more effective than fibroblasts in maintaining B cell viability by blocking spontaneous apoptosis. Moreover, the mechanism involved the activity of two surface molecules constitutively expressed by RA-NLC, CD106, and CD157. As mentioned above, CD157 expressing bone marrow stromal cells previously have been shown to enhance proliferation of a pre-B cell line (16), but this is the first indication that CD157 may have a functional impact on mature peripheral B cells. Although recombinant soluble CD157 alone had no effect on spontaneous B cell apoptosis, together with RA-NLC, enhanced protection from apoptosis was noted. This finding, along with the data from blocking studies with antibodies to CD157, strongly implied that RA-NLC protected B cells from apoptosis by delivering two signals to B cells, one of which may be provided by CD157 engaging an unknown ligand on the B cell.

The second signal that protected B cells from apoptosis was provided by CD106, a molecule that was also constitutively expressed by RA-NLC and could be further upregulated by IFN-γ. An interaction between CD106 expressed by the RA-NLC and its ligand, CD49d/CD29 ($\alpha_4\beta_1$, VLA-4) expressed by the B cell, appeared to be essential for maintenance of B cell viability and prevention of apoptosis. Previous evidence has shown that CD106 is essential for other functions of nurse cells such as the induction of pseudoemperipolesis (7). Moreover, CD49d/CD29 engagement has recently been shown to provide a costimulatory signal to human B cells (50). Thus, interactions between RA-NLC–expressed CD106 and B cell–expressed CD49d/CD29 are reasonable candidates to provide viability promoting signals to B cells.

Although RA-NLC failed to support initial B cell proliferation, they supported increased Ig production by otherwise unstimulated peripheral B cells or B cells activated with anti–CD3-stimulated T cells. Of note, RA-NLC were much more effective than fibroblasts at stimulating resting B cells to produce Ig. Although the mechanism of this phenomenon is unclear, it does not appear to be related to the CD40 signaling pathway as the RA-NLC failed to express CD40 ligand (CD154), even after stimulation with IFNγ. It is possible that the molecules involved in the induction of Ig production are the same as those promoting viability, namely either CD157, CD106, or both. The possibility that these molecules provide signals to mature B cells that facilitate Ig production is consistent with the expression of CD157 by reticular cells in spleen and lymph node (44) and the recently described role of signaling via CD49d/CD29 in facilitating Ig production by peripheral B cells (50). This possibility is currently being examined. Regardless of the mechanism, the data are consistent with the conclusion that NLC in the rheumatoid synovium and bone marrow play a role in the local and systemic overreactivity of B cells characteristic of RA.

RA-NLC also supported the outgrowth of EBV-transformed B lymphoblastoid cells from normal B cells. Of importance, the RA-NLC were not infected with EBV, as has been reported for some synovial membrane-derived fibroblasts (51). Moreover, RA-NLC did not facilitate growth of EBV-transformed B lymphoblastoid cell lines, suggesting that they facilitated outgrowth of latently transformed B cells from peripheral blood. The precursor frequency of B cells undergoing long-term growth when cultured on RL-NLC (1:12,500) was comparable to the frequency of B cells latently infected with EBV in adult peripheral blood (52, 53) supporting this conclusion. The mechanism whereby RA-NLC promote the outgrowth of EBV-transformed B lymphoblastoid cell lines from human peripheral B cells is unknown, but is likely to involve more than nonspecific support of viability, as human fibroblasts supported B cell viability somewhat, but failed to facilitate the outgrowth of EBV-transformed B lymphoblastoid cell lines. Whether similar surface molecules are involved in this process as were involved in the maintenance of B cell viability is currently unknown, but is the subject of ongoing investigation.

In rheumatoid synovium, germinal center-like structures containing B cells aggregated around cells resembling follicular dendritic cells (FDC) develop (12). The exact lineage of these FDC-like cells is unclear, but they and FDC in secondary lymphoid organs have certain characteristics in common with RA-NLC. For example, synovial FDC-like cells express CD106 at high levels (12, 54). Constitutive expression of CD106 appears to be a feature of both NLC and FDC. In this regard, some (55) but not all (56) FDC lines derived from human tonsil express high levels of CD106. Importantly, CD106 plays a critical role in the interaction of FDC with B cells both in vivo and in vitro (55, 57, 58). Moreover, at least one FDC-like line has been shown to express CD157 (55). In addition, FDC-like lines are similar to nurse cell lines in vitro, in that both can promote pseudoemperipolesis (59). Finally, both FDC and nurse cells can promote B cell viability. Thus, an FDC-like line promoted the viability of tonsillar B cells (56), whereas freshly prepared FDC promoted the viability of germinal center tonsillar B cells in a CD49d/CD29-dependent manner (60). There are, however, differences between FDC lines and RA-NLC, including the cytokines produced (61) and whether they interact uniquely with B cells (58, 61) or with both T and B cells (59), as is typical of nurse cells. In addition, FDC lines appear to support emperiopolesis (56, 59, 60) as well as the pseudoemperipolesis (59) typical of nurse cells (6–9). Despite these discrepancies, the results suggest the possibility that RA-NLC and FDC-like cells at inflammatory sites or even in secondary lymphoid organs have many overlapping functional and phenotypic features. However, RA-NLC fail to express CD21 or CD35, characteristic markers of classic FDC (62–64). The absence of these markers on RA-NLC may be related to the observation that although CD21 and CD35 are expressed by FDC in situ, they are rapidly lost when these cells are cultured in vitro (55, 56, 59, 61). Of note, these markers can be induced on synoviocytes by stimulation with the proinflammatory cytokines TNF-α and IFNγ (12). In this study, IFNγ upregulated expression of both CD106 and CD157 by RA-NLC. If this cytokine, in conjunction with TNF-α also upregulated expression of CD21 and CD35 by RA-NLC, the cells would then exhibit the phenotype of synovial FDC. In conjunction with the capacity of RA-NLC to facilitate B cell functional activity, cytokine-activated RA-NLC may well account for the aggregates of B cells and CD106 expressing FDC-like cells (54) and the local activation of B cells and immunoglobulin production characteristic of rheumatoid synovitis (36, 37).

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Bidirectional Regulation of Human B Cell Responses by CD40-CD40 Ligand Interactions[1]

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Positive and negative effects of CD40 ligation on human B cell function were suggested by the observation that mAb to CD40 ligand partially blocked the suppressive influences of anti-CD3-stimulated control $CD4^+$ T cells, as well as the B cell stimulatory effects of anti-CD3 activated mitomycin C-treated $CD4^+$ T cells. To examine the negative effects of CD40 ligation in greater detail, B cells were cultured with anti-CD3 activated mitomycin C-treated $CD4^+$ T cells that expressed optimal levels of CD40 ligand; additional recombinant human CD40 ligand significantly suppressed Ig production, but not proliferation. In contrast, when B cells were stimulated with SAC (formalinized Cowan I strain *Staphylococcus aureus*) and IL-2 in the absence of T cells, small amounts of recombinant CD40 ligand-stimulated Ig production, whereas larger quantities directly suppressed Ig secretion. The suppressive action of CD40 ligation on Ig production was most apparent after initial B cell activation. Moreover, IgD^- memory B cells were significantly more sensitive to inhibition by CD40 ligation than IgD^+ naive B cells. Engagement of CD40 not only suppressed Ig secretion by IgD^- memory B cells, but also expression of CD38. Finally, activated B cells acquired the capacity to down-regulate CD40 ligand expression by stimulated $CD4^+$ T cells more effectively than resting B cells. These results indicate that during T cell-B cell collaboration, engagement of CD40 can influence Ig production both positively and negatively, depending on the density of CD40 ligand as well as the stage of B cell activation and differentiation. ***The Journal of Immunology,* 1997, 158: 4620–4633.**

A variety of receptor-ligand pairs have been reported to play important roles in contact-dependent T-B collaboration. Among these, CD40-CD40 ligand interaction plays a central role (1). The importance of this interaction has been confirmed by in vivo studies that have documented that interference with CD40 signaling by targeted gene disruption or by administration of mAb inhibited both primary and secondary immune responses to T cell-dependent Ags (2, 3) and prevented the formation of germinal centers as well as the generation of memory B cells (4–6). Many in vitro studies also have demonstrated that ligation of CD40 induces B cell activation, resulting in proliferation and secretion of Ig, as well as Ig heavy chain switch recombination in the presence of appropriate cytokines (7–10), and also leading to the generation of memory B cells (11).

