by anti-B7-1 mAb stimulus or the inhibition in the DNA synthesis. Therefore, we co-cultured LPS-stimulated B cells with anti-B7-1 mAb and then monitored the viability of cells by propidium iodide staining. It was ascertained that anti-B7-1 mAb signaling did not alter the viability of the cells (Fig. 5). No change in the expression of Fas and FasL was also observed on B cells (data not shown).

Effect of Cross-linking of CD80, CD86, and CD40 Molecules on B Cell Lymphoma

Inhibition of the Proliferation of WEHI-279 by Anti-B7-1 and CD40 Abs—Because the CD40 molecule has differential effect on normal B cells and B cell lymphomas (27), we evaluated the signaling capacity of CD80, CD86, and CD40 molecules in the proliferation of WEHI-279 B cell lymphoma (Fig. 6). Interestingly, anti-B7-1 and CD40 mAbs but not anti-B7-2 mAb could significantly down-regulate the growth of WEHI-279 cells. The dose of 1.0 μg/ml anti-B7-1 mAb was highly potent in inhibiting the proliferation of 500 and 5000 cells up to 43 and 29%, respectively (Fig. 6, *A* and *B*). Similarly, the extent of anti-CD40 mAb mediated inhibition was comparable with the effect induced by anti-B7-1 mAb. It has also been reported previously that anti-CD40 mAb renders WEHI-279 cells sensitive for Fas-mediated killing (14). However, for the first time we have
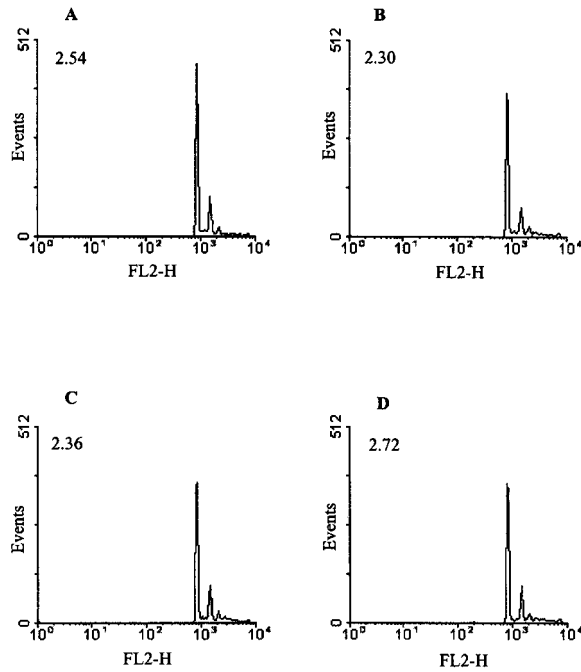
FIG. 5. **Cross-linking of B7-1 do not induce any change in the viability of B cells.** B cells were incubated with LPS (*A*), control RtIg (*B*), anti-B7-1 Ab (*C*), and anti-B7-2 Ab (*D*) for 48 h and then with anti-Fas Ab for 14 h. The cells were stained with propidium iodide and apoptosis was monitored by FACS analysis (for details, see "Experimental Procedures"). The data shown in *parentheses* represent the percentage of the positive apoptotic cells. The figure is representative of at least three experiments.