Most of these studies have focused on the positive influences of CD40 ligation. Recent evidence, however, has also suggested that CD40 engagement may inhibit B cell responses under certain circumstances. In this regard, it has been demonstrated that mAb to CD40 inhibited the growth of certain B cell lymphomas (12–14). Signaling through CD40 has also been shown to induce the death of transformed cells of mesenchymal and epithelial origin (15). Moreover, intense signaling through CD40 was noted to induce apoptosis of Ig secreting hybridoma by a Fas- and TNF-α-independent mechanism (16). Finally, CD40 ligation inhibited the differentiation of tonsillar B cells into plasma cells or Ig-secreting cells in Ag-specific (17, 18) and Ag-nonspecific responses (11). In these reports, the negative function of CD40 ligation was investigated in cultures of tonsillar B cells, murine B cells, B cell lines, or B cell hybridomas. The possibility that CD40 ligation during T cell-B cell collaboration might limit responses of circulating B cells has not previously been explored.

Here, we show evidence that CD40-CD40 ligand interaction either enhances or suppresses Ig production by normal human peripheral B cells during T cell-B cell collaboration. The functional impact of engagement of CD40 is dependent on the density of CD40 ligand expressed by activated $CD4^+$ T cells, as well as on the stage of B cell activation and differentiation. The data show that low concentrations of CD40 ligand enhanced Ig production by B cells, whereas high concentrations suppressed Ig production, but not proliferation of B cells. IgD^- memory B cells were far more sensitive to the suppressive influences of CD40 ligation than IgD^+ naive B cells. IgD^- memory B cells responded to ligation of CD40 by producing less IgG and IgA and also expressing a lower intensity of CD38. Regulation of the impact of CD40 ligation was noted in that activated B cells strongly suppressed CD40 ligand expression by activated $CD4^+$ T cells. These results indicate that CD40 ligation mediates complex, often opposing effects on B cells and B cell subpopulations and that B cells have the capacity to escape the suppressive action of CD40 ligation during T cell-B cell collaboration.

Materials and Methods

Monoclonal Abs, cytokines, and reagents

Various Ab were used, including 64.1, a mouse IgG2a mAb directed at the CD3 molecular complex on mature T cells (19); 4D9-8, a mouse IgG1

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[3] Abbreviations used in this paper: SAC, formalinized Cowan I strain *Staphylococcus aureus*; MFI, mean fluorescence intensity; PE, phycoerythrin.

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mAb against the human CD40 ligand molecule that was a kind gift of Dr. R. J. Noelle (Dartmouth Medical School, Hanover, NH); MOPC, a control mouse IgG1 mAb; MR1, a hamster IgG mAb against mouse CD40 ligand; 145-2C11, a hamster IgG mAb against mouse CD3; OKT8 (American Type Culture Collection (ATCC), Bethesda, MD), a mouse IgG2a mAb directed at the CD8 molecule; L243 (ATCC), a mouse IgG2a mAb directed at monomorphic HLA-DR determinants; Coulter clone B4-BIOTIN, a biotinylated mouse IgG1 mAb directed at human CD19 (Coulter Corp., Miami, FL); a PE-conjugated mouse IgG1 mAb directed at human CD19 (Sigma Chemical Co., St. Louis, MO); a sheep anti-mouse IgG1 FITC conjugate (The Binding Site, Birmingham, U.K.); an FITC-conjugated mouse IgG1 mAb directed at human CD38 (Caltag Laboratories, Burlingame, CA); a control FITC-conjugated mouse IgG1 mAb (Caltag Laboratories); and an FITC-conjugated goat $F(ab')_2$ anti-human IgD Ab (Caltag Laboratories, So. San Francisco, CA).

Purified rIL-2 was obtained from Hoffmann-La Roche (Nutley, NJ) and used at 20 U/ml. Formalinized Cowan I strain *Staphylococcus aureus* (SAC, Pansorbin) was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and was used at a final concentration of 1/60,000 (v/v).

Expression of murine or human CD40 ligand by baculovirus-infected Sf9 cells and preparation of Sf9 cell membranes

Sf9 cells were incubated at 26°C with wild-type AcMNPV or human or murine CD40 ligand-encoding recombinant baculovirus (kind gifts of Dr. Marilyn Kehry, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, or Dr. Katheryn Meek, University of Texas Southwestern Medical Center at Dallas, respectively). After a 72-h incubation, cells were harvested, washed twice in PBS, and resuspended in cold homogenization buffer (0.2 M Tris/Cl, pH 7.4) and Pefabloc SC (AEBSF hydrochloride, Boehringer Mannheim GmbH, Mannheim, Germany). Cells were disrupted at 4°C by a Polytron (Brinkman, Westbury, NY) homogenizer at speed 7. Seven to ten 15-s homogenizations were usually found to be sufficient to break >95% of the cells. Afterward, the homogenate was centrifuged at $200 \times g$ for 3 min, and the supernatant was collected and centrifuged again at $7{,}000 \times g$ for 10 min. The membrane pellet was resuspended in PBS at a final concentration of 20 to 25×10^6 cell equivalents per milliliter based on the starting cell number. The membrane suspension was kept at −80°C until used. The final concentration of total protein in the membrane suspension was approximately 0.5 $\mu g/\mu l$. These experiments were conducted with three separate preparations of human CD40 ligand expressing Sf9 membranes and one preparation of mouse CD40 ligand expressing Sf9 membranes. Each lot of membranes was shown to have stimulatory capacity for human B cells using previously described methods (20, 21).

The level of functionally active CD40 ligand expressed by Sf9 cell membranes was determined by a competitive binding assay using a previously described CD40 ligand-CD8 construct as a standard (16). In this assay, Ramos B lymphoma cells were incubated with various concentrations of the CD40 ligand-CD8 construct, and the degree of binding was determined with an FITC-conjugated mAb to CD8 (YTS169.4, ATCC) and analysis by flow cytometry. The capacity of various amounts of membranes prepared from wild-type or recombinant CD40 ligand-expressing baculovirus-infected Sf9 cells to block binding of the CD40 ligand-CD8 construct by Ramos cells was then assessed. Using this approach, it was determined that 2, 4, 6, and 8 μl of CD40 ligand-expressing Sf9 membranes bound 10, 25, 40, and 60%, respectively, of CD40 expressed by 1×10^5 Ramos cells.

Culture medium

All cultures were conducted in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with penicillin G (200 U/ml), gentamicin (10 μg/ml), L-glutamine (0.3 mg/ml), and 10% FBS (Life Technologies).

Cell preparation

PBMC were isolated from heparinized blood of healthy adult volunteers by centrifugation over sodium diatrizoate/Ficoll gradients (Pharmacia, Inc., Piscataway, NJ). B cells were purified by two different procedures. First, the CEPRATE LC Kit (CellPro, Bothell, WA), a disposable cell separation system for isolating specific cell populations, was used to prepare $CD19^+$ B cells following the manufacturer's protocol. Briefly, PBMC were reacted with subsaturating concentrations of biotinylated mouse anti-human CD19 mAb (Coulter) at 4°C for 25 min, and after washing, applied to a column containing avidin-coated beads. After unbound cells passed through the column, bound B cells were recovered by mechanical manipulation of the avidin bead column. The resultant population of B cells contained >97% $CD19^+$ B cells. Peripheral B cells prepared in this manner from normal donors were almost entirely small resting cells as determined by flow cytometric analysis of side and forward scatter. The population was routinely devoid of activated $CD69^+$ B cells.

A second procedure was also used to purify B cells from PBMC by negative selection as previously described (22). Briefly, PBMC were depleted of monocytes and NK cells by incubation with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co.) in serum-free RPMI 1640 as described (23). The treated cell population was washed and incubated with neuraminidase-treated SRBC (24). The rosetting and nonrosetting populations were then separated by centrifugation on diatrizoate/Ficoll gradients. The nonrosetting cells were obtained from the interface and were again rosetted and centrifuged on diatrizoate/Ficoll gradients to remove residual T cells. The resultant population of B cells contained <2% esterase-positive monocytes and <1% T cells as determined by staining with OKT3 and OKT11, pan-T cell mAb, followed by flow cytometric analysis. The cells were additionally characterized as containing >90% $CD19^+$ B cells and no Leu $11b^+$ NK cells.

In some experiments, IgD^+ or IgD^- B cells were separated from purified B cells by flow cytometry. Purified B cells were incubated with saturating concentrations of a FITC-conjugated goat $F(ab')_2$ anti-human IgD Ab for 30 min at 4°C, washed with PBS, and resuspended at a concentration of 5×10^6/ml in PBS containing 2% FBS. Cells were sorted at rates of 2000 to 2500 cells per second using a fluorescence-activated cell sorter (FACStar^{Plus}, Becton Dickinson, San Jose, CA). The threshold was set to abort all dead cells and debris as determined by light scattering. After sorting, IgD^+ or IgD^- B cell populations were washed with PBS and resuspended in RPMI-10% FBS. The viability of each cell population was >98%.

The sedimented rosette-forming cells were treated with isotonic NH_4Cl to lyse the SRBC and were passed over a nylon wool column. Afterward, purified $CD4^+$ T cells were prepared from the rosette-forming cells by negative selection, using a panning technique (25) to deplete contaminating $HLA\text{-}DR^+$ cells and $CD8^+$ T cells. Cells were reacted with saturating concentrations of the anti-HLA-DR mAb, L243, and OKT8. After washing, the cells were incubated on goat anti-mouse Ig (GaMIg) (Cappel Laboratories, Inc., Cochranville, PA)-coated panning dishes. After a 70 min incubation at 4°C, the nonadherent cells were gently aspirated and were panned a second time on another GaMIg-coated panning dish, after which the nonadherent cells were aspirated. The $CD4^+$ T cell population obtained in this manner contained <0.1% esterase-positive cells, <1% $CD8^+$ T cells, and >96% $CD4^+$ T cells.

In some experiments, $CD4^+$ T cells were treated with mitomycin C before culture. This was accomplished by suspending them in culture medium at approximately 5×10^6/ml and incubating them on a rotator for 50 min at 37°C with mitomycin C at a concentration of 40 μg/ml. Afterward, the cells were washed four times and suspended in culture medium for use.

Culture conditions

All cultures were conducted in 96-well microtiter plates with round-bottom wells (Costar, Cambridge, MA). When B cells were activated with $CD4^+$ T cells, microtiter wells were coated with mAb to CD3 before cells were added, as previously described (26, 27). Briefly, anti-CD3 mAb 64.1 was dissolved in Tris-buffer (50 mM, pH 9.5) at a concentration of 4 μg/ml, unless otherwise specified, and 50 μl was placed in each of the wells of 96-well microtiter plates with round-bottom wells (Costar) and incubated at 37°C for 2 h. The wells were then washed twice with RPMI to remove nonadherent mAb before addition of cells. Approximately 14 to 20% of the added mAb adhered to the wells (27). B cells (2.5×10^4/well) were incubated with various numbers of $CD4^+$ T cells. Routine cultures were conducted in duplicate in a total volume of 200 μl. The cells were incubated routinely for 10 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

In some experiments, a two-step assay of B cell stimulation was conducted. B cells were activated for various periods of time (usually 48 h) with $CD4^+$ T cells stimulated by immobilized mAb to CD3 or with SAC plus IL-2 in the presence or absence of membrane-expressed CD40 ligand or control membranes. At the end of the initial culture, activated B cells were separated from $CD4^+$ T cells by rosetting with neuraminidase-treated SRBC, or B cells stimulated by SAC and IL-2 were harvested. The B cells were washed and recultured with $CD4^+$ T cells in anti-CD3 mAb-coated microtiter wells or were recultured with IL-2 in the presence or absence of membrane-bound CD40 ligand. The total combined length of the first and second cultures was 10 days.

Flow cytometric analysis

The cells were cultured for various lengths of time with various stimuli as indicated, and after washing with PBS containing 2% normal human

serum and 0.1% sodium azide, cells were reacted with saturating concentrations of various mAb at 4°C for 30 min. For staining with 4D9-8 (anti-CD40 ligand), secondary FITC-conjugated sheep anti-mouse IgG1 (The Binding Site) was used. To detect CD40 ligand expression on $CD4^+$ T cells cultured with B cells, cells were reacted with 4D9-8, secondary FITC-conjugated Ab and PE-conjugated anti-CD19. Afterward, CD40 ligand expression on the CD19-negative cell population was analyzed. Analysis was performed using the FACScan (Becton Dickinson) flow cytometer. Dead cells were eliminated by propidium iodide staining. Density of staining was expressed as the change in mean fluorescence intensity (ΔMFI) for staining, with the mAb of interest calculated by subtracting the MFI of staining with the control mAb.

Measurement of Ig production

At the end of 10 days of incubation, the supernatants from each culture well were aspirated and assayed for Ig content. Ig in the culture supernatants was quantitated using isotype-specific ELISAs as previously described (28). Briefly, microtiter plates (Cooke, Dynatech Laboratories, Alexandria, VA) were coated with affinity-purified goat Abs to human α-, μ-, or γ-chains (Tago, Camarillo, CA), diluted in borate-buffered saline by incubating at 37°C for 2 h, and kept at 4°C overnight. Plates were then washed with Tween-20 saline and incubated with serial dilutions of standard Ig preparation (IgA and IgG, Behring Diagnostics, La Jolla, CA; IgM, Tago) or diluted culture supernatants in PBS containing 0.5% Tween-20 (Sigma Chemical Co.) at 37°C for 2 h. Bound Ig was detected with peroxidase-conjugated affinity-purified isotype-specific Abs (Tago) in the presence of *o*-phenylenediamine dihydrochloride (Sigma Chemical Co.) as substrate. Absorbance was determined at a wavelength of 490 nm on the EL312 Biokinetics Reader (Bio-Tek Instruments, Winooski, VT). The amount of Ig in the supernatants was calculated by comparison with a standard curve by using the KinetiCalc program (Bio-Tek Instruments). Triplicates for each culture condition were performed and differed by less than 10%. The sensitivities of the specific assays were 12 ng/ml for IgA and IgG and 24 ng/ml for IgM.

Measurement of B cell DNA synthesis

To examine B cell DNA synthesis, the cells were incubated for 4 days at 37°C with 1 μCi [^{3}H]thymidine (6.7 Ci/mM; ICN Pharmaceuticals, Inc., Irvine, CA) present for the last 18 h. The cells were then harvested onto glass fiber filter paper, and [^{3}H]thymidine incorporation was determined by liquid scintillation spectroscopy.

Assay of B cell growth

To assay cellular growth, a rapid colorimetric method was used (29). Briefly, B cells were cultured with SAC and IL-2 in the presence or absence of recombinant CD40 ligand. Afterward, B cells were harvested, washed, and then recultured with IL-2 in the presence or absence of recombinant CD40 ligand for the indicated length of time, after which 180 μl of the supernatant was removed without disturbing the pellet. Ten microliters of a yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.), and 100 μl of PBS were added to the remaining pellet and incubated at 37°C for 4 h, after which time 100 μl of 0.1 N HCl/isopropanol alcohol was added to terminate the reaction and dissolve the formazan product. The contents of the well were pipetted gently until mixed. The absorbance was read at 570 and 630 nm on the EL312 Biokinetics Reader (Bio-Tek Instruments).