demonstrated that anti-CD40 and anti-B7-1 mAbs show synergistic effect. Both the Abs, when added into the cultures, inhibited the proliferation of WEHI-279 by 83 and 48% for the two doses of the cells used in the experiments, respectively (Fig. 6, *A* and *B*). The control cultures containing RtIg did not induce any significant change in the growth of the cells.
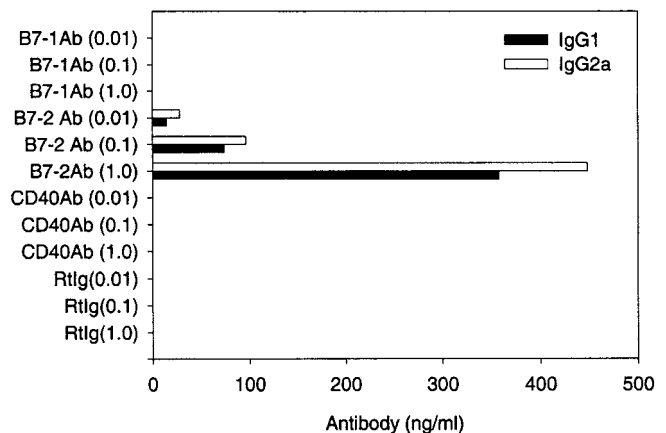
Induction of the Secretion of IgG1 and IgG2a Isotypes by Anti-B7-2 mAb-stimulated WEHI-279 Cells—Because anti-B7-1 and -B7-2 mAbs could regulate the proliferative response in B cells and WEHI-279 lymphoma and the differentiation events in B cells, we were therefore curious to know whether these antibodies could exert any impact on the secretion of IgG1 and IgG2a by WEHI-279 cells. Accordingly, we monitored the secretion of IgG1 and IgG2a isotypes, in the cultures of WEHI-279, stimulated with antibodies to CD80 (B7-1), CD86 (B7-2), and CD40 molecules. WEHI-279 cells cultured with medium alone or with LPS failed to show detectable level of IgG-isotypes. Most surprisingly, anti-B7-2 mAb could significantly induce WEHI-279 cells to secrete IgG1 and IgG2a (Fig. 7). However, anti-B7-1 and CD40 mAbs completely failed to stimulate WEHI-279 cells to produce IgG isotypes. Similar effects were also observed in the case of A20 lymphoma (data not shown). The control cultures comprising WEHI-279 cells and RtIg could not induce any change.

Cross-linking of CD80 and CD40 but Not CD86 Induces the Expression of Fas and FasL on WEHI-279

Because anti-B7-1 and CD40 mAbs exerted decline in the growth of WEHI-279, it became essential for us to monitor the cause of this response. We therefore did flow cytometry to explore the possibility of the expression of Fas and FasL on WEHI-279 cells incubated with anti-B7-1, B7-2, and CD40 Abs (Fig. 8). Importantly, anti-B7-1 and CD40 mAbs but not anti-B7-2 mAb could successfully augment the expression of Fas and FasL on WEHI-279 cells (Fig. 8, *a* and *b*). The control
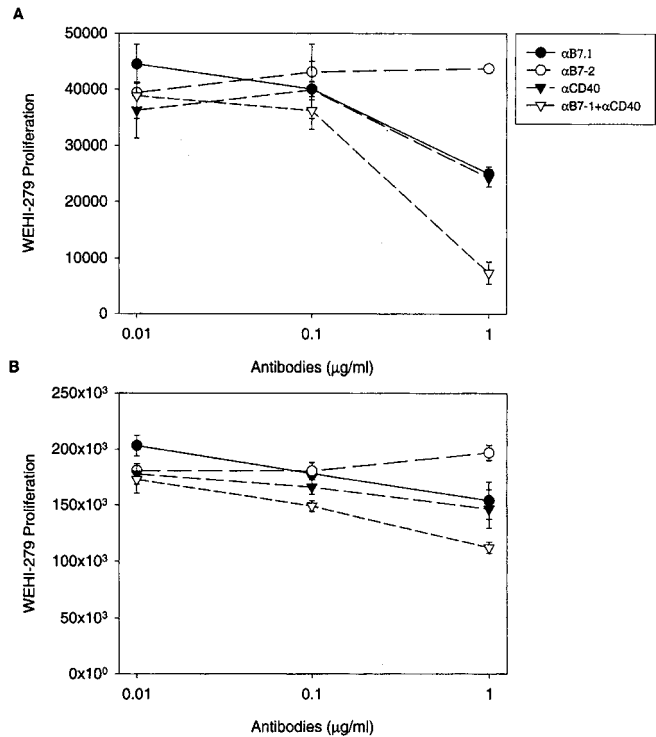
FIG. 6. **Anti-B7-1 and CD40 Abs arrest the growth of WEHI-279 lymphomas.** Anti-B7-1, anti-B7-2, and CD40 Abs (1.0 μg/ml) were incubated with 5×10^2 cells/well (*A*) and 5×10^3 cells/well (*B*) of WEHI-279 lymphoma. The cultures were kept for 72 h, and the proliferation was monitored at the last 6 h of cultures by [³H]thymidine incorporation. WEHI-279 cultured with medium alone showed 43,890 ± 3485 cpm and 21,7177 ± 7532 cpm for 5×10^2 cells/well and 5×10^3 cells/well, respectively. In control cultures, RtIg did not register any detectable change. Results expressed are the mean ± S.D. of three experiments.