Results

Expression of CD40 ligand by anti-CD3-stimulated $CD4^+$ T cells

Initial experiments investigated CD40 ligand expression by anti-CD3-stimulated $CD4^+$ T cells in the absence or presence of B cells. As shown in Figure 1 and comparable with previously published results (30), CD40 ligand expression by activated control $CD4^+$ T cells in the absence of B cells reached a maximum after 24 h of stimulation and thereafter began to decline. CD40 ligand expression was still detected at 72 h, albeit diminished in intensity. Afterward, the expression continued to decrease to basal levels and was not detected at 144 h or later. The expression of CD40 ligand by activated mitomycin C-treated $CD4^+$ T cells also reached a maximum at 24 h, but the density was significantly lower than that expressed by activated control T cells. CD40 ligand expression by activated mitomycin C-treated T cells became undetectable by 72 h. The stability of CD40 ligand expression by each activated T cell population was examined by adding cycloheximide at 24 h after stimulation to block de novo protein synthesis. The $T_{1/2}$ of CD40 ligand expressed by activated control $CD4^+$ T cells or mitomycin C-treated $CD4^+$ T cells, which was calculated from the ΔMFI of each sample between 24 h and 48 h, was comparable (4.7 and 6.4 h, respectively).

B cells have been reported to down-regulate CD40 ligand expression by T cells (30). Experiments were conducted, therefore, to determine whether coculture with B cells would alter expression of CD40 ligand by the T cell populations differentially. As shown in Figure 1, CD40 ligand expression by both of the activated $CD4^+$ T cell populations was markedly diminished in the presence of B cells, although the density of CD40 ligand expressed by control $CD4^+$ T cells during the initial 48 h remained greater. These results indicate that the major difference between activated control and mitomycin C-treated $CD4^+$ T cells was the much greater expression of CD40 ligand by the former during the initial 48 to 72 h of culture.

Monoclonal Ab to CD40 ligand enhances Ig production by B cells cultured with anti-CD3-stimulated control $CD4^+$ T cells

The next experiments were designed to examine the effects of mAb to CD40 ligand on B cell Ig production induced by anti-CD3-stimulated control or mitomycin C-treated $CD4^+$ T cells. As seen in Figure 2, anti-CD3-stimulated mitomycin C-treated $CD4^+$ T cells induced very large amounts of Ig. In contrast, anti-CD3-stimulated control $CD4^+$ T cells induced the production of minimal amounts of Ig and inhibited the production of Ig supported by anti-CD3-stimulated mitomycin C-treated $CD4^+$ T cells as previously described (31). When mAb to CD40 ligand was added at the initiation of culture, B cell Ig production induced by anti-CD3-stimulated mitomycin C-treated $CD4^+$ T cells was inhibited. This effect was noted only when the mAb was added at the initiation of culture. In contrast, mAb to CD40 ligand enhanced Ig production by B cells cultured with anti-CD3-stimulated control $CD4^+$ T cells. This effect was most apparent when mAb to CD40 ligand was added between 24 and 48 h of culture. Suppression of Ig production in cultures of B cells stimulated with mitomycin C-treated and control T cells was also partially relieved when mAb to CD40 ligand was added between 24 and 48 h of culture. mAb to CD40 ligand had no effect on Ig production when added 72 to 96 h after stimulation or later.

Supplemental rCD40 ligand suppresses B cell Ig production

The next experiments were conducted to determine whether excess CD40 ligand would alter Ig production in cultures of B cells and activated mitomycin C-treated $CD4^+$ T cells. As a control, the effect of the same amount of exogenous CD40 ligand on Ig production by purified B cells stimulated with SAC and IL-2, but no T cells, was examined. Preliminary experiments determined the number of activated mitomycin C-treated T cells that induced the maximal amount of Ig production. As shown in Figure 3*A*, when B cells were cultured with activated mitomycin C-treated $CD4^+$ T cells, Ig production was dramatically suppressed by the addition of Sf9 membranes expressing recombinant human CD40 ligand at the initiation of culture. The apparent suppression of IgM production was not related to Ig class switching because production of IgG and IgA was also suppressed. When B cells were stimulated with SAC and IL-2 in the absence of T cells, small concentrations of recombinant human CD40 ligand dramatically enhanced Ig production, whereas larger concentrations suppressed Ig secretion

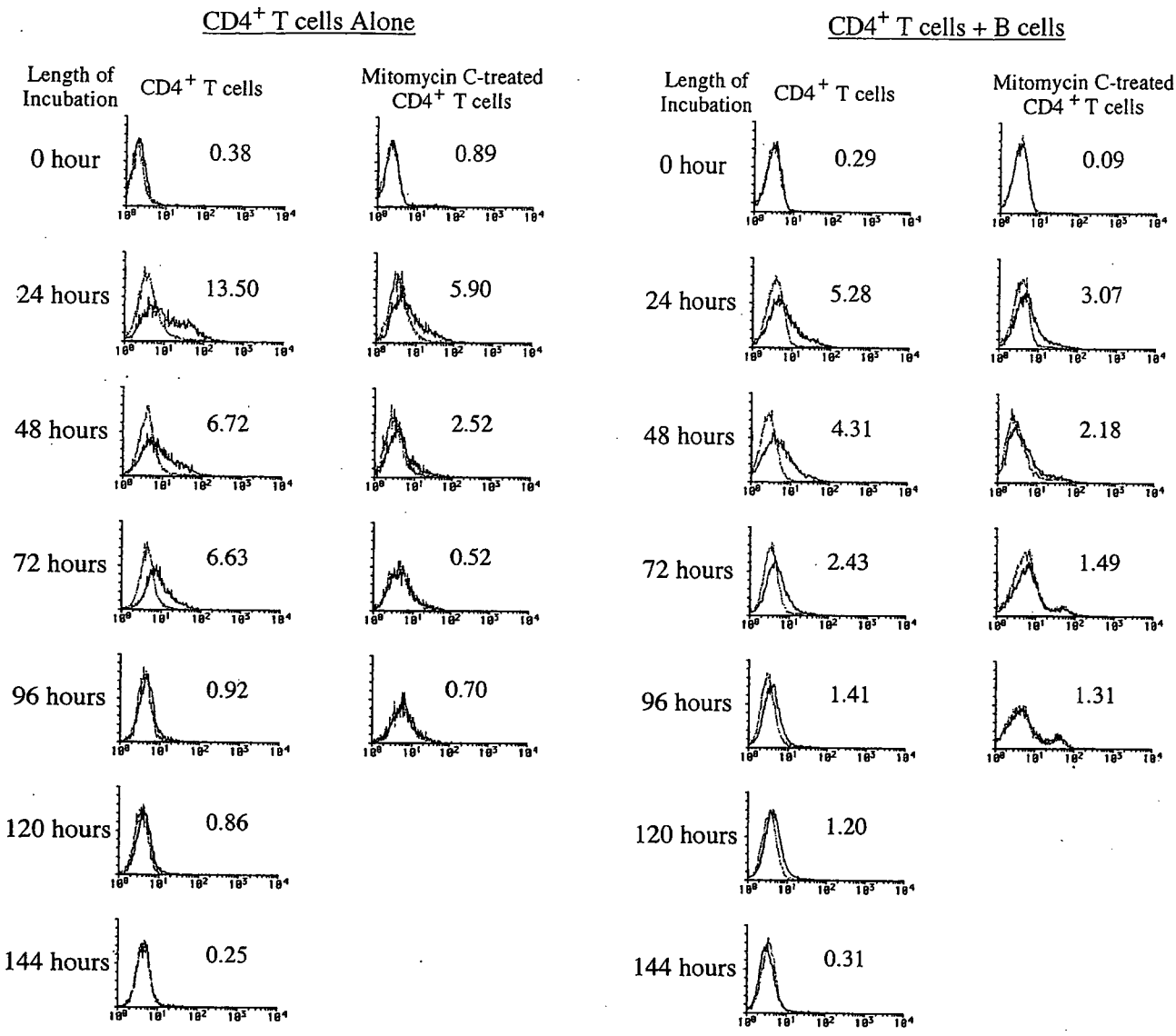


FIGURE 1. Kinetics of CD40 ligand expression by anti-CD3-stimulated $CD4^+$ T cells cultured with or without B cells. Either control $CD4^+$ T cells or those treated with mitomycin C before stimulation (1×10^5/well) were incubated with or without B cells (2.5×10^4/well) in anti-CD3 (200 ng/well)-coated wells for the length of time indicated. Then, cells were stained with CD19-PE mAb and either 4D9-8 or a control mAb followed by anti-mouse IgG1-FITC. CD40 ligand expression by CD19-negative cells was analyzed by flow cytometry. Numbers in each histogram indicate the ΔMFI for staining with 4D9-8 calculated by subtracting the MFI of staining with the control mAb.

(Fig. 3*B*). To insure the specificity of the inhibitory effect, similar experiments were conducted with recombinant murine CD40 ligand, which is known to stimulate human B cells (20, 21). Murine CD40 ligand also caused marked suppression of T cell-dependent Ig production that was completely reversed by a mAb specific for the murine CD40 ligand (Fig. 4). Finally, in contrast to the effect on Ig production, recombinant CD40 ligand did not suppress T cell-dependent B cell [^{3}H]thymidine incorporation and modestly, but significantly, stimulated [^{3}H]thymidine incorporation by purified B cells (Fig. 5). Larger concentrations of CD40 ligand induced less [^{3}H]thymidine incorporation by highly purified B cells.

The effect of supplemental rCD40 ligand is dependent on the quantity of physiologic CD40 ligand expressed by activated $CD4^+$ T cells

The relationship between CD40 ligand expression by $CD4^+$ T cells and the intensity of stimulation by anti-CD3 was examined by activating mitomycin C-treated $CD4^+$ T cells with various concentrations of immobilized anti-CD3 in the presence of B cells. Low concentrations of anti-CD3 (3 ng/well) induced minimal, if any, CD40 ligand expression, whereas maximal CD40 ligand expression was noted with 9 ng/ml or more of anti-CD3 (data not shown). To relate this to the functional outcome of T cell-B cell collaboration, recombinant CD40 ligand was added to cultures stimulated with various concentrations of anti-CD3, and the impact on Ig production was examined (Fig. 6). When a low concentration of anti-CD3 (3 ng/well) was used as a stimulus, the addition of CD40 ligand enhanced Ig production by six- to eightfold. When higher concentrations of anti-CD3 were employed to stimulate the $CD4^+$ T cells, the addition of the same concentration of recombinant CD40 ligand suppressed Ig production. These results are consistent with the conclusion that the impact of supplemental CD40 ligand is dependent on the level of endogenous CD40 ligand expression.

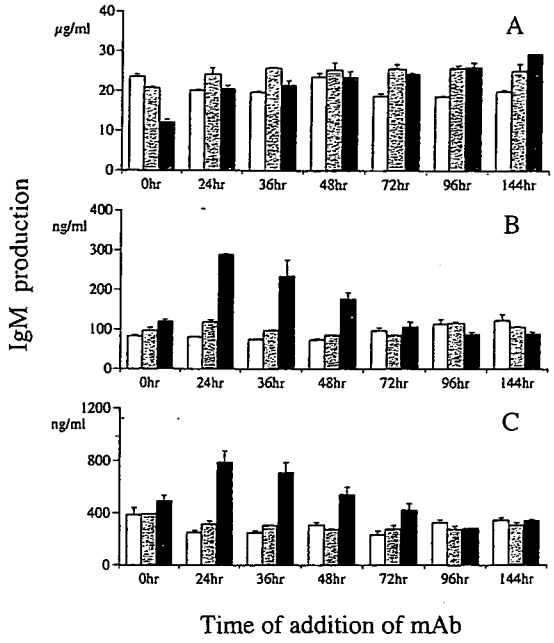


FIGURE 2. The role of CD40-CD40 ligand interaction in T cell-dependent Ig secretion by B cells. B cells (2.5×10^4/well) were cultured with (A) anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells, (B) anti-CD3 (200 ng/well)-stimulated control $CD4^+$ T cells, or (C) a 1/1 mixture of both populations (1×10^5/well). Either 4D9-8 (black), a control mAb (gray), or no mAb (white) was added at a final concentration of 10 μg/ml at various times after stimulation. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data from three experiments are shown, expressed as mean ± SEM. Data shown are for analysis of IgM secretion. Similar results were noted when IgG or IgA secretion was analyzed.

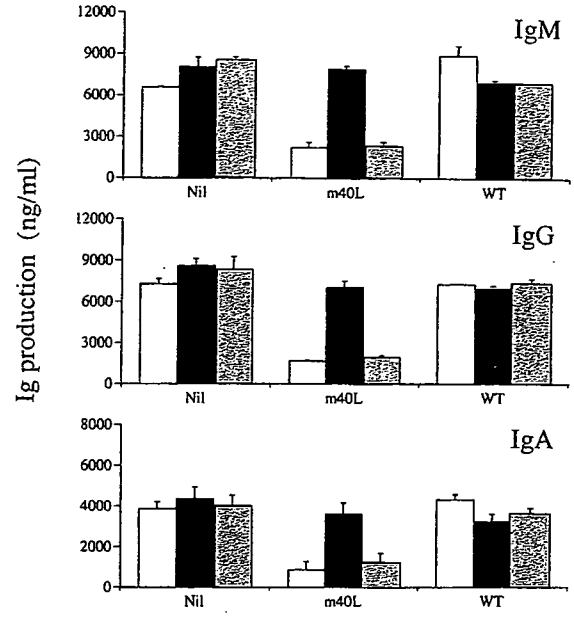


FIGURE 4. mAb to murine CD40 ligand (MR1) completely blocks the suppressive effect of recombinant murine CD40 ligand on Ig secretion by B cells. B cells (2.5×10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells (1×10^5/well) in the presence of 6 μl of membranes from Sf9 cells infected with either recombinant murine CD40 ligand or wild-type baculovirus. Either an anti-mouse CD40 ligand mAb (MR1, black), a control mAb (145-2C11, gray), or no mAb (white) was added at a concentration of 1 μg/ml where indicated. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.

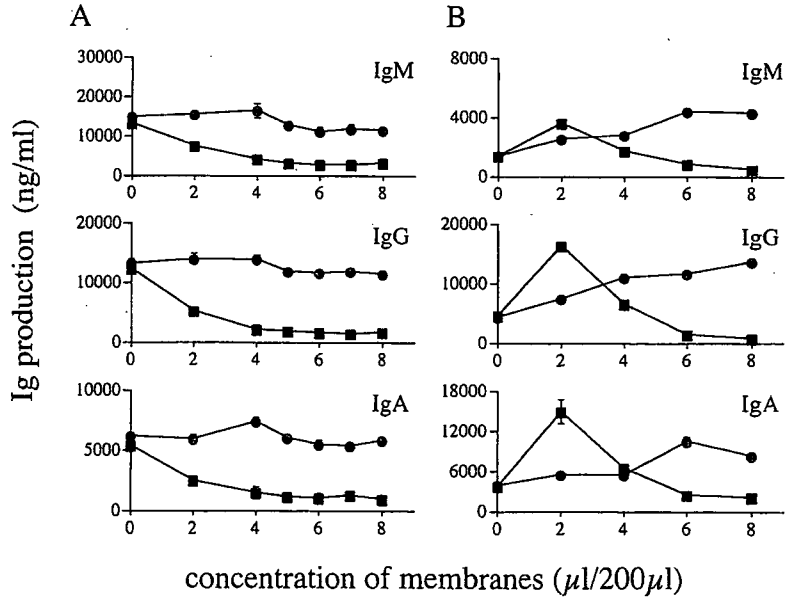


FIGURE 3. Supplemental CD40 ligand suppresses Ig secretion by B cells in a dose-dependent manner. B cells (2.5×10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells (2×10^5/well) (*A*) or with SAC + IL-2 (*B*) in the presence of various concentrations of membranes from Sf9 cells infected with recombinant human CD40 ligand (squares) or wild-type (circles) baculovirus. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data from three experiments are shown, expressed as mean ± SEM.