FIG. 7. **Signals by anti-B7-2 Ab but not anti-B7-1 and CD40 Abs induces the production of IgG1 and IgG2a isotypes by WEHI-279 cells.** WEHI-279 (5×10^3 cells/well) was cultured with different concentrations of anti-B7-1 (0.01–1.0 μg/ml), anti-B7-2 (0.01–1.0 μg/ml), and CD40 (0.01–1.0 μg/ml) Abs; after 5 days, the SNs from the control and experimental wells were collected and IgG1 and IgG2a were measured as mentioned in legend to Fig. 3. WEHI-279 cells cultured with medium alone or with control RtIg could not evoke the secretion of the isotypes. The data are representative of three similar experiments.

cultures involving RtIg did not display any change in Fas and FasL expression.

Cross-linking of CD80 Induces WEHI-279 Cells to Undergo Apoptosis

We finally analyzed the functional activity of Fas and FasL induced on the surface of WEHI-279 cells by propidium iodide

a

b
Fas Ligand
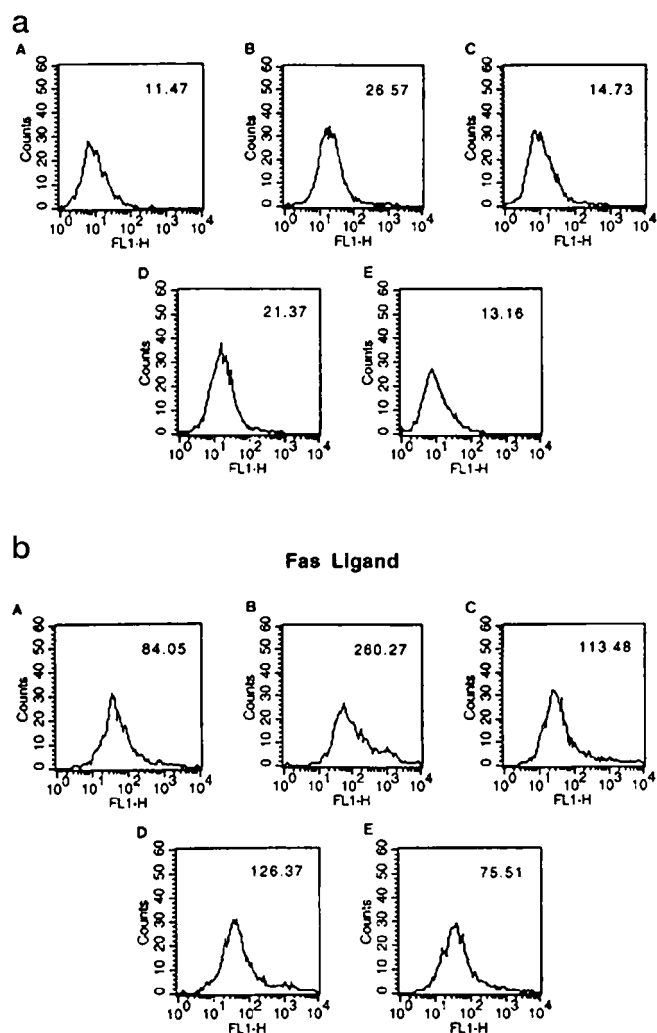
FIG. 8. **Anti-B7-1 and CD40 Abs but not anti-B7-2 Ab induces the expression of Fas and FasL on WEHI-279 lymphomas.** The cells were incubated for 48 h with medium (*A*), anti-B7-1 (*B*), B7-2 (*C*), or CD40 (*D*) Abs and control RtIg (*E*). The expression of Fas (*a*) and FasL (*b*) was evaluated by staining with their respective Abs and analyzed by FACS. The data shown in the *parentheses* depict MFI. The figure is representative of at least three experiments.
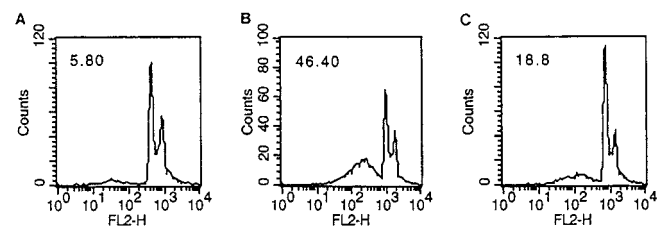
FIG. 9. **Cross-linking by anti-B7-1 Ab induces apoptosis of WEHI-279 lymphoma.** The cells were incubated with medium (*A*), anti-B7-1 Ab (*B*), or anti-B7-2 Ab (*C*) for 48 h and then with anti-Fas Ab for 14 h. The cells were stained with propidium iodide, and apoptosis was monitored by FACS. The data shown in *parentheses* represent percentage of positive apoptotic cells. The figure is representative of at least two experiments.