The suppressive effect of CD40 ligand on Ig production by B cells requires its presence between 48 to 96 h of culture with B cells

The next experiments were designed to determine the stage at which CD40 ligand exerted its suppressive effects on Ig production by B cells cultured with anti-CD3-stimulated mitomycin C-treated $CD4^+$ T cells. The results shown in Figure 3*A* determined the concentration of recombinant CD40 ligand that induced maximal suppression of Ig production, and this amount of CD40 ligand was added at different times after initiation of cultures of B cells with

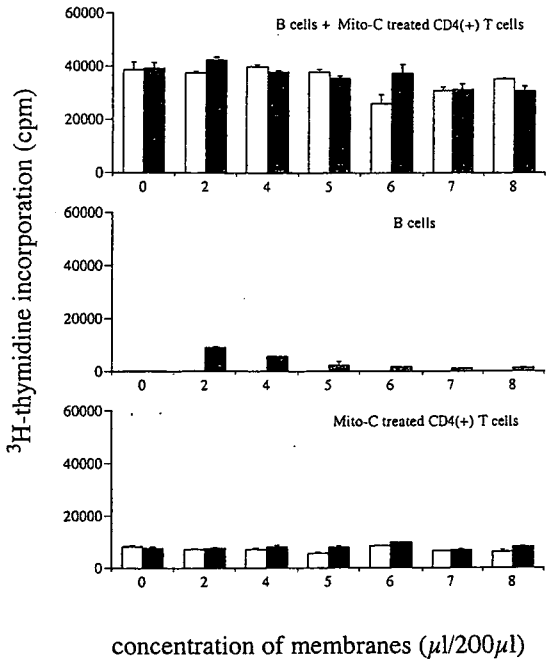


FIGURE 5. The effect of supplemental CD40 ligand on DNA synthesis of B cells cultured with anti-CD3 activated CD4$^+$T cells. B cells (2.5×10^4/well) were cultured alone or with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated CD4$^+$ T cells (1×10^5/well). Various concentrations of membranes from Sf9 cells infected with recombinant human CD40 ligand (solid) or wild-type (open) baculovirus were added. [^{3}H]thymidine incorporation was assessed after a 96-h incubation. Data are expressed as mean ± SEM.

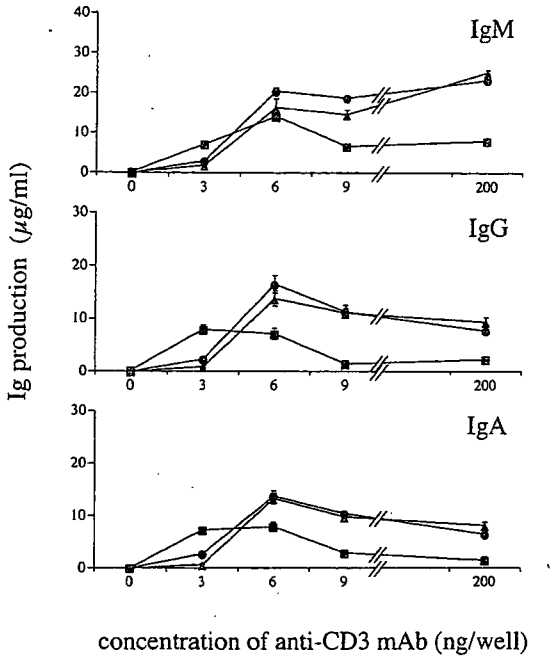


FIGURE 6. The impact of recombinant CD40 ligand on Ig secretion by B cells depends on the density of CD40 ligand expressed by activated T cells. B cells (2.5×10^4/well) were cultured with mitomycin C-treated CD4$^+$ T cells (7.5×10^4/well) stimulated with various concentrations of anti-CD3. Membranes (2 μl) of Sf9 cells infected with either recombinant human CD40 ligand (squares), wild-type (circles) baculovirus, or no membranes (triangles) were added at the initiation of the cultures. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.

anti-CD3-stimulated mitomycin C-treated CD4$^+$ T cells. As seen in Figure 7, Ig production was strongly suppressed when recombinant CD40 ligand was added at day 0, 2, and 4. In contrast, when CD40 ligand was added at day 6 or later, no effect on Ig production was noted.

To confirm that CD40 ligand suppressed B cell responses after initial activation, B cells were separated from anti-CD3-stimulated mitomycin C-treated CD4$^+$ T cells after either 48 h or 96 h of stimulation, and recultured with IL-2 in the presence or absence of recombinant CD40 ligand. As shown in Figure 8, recombinant CD40 ligand clearly suppressed Ig production by B cells that had been activated by coculture with T cells for 48 h. Of note, when B cells had been activated for 96 h, they became resistant to the suppressive effects of CD40 ligand and again became responsive to the positive effects of CD40 ligation with enhancement of Ig production observed. These results are consistent with the conclusion that the effect of CD40 ligation varies profoundly based on the length of stimulation of the B cell.

To elucidate the suppressive role of CD40 ligation, purified B cells were activated with SAC and IL-2 in the absence of T cells. Activated B cells were harvested after 48 h and recultured with IL-2. CD40 ligand was added to either the initial cultures or the subsequent cultures of activated B cells or both. As shown in Table I, the addition of CD40 ligand to the first culture enhanced production of IgM, IgG, and IgA. In contrast, when CD40 ligand was added to the subsequent culture of activated B cells, only production of IgM was enhanced. Finally, when CD40 ligand was present in both the initial and subsequent cultures, suppression of the production of all Ig isotypes was noted compared with that found in cultures containing CD40 ligand in the initial culture only. In all circumstances, the degree of suppression was greater when larger amounts of CD40 ligand were added to the subsequent culture (data not shown). Finally, addition of CD40 ligand at the initiation of culture or 48 h later substantially increased the number of metabolically active cells found after a 10-day incubation (Table I). These results are consistent with the conclusion that CD40 ligand suppressed Ig production, but not growth of activated B cells, and that inhibition of Ig production by later exposure to CD40 ligand was enhanced when the B cells had been initially activated in the presence of CD40 ligand. Moreover, production of IgM appeared to be regulated differently from production of IgG and IgA.

CD40 ligand exerts a greater suppressive effect on Ig production by IgD$^-$ memory B cells

The next experiments were conducted to investigate the differential effects of CD40 ligation on IgM vs IgG and IgA production. In cultures of human B cells with various polyclonal B cell activators, IgM production largely derives from IgM$^+$IgD$^+$ naive B cells, whereas IgG and IgA is produced by IgD$^-$ postswitch memory B cells (32). Therefore, the possibility that CD40 ligand exerted a differential regulatory effect on naive vs memory B cells was examined. Unfractionated B cells, IgD$^+$ naive B cells or IgD$^-$ memory B cells were stimulated with SAC and IL-2 in the presence or absence of recombinant CD40 ligand. As seen in Figure 9, recombinant CD40 ligand enhanced IgM production by SAC + IL-2-stimulated IgD$^+$ naive B cells and induced small amounts of IgG production in some experiments (0 ng/ml vs 50 ng/ml in cultures containing wild-type and CD40 ligand membranes, respectively).