staining. Interestingly, WEHI-279 cells incubated with anti-Fas mAb in the absence of CD80 cross-linking showed a MFI of 5.80 (Fig. 9*A*). However, cross-linking of CD80 into the cultures exhibited 8.00-fold increase (MFI of 46.40) in the number of cells undergoing apoptosis (Fig. 9*B*). CD86 cross-linking could induce only marginal change (Fig. 9*C*). We also observed the DNA-ladder formation when CD80 was cross-linked (data not shown).

a

b
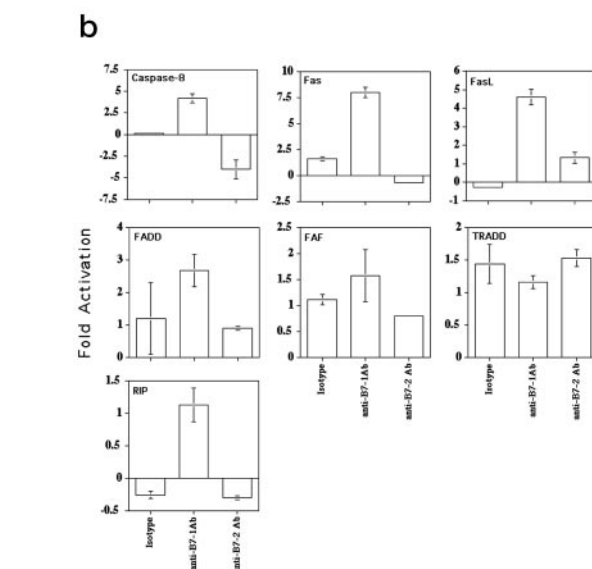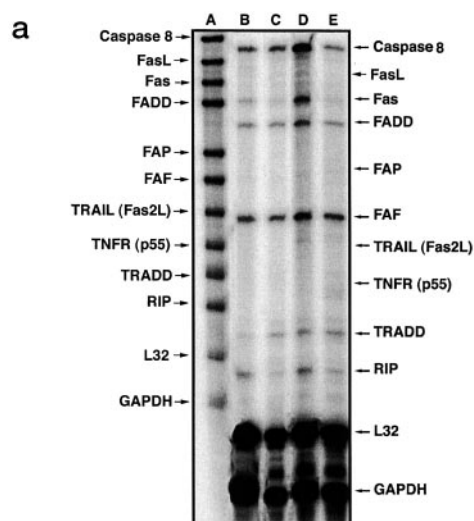
FIG. 10. **Signaling through CD80 enhances the expression of pro-apoptotic molecules.** A multiprobe ribonuclease protection assay was performed to detect the expression of apoptosis-regulatory molecules in WEHI-279 cells. Five micrograms of RNA was analyzed in each *lane*. *Lane A*, probe; *lanes B–E*, cells cultured with medium (*B*), RtIg (*C*), anti-CD80 Ab (*D*), or anti-CD86 Ab (*E*). Glyceraldehyde-3-phosphate dehydrogenase and L32 were used to serve as housekeeping gene controls. The results shown are representative of two experiments (*A*). Histogram for the normalized quantity for each band was obtained by dividing with L32 housekeeping gene control. The -fold activation was calculated by dividing the value of normalized quantity of the experiment with WEHI-279 cells cultured with medium alone. The results expressed as mean ± S.D. are obtained from two experiments (*B*).

Cross-linking of CD80 Enhances the Expression of Pro-apoptotic Molecules, whereas CD86 Up-regulates the Level of Anti-apoptotic Molecules

Finally, we tested the involvement of the apoptosis-regulatory molecules representative from several classes of proteins, namely caspase-3, caspase-8, Fas, FasL, Fas-associated death domain, Fas-associated phosphatase, Fas-associated factor, TNF-related apoptosis-inducing ligand (Fas2L), tumor necrosis factor (TNF) receptor (p55), TNF receptor-associated death domain, receptor inhibitory protein, Bak, Bax, Bad, Bcl-w, Bfl-1, Bcl-x$_L$, and Bcl-2 (Figs. 10–12). The expression of the molecules was analyzed by flow cytometry (Fig. 8), ribonuclease protection assay (RPA) (Figs. 10 and 11), and Western blotting (Fig. 12). Anti-CD80 Ab mediated signaling signifi-
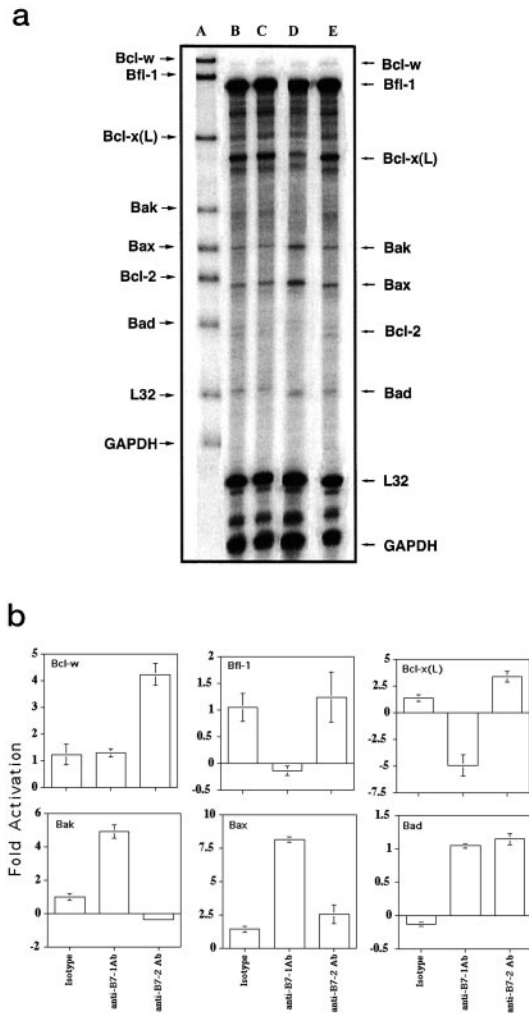
FIG. 11. **Signaling through CD86 enhances the expression of anti-apoptotic molecules.** A multiprobe ribonuclease protection assay was performed to detect the expression of apoptosis-regulatory molecules in WEHI-279 cells. Five micrograms of RNA was analyzed in each track. *Lane A*, probe; *lanes B–E*, cells cultured with medium (*B*), RtIg (*C*), anti-CD80 Ab (*D*), or anti-CD86 Ab (*E*). Glyceraldehyde-3-phosphate dehydrogenase and L32 were used to serve as housekeeping gene controls. The results shown are the representative of two assays (*A*). Histogram for the normalized quantity for each band was obtained by diving with L32 housekeeping gene control. The -fold increase or decrease was calculated by dividing the value of normalized quantity of test samples with that of WEHI-279 cells cultured with medium alone. The results expressed as mean ± S.D. are obtained from two experiments (*B*).
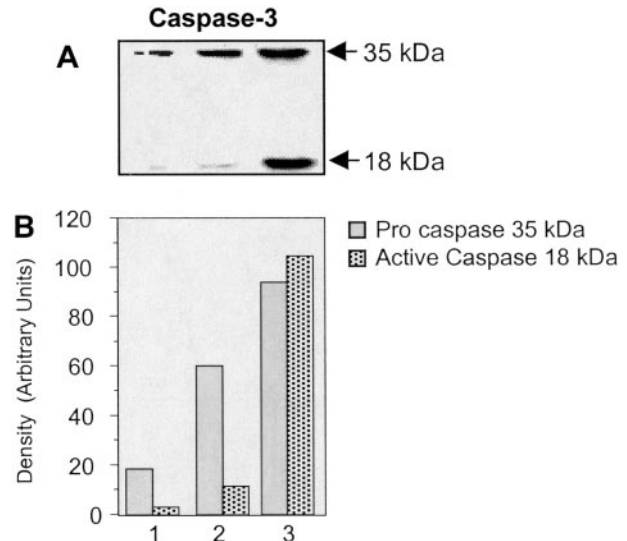

FIG. 12. **Signaling through CD80 up-regulates the expression of caspase-3.** The cell extracts obtained from the WEHI-279 cells cultured with medium (*lane 1*), RtIg (*lane 2*), and anti-CD80 Ab (*lane 3*) was resolved on 12% polyacrylamide gel and was assayed by Western blotting using anti-caspase-3 Ab (*A*). The histogram represents the density of expression levels (arbitrary units) obtained by analyzing with NIH Image software (*B*). The results shown are obtained from a single experiment.