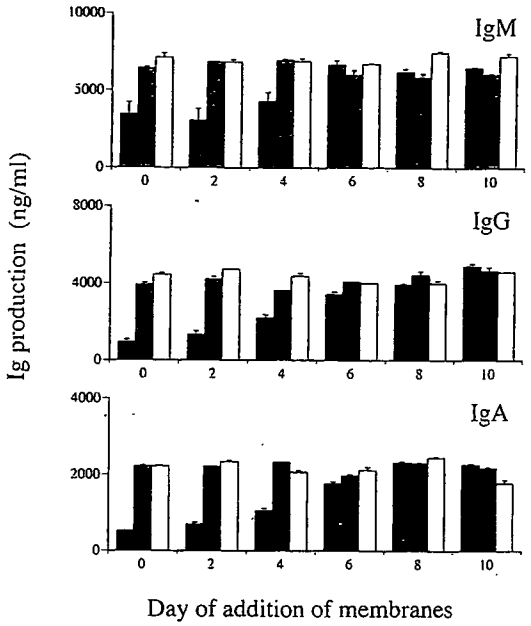


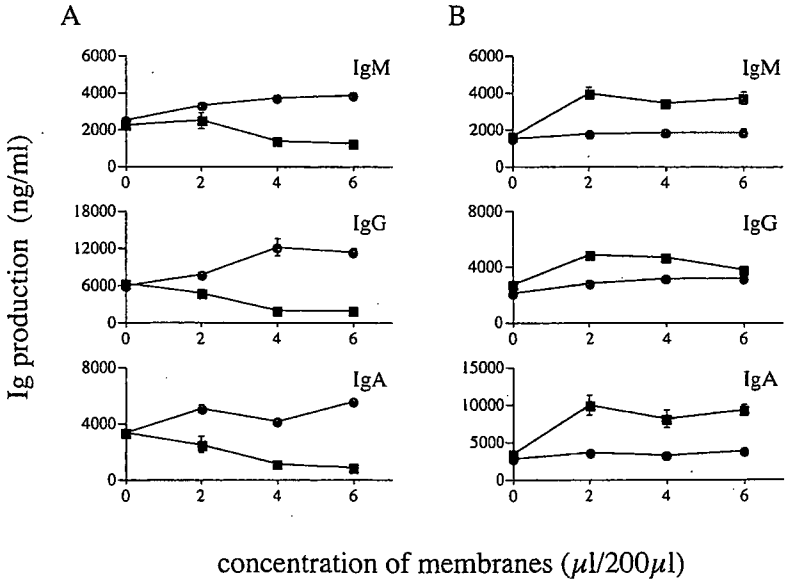
FIGURE 7. Kinetics of the suppression of B cell responses by CD40 ligand. B cells (2.5 × 10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells (1 × 10^5/well). Membranes (6 μl) of Sf9 cells infected with either recombinant human CD40 ligand (black) or wild-type baculovirus (gray) or no membranes (white) were added at the time of stimulation or afterward. The supernatants were harvested after 10 days of culture and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.

Table I. *Differential regulation of Ig secretion by early and later addition of CD40 ligand*

Addition to First Culture	Addition to Second Culture: Nil	40L	WT
		IgM (ng/ml)	
Nil	1280 ± 34	5960 ± 116	1800 ± 70
40L	4010 ± 47	2040 ± 46	6290 ± 910
WT	2210 ± 15	4790 ± 371	2210 ± 140
		IgG (ng/ml)	
Nil	1240 ± 22	1740 ± 163	2010 ± 78
40L	5830 ± 430	1210 ± 55	9880 ± 744
WT	1640 ± 106	1130 ± 169	1530 ± 134
		IgA (ng/ml)	
Nil	453 ± 16	942 ± 34	742 ± 26
40L	3710 ± 185	1030 ± 85	6580 ± 725
WT	509 ± 29	800 ± 53	488 ± 7.0
		MTT (OD × 10^{-3})	
Nil	122 ± 10.7	240 ± 6.0	99.7 ± 2.6
40L	160 ± 4.5	194 ± 3.3	158 ± 6.2
WT	93.5 ± 0.5	207 ± 0.5	101 ± 4.6

[a] B cells (2 × 10^5/well) were cultured for 48 h with SAC (1:60,000) and IL-2 (20 U/ml) in the presence of membranes (6 μl) of Sf9 cells infected with either recombinant human CD40 ligand (40L) or wild type (WT) baculovirus. Afterward, cells were harvested from the wells and cultured (5 × 10^4/well) with IL-2 (20 U/ml) in the presence of either membrane (4 μl). The supernatants were harvested after a total culture of 10 days and Ig content was analyzed by isotype-specific ELISA. The number of metabolically active cells was assessed by MTT assay after a total length of culture of 10 days. OD at the initiation of the second culture was 102, 110, and 91 × 10^3, for Nil, 40L, and WT in the first culture, respectively. Data presented are representative of at least three independent experiments with similar results.

FIGURE 8. Sensitivity of B cells to suppression by CD40 ligand is dependent on the stage of B cell activation. B cells (5 × 10^4/well) were cultured with anti-CD3 (200 ng/ml)-stimulated mitomycin C-treated $CD4^+$ T cells (1 × 10^5/well) for either 48 h (*A*) or 96 h (*B*). Afterward, cells were harvested from the wells, and B cells were separated from T cells by rosetting with neuraminidase-treated SRBC. The activated B cells (5 × 10^4/well) were cultured in the presence of IL-2 (20 U/ml). Various concentrations of membranes of Sf9 cells infected with either recombinant human CD40 ligand (squares) or wild-type baculovirus (circles) were added where indicated. The supernatants were harvested after a total culture of 10 days, and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.



By contrast, the same concentration of CD40 ligand suppressed both IgM and IgG production by IgD^- memory B cells. With all of the concentrations of CD40 ligand tested, Ig production by IgD^- memory B cells was suppressed (Fig. 9*B*). Only when extremely low concentrations of CD40 ligand were employed, below the concentration capable of stimulating IgD^+ naive B cells, was a positive response from IgD^- memory B cells observed (data not shown). Finally, as shown in Figure 9*C*, increased production of IgM was noted when IgD^+ naive B cells were stimulated with CD40 ligand at the initiation of culture or 48 h later. Only when IgD^+ naive B cells were stimulated with CD40 ligand at both the initiation and at 48 h was inhibition noted. In contrast, CD40 ligand suppressed responses of IgD^- memory B cells when added either at the initiation of culture, at 48 h, or at both times. These results are consistent with the conclusion that IgD^+ naive and IgD^- memory B cells are markedly different in their responses to CD40 ligation, with the former poised toward stimulation and the latter toward suppression by this interaction.

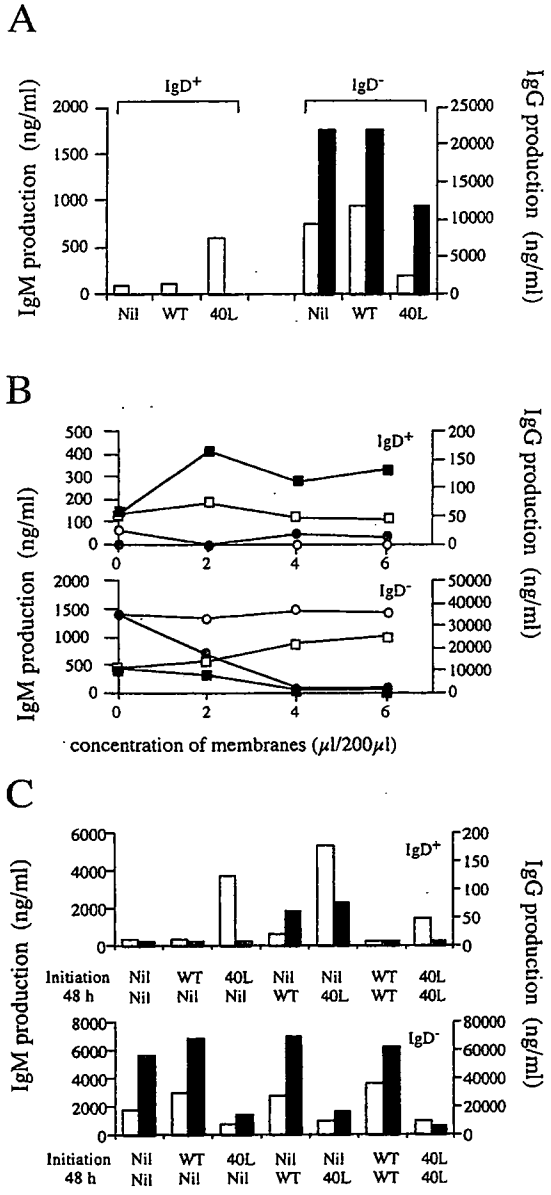


FIGURE 9. Difference of the effect of CD40 ligand on Ig secretion by IgD^+ or IgD^- B cell subpopulations. *A*, IgD^+ (*left* bars) or IgD^- (*right* bars) B cells (5×10^4/well) were cultured with SAC (1:60,000) and IL-2 (20 U/ml) in the absence or presence of membranes (2 μl) of Sf9 cells infected with either recombinant human CD40 ligand or wild-type baculovirus added at the initiation of culture. After a 10-day culture, IgM production (open bars) and IgG production (closed bars) were assessed. *B*, IgD^+ (*upper panel*) or IgD^- (*lower panel*) B cells (5×10^4/well) were cultured with SAC and IL-2 in the absence or presence of various concentrations of membranes of Sf9 cells infected with either recombinant human CD40 ligand (closed symbols) or wild-type baculovirus (open symbols) added at the initiation of culture. After a 10-day incubation, IgM (squares) and IgG (circles) were assessed. *C*, IgD^+ (*upper panel*) or IgD^- (*lower panel*) B cells were cultured with SAC and IL-2 alone or in the presence of 2 μl of membranes from Sf9 cells infected with either recombinant human CD40 ligand or wild-type baculovirus added at the initiation of culture or 48 h later, as indicated. After a 10-day incubation, IgM (open bars) and IgG (closed bars) secretion was assessed.