cantly and reproducibly up-regulated the levels of caspase-3, caspase-8, Fas, FasL, Bak, and Bax (Figs. 10–12). Maximum increase was observed in the case of Bax (8.16-fold) followed by Fas (8.0-fold), Bak (4.95-fold), FasL (4.6), and caspase-8 (4.16) (Figs. 10 (*a* and *b*) and 11 (*a* and *b*)). Further, stimulation through CD80 molecule could also decrease the expression of anti-apoptotic molecules Bcl-x(L) (4.95). The expression of other pro-apoptotic (namely Fas-associated death domain, Fas-associated phosphatase, Fas-associated factor, TNF-related apoptosis-inducing ligand, TNF receptor, TNF receptor-associated death domain, receptor inhibitory protein, and Bad) and anti-apoptotic (Bcl-w, Bfl-1, Bcl-2) molecules remained unaltered. In contrast, CD86 mediated signaling significantly enhanced the expression of anti-apoptotic molecules Bcl-w (4.23-fold) and Bcl-x$_L$ (3.36-fold) (Fig. 11, *a* and *b*). Potent decrease in the level of pro-apoptotic marker caspase-8 (4.04-fold) and marginal down-regulation of Bak was also noticed.

DISCUSSION

An array of costimulatory molecules, *e.g.* ICAM-1, LFA-1, VCAM-1, HSA, CD40, and B7 (1–5), are expressed on the surface of B cells. Their role has gained considerable significance in the activation of T cells, but very little is known in connection with the activation of B cells (7, 8). The exact mechanism of sequence of signals provided by different costimulatory molecules in the stimulation and inhibition of B cells is largely unknown.

In the present study, we have attempted to analyze the distinct role of CD80 and CD86 costimulatory molecules in the activation and differentiation of B cells. The following five major findings have emerged from this study. 1) Cross-linking by anti-B7-1 mAb (16-10A1) inhibited the proliferation and differentiation of B cells; 2) anti-B7-2 mAb (GL1) promoted the growth and differentiation of B cells; 3) anti-B7-1 and CD40 mAbs acted synergistically and declined the growth of B cell lymphomas; 4) induction of secretion of IgG isotypes by WEHI-279 cells was triggered with anti-B7-2 mAb; 5) anti-B7-1 mAbs induced apoptosis in B cell lymphoma by enhancing the expression of caspase-3, caspase-8, Fas, FasL, Bak, and Bax and decreasing the expression of anti-apoptotic molecules Bcl-x(L).

Our findings suggest that CD80 and CD86 costimulatory molecules are responsible for delivering the signals essential for the inhibition and expansion of B cells, respectively. Hence it may be inferred that anti-B7-1 mAb provides a type of inhibitory signals to B cells similar to those delivered by CTLA-4 to T cells (28). Different studies have suggested a role of CD28 and CTLA-4 in the regulation of Ig synthesis. Defective production of switched Ig isotypes is seen both in mice treated with soluble CTLA-4 and in mice transgenic for CTLA-4 (16, 29, 30). CD28-deficient mice have reduced basal levels of IgG1 and IgG2a and diminished immunoglobulin class switching (15). Further, *in vivo* blocking of CD86 with anti-B7-2 mAb has shown the arrest of the germinal center formation and resulted in the reduction in primary antibody production (31). These results are in line with our observations about the negative signal transduction through CD80. To best of our knowledge, this is the first report regarding the role of CD80 and CD86

molecules in influencing the B cell activity. In the present study, we used a T-independent system and preferred to ligate CD80 and CD86 with their respective mAbs to rule out the possibility of interference of other accessory signals when B cell interacts with T cell. As compared with CD28, CTLA-4 molecule binds to CD80 with 20-fold higher avidity. It is also known that signaling through CTLA-4 can function to suppress the production of cytokines produced by T-helper cells that helps the differentiation of B cells (32). CD86 is also known to bind to CTLA-4 but with low affinity as compared with CD80. Similarly, interaction of CD86 with CD28 has high affinity, and delivers positive signals for T cell activation. Further, the property of CD80 in delivering inhibitory signals may also be related to its slower off-rate for CTLA-4 binding, thereby providing ample time to deliver signaling events necessary for restraining B cells from being activated (33).

The kinetics of rapid and high level of expression of CD86 in early immune responses and the delayed expression of CD80 on antigen presenting cells can be viewed as necessary activation and inhibitory signals, respectively, delivered by these molecules during the immune response (34).

Another important conclusion that can be drawn from our data is that the IL-4- and IFN-γ-induced secretion of IgG1 and IgG2a, respectively, could be inhibited by anti-B7-1 Ab. IL-4 and IFN-γ acted synergistically with anti-B7-2 Ab and augmented the production of IgG1 and IgG2a, respectively. Interestingly, IL-4 could inhibit the secretion of IgG2a induced by anti-B7-2 Ab but IFN-γ failed to obstruct the secretion of IgG1, even though the latter has antagonistic property for IL-4. At present, this activity of IL-4 and IFN-γ is difficult to explain. However, it has been reported that IL-4 induces the secretion of IgG1 by LPS-activated B cells but inhibits the formation of IgG2a. In contrast, IFN-γ promotes the yield of IgG2a and down-regulated the production of IgG1 (17).

In the present study, B7-1 and B7-2 served as counter-receptors that transduced distinct signals to B cells upon engagement with their respective antibodies. The differential signaling effect may also be because of the dissimilarity in the structure and, in particular, the nature of their cytoplasmic tail (18). Especially B7-2 has a significant cytoplasmic tail, which has a markedly different cytosolic domain from that of B7-1 and contains a tyrosine residue. Further, unlike B7-1, the cytoplasmic domain of B7-2 contains three potential sites for phosphorylation by protein kinase C (35–37). The structure of B7-1 has a short cytoplasmic tail and does not predict intrinsic tyrosine kinase activity; therefore, if B7-1 must transduce signals, specific tyrosine kinases must associate with the receptor (38, 39). Earlier studies have suggested the transmembrane signaling through B7/BB1 expressed on T lymphocytes (40). Finally, it has been observed recently that the two regions in CD80 cytoplasmic tail regulate T cell costimulation and CD80 redistribution. A 30-kDa phosphoprotein has been found to be associated with the cytoplasmic tail of CD80 after cell activation, suggesting it may play a role in CD80 function (41). In line with these observations, it has been demonstrated that CD80 and CD86 can mediate differential signal transduction, and such signaling could distinctly regulate the B cell's function as an effector cell (7, 8, 19, 20).

Our experiments also demonstrate that the augmentation or inhibition of B cell activity is a concerted effect of the signaling by B7 molecules and cytokines. It was still of concern that this activity may be because of survival/death status only, and all changes in levels of Ig production and costimulation simply lead to further enhancement or decrease in B cell survival. To address the issue, the experiments were carried to study the role of anti-B7 Abs in affect-

ing B cell viability and trans-membrane signaling. The triggering of B cells by anti-B7-1 or anti-B7-2 mAbs could not cast any impact on the viability of normal B cells, as evidenced by propidium iodide staining. Further, CD80 cross-linking could not induce Fas or FasL expression on the surface of B cells. Furthermore, we established categorically that this event of B cell activity is because of signaling, as evidenced by rapid alteration in phosphorylation of tyrosine residues, and not because of overproliferation/death by CD86 cross-linking (data not shown). This was further substantiated by differential regulation of intracellular expression of IgG1 and IgG2a by CD80 and CD86 ligation. These experiments very clearly demonstrate that isotype production is not a mere function of proliferation/survival of B cell, rather it is because of delivery of stimulatory or inhibitory signals through CD80 and CD86 costimulatory molecules, respectively.

To further provide weight to our findings of the regulatory capacity of anti-B7-1 and -B7-2 Abs in the activation of B cells, we utilized B cell lymphomas, WEHI-279, which express CD80, CD86, and CD40 molecules. More surprisingly, anti-CD80 and CD40 Abs could significantly arrest the proliferation of WEHI-279. Thus it may be concluded that the signaling via CD80 and CD40 molecules may be responsible for inhibiting the growth of B cell lymphomas. In contrast, costimulation through CD86 may help lymphomas to proliferate and secrete antibodies that may help lymphomas to evade immune surveillance. Further, triggering through CD80 and CD40 may help the regression of the lymphomas in the early stages. Although signaling through CD80 retards the growth of B cell lymphomas by inducing the expression of Fas and FasL and in turn induces apoptosis, it fails to induce Fas and FasL or apoptosis in normal B cells. At present, it is difficult to explain why ligation of B7-1 with anti-B7-1 has a differential effect on normal *versus* tumor B cells. This could be because of the involvement of the distinct domains of CD80 in normal B cells and B lymphomas. Moreover, this observation is in agreement with the fact that, in the case of CD40 molecule, antibodies to CD40 stimulate normal B-lymphocytes but inhibit proliferation of B cell lymphoma (27). Further, binding of CD40 ligand to CD40 on a murine B lymphoma induces B7-1 and inhibits cell growth (12, 14). The dual nature of signaling events has also been reported in the case of BCR triggering. It has been shown that BCR signaling induces apoptosis in germinal center B cells but not in peripheral B cells. Mature germinal center B cells can re-acquire sensitivity to BCR-induced cell death following CD40 ligation. In contrast, neither virgin nor memory B cells become susceptible to antigen receptor-triggered apoptosis upon CD40 stimulation (42).