It has previously been suggested that CD40 ligation favors the development of $CD38^-$ memory cells from germinal center B cells and restricts the development of $CD38^+$ plasma cells (11). Since peripheral blood IgD^+ naive and IgD^- memory B cells represent pre- and post-germinal center cells, respectively, it was of interest to determine the impact of CD40 ligation on CD38 expression by these cells. As shown in Figure 10, SAC + IL-2 increased expression of CD38 on B cells. This effect was blocked by CD40 ligand. Even when CD38 expression was up-regulated by stimulation with SAC + IL-2 in the absence of CD40 ligand, late addition of CD40 ligand caused rapid down-modulation. When B cells were separated into IgD^+ or IgD^- subpopulations, $CD38^+$ cells were found in both the IgD^+ and IgD^- B cell populations. Although SAC + IL-2 induced CD38 expression by both B cell populations, $CD38^{bright}$ cells were only found in the activated IgD^- memory B cell population (Fig. 11). Whereas CD40 ligand down-regulated CD38 expression by activated IgD^- memory B cells, it enhanced expression by activated IgD^+ naive B cells.

Activated B cells down-regulate CD40 ligand expression by $CD4^+$ T cells more effectively

The final experiments examined the capacity of activated B cells to influence CD40 ligand expression by anti-CD3-stimulated $CD4^+$ T cells. CD40 ligand expression by $CD4^+$ T cells was assessed in cultures supplemented with either resting B cells or B cells that previously had been activated with anti-CD3-stimulated $CD4^+$ T cells. As seen in Figure 12, CD40 ligand expression by activated $CD4^+$ T cells was down-regulated by coculture with B cells and much more markedly down-regulated by coculture with activated B cells. However, when the activated B cells were removed from culture, the $CD4^+$ T cells rapidly re-expressed CD40 ligand. On the other hand, when $CD4^+$ T cells were first activated to express CD40 ligand, coculture with activated but not resting B cells rapidly down-modulated CD40 ligand expression.

Discussion

Engagement of B cell CD40 by CD40 ligand expressed by activated T cells plays an essential role in the activation, proliferation, and differentiation of B cells during T cell-B cell collaboration (1–11). Initial studies focused on the stimulatory effects of this interaction on B cell function. However, more recent results have indicated a more complex role of CD40 engagement on B cell function, with the possibility that signals provided through CD40 might inhibit B cell growth and Ab formation (12–14, 16) or induce apoptosis of B cells (16) at specific stages of maturation and/or differentiation. The current studies, therefore, examined the positive and negative influences of ligation of CD40 on the function of human peripheral B cells. As peripheral blood B cells contain both IgM^+IgD^+ naive B cells that have not yet been expanded in germinal centers and IgD^- memory B cells that have previously been stimulated by Ag (32), the use of this population permitted an analysis of the differential sensitivity of these subsets to signals conveyed by CD40 ligation. To ensure that CD40 engagement was provided in a physiologically relevant manner, recombinant membrane-bound human CD40 ligand expressed by membranes of baculovirus-infected Sf9 cells was employed. It was anticipated that this form of CD40 ligand would mimic engagement of CD40 accomplished by activated T cells most closely (20, 21), as the avidity and three-dimensional organization of the interaction should be comparable and effects mediated by Fc receptor binding of mAb and hypercross-linking by multimeric constructs would be avoided. The physiologic relevance of this approach was emphasized by the observation that both the positive and negative effects of CD40 ligation were detected in cultures of intact anti-CD3 activated $CD4^+$ T cells and B cells.

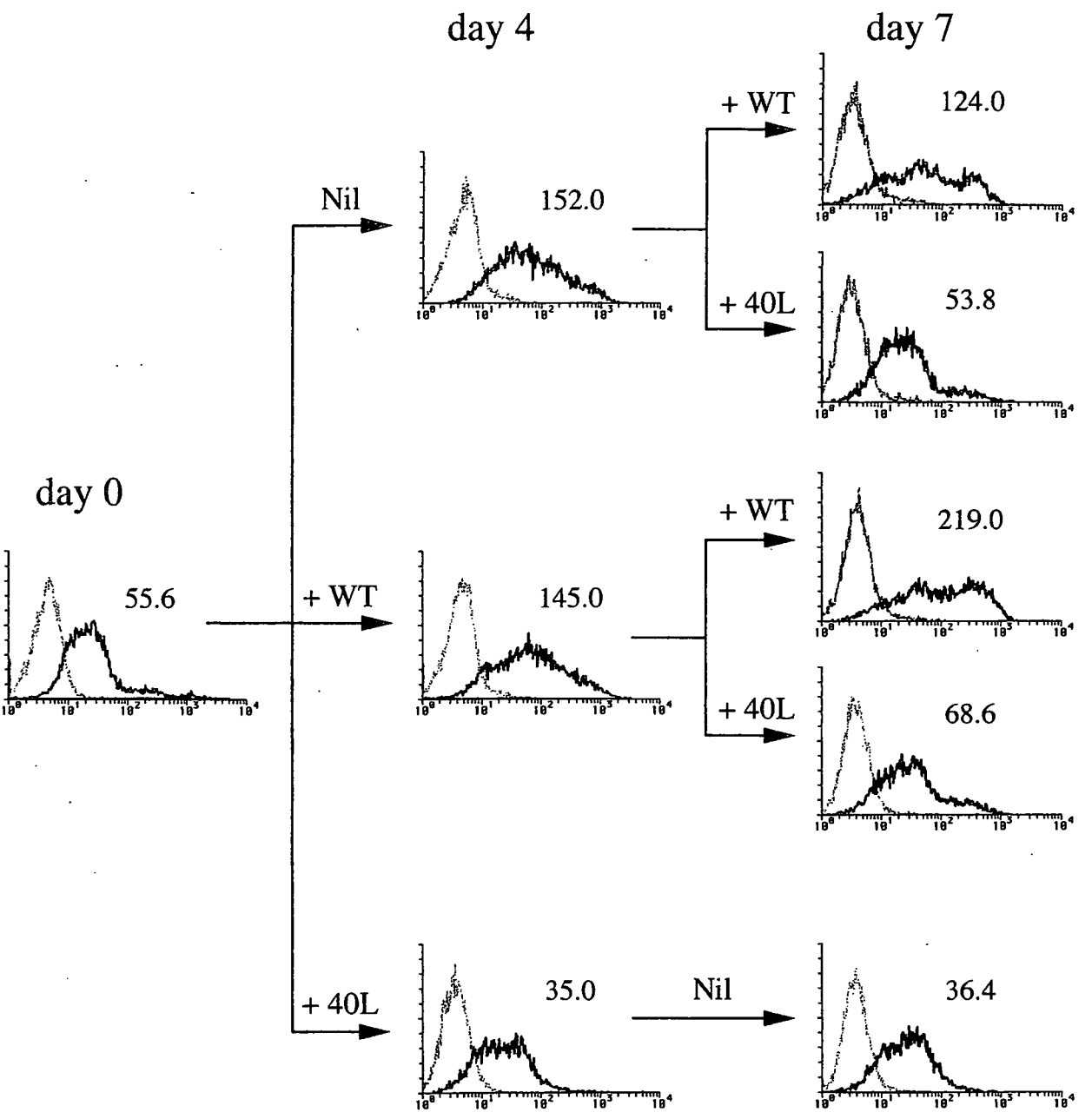


FIGURE 10. CD38 expression is decreased by engagement of CD