Apart from demonstrating the expression of Fas and FasL by FACScan and their involvement in inducing apoptosis, we also monitored by ribonuclease protection assay the involvement of CD80 and CD86 molecules in the regulation of the activation of several pro- and anti-apoptotic molecules in WEHI-279 B cell lymphoma. We have observed that signaling through CD80 molecule augmented the levels of pro-apoptotic molecules, *i.e.* caspase-3, caspase-8, Fas, FasL, Bak, and Bax. This suggests that CD80 signaling induces apoptosis in WEHI-279 via mechanism involving pro-apoptotic molecules caspase-3, caspase-8, Fas, FasL, Bak, and Bax, thus rendering WEHI-279 cells more vulnerable to apoptosis and therefore restricting the progression of the lymphoma. The T cell co-stimulatory molecule CD28, which has a high and low binding affinity for CD86 and CD80, respectively, promotes T cell survival by up-regulating Bcl-x(L) and down-regulating FasL expression (43). Based on these results and our findings, it may be postulated that there

may exist a probability that, when CD28 is ligated with CD86, it may deliver a protective signal necessary for the clonal expansion of B cells. In contrast to this, when CTLA-4 gets engaged with CD80, it may deliver a lethal signal responsible for controlling the clonal expansion of B cell and B cell lymphomas. Further, overexpression of caspases is sufficient to cause apoptosis and Bax have been correlated with disease progression and shorter survival of B cell chronic lymphocytic leukemia patients (44, 45). In contrast, signaling through CD86 increased the expression of anti-apoptotic molecules Bcl-w and Bcl-x(L). Thus, there may be a possibility that ligation of B7-2 on WEHI-279 may promote their survival by increasing the expression of anti-apoptotic proteins Bcl-w and Bcl-x(L). It has also been reported earlier that increased expression of Bcl-x(L) but not Bcl-2 could prevent the apoptosis in B cell lymphoma WEHI-231 (46). Further, it has been reported that signaling through CD40 up-regulated Bcl-x(L) and Bfl-1 and protected B cell lymphoma from apoptosis (47).

Recent studies have highlighted the lack of costimulatory molecules as a predominant reason for the inefficient tumor rejection (13, 14, 48). Moreover, if the lymphomas express CD80, signaling through this molecule may impede their growth by inducing the expression of pro-apoptotic and down-regulating the levels of anti-apoptotic molecules and thereby facilitating apoptosis (49, 50). It is important to mention here that CD80-mediated delivery of inhibitory signals may not only be responsible for apoptosis of CD80-bearing tumor cells, but may also be responsible in the regulation of immune responses and maintaining self-tolerance in B cells. Thus, in the case of tumors, ligation of B7-1 by anti-CD80 Ab may also deliver effective anti-tumor immunity.

In conclusion, we report here that selective inhibition of B cell proliferation and differentiation via CD80 signaling may be viewed as a novel strategy to restrict the undesired stimulation of B cell and progression of B cell lymphomas. The triggering through B7-2 may play a crucial role in the activation of B cells. This observation may therefore explain why there is the existence of both the isomers on a single cell. Taken together with the data from others, these observations allow us to propose a sequential positive and negative feedback model between B and T cells. The predominance of the signals transduced through CD28 and CTLA-4 govern the outcome of a T cell immune response. As the CD80 and CD86 have been found to have differences in their ability to bind CD28 and CTLA-4, the predominant engagement of CD86 with CD28 leads to proliferation of T cells, whereas stronger avidity of CTLA-4 with CD80 delivers a down-regulatory signal for T cell responses (29). On the other hand, our results show that stimulation through CD80 and CD86 can modulate the humoral response by transducing positive and negative signals in B cells and may control the progression of B cell lymphomas.

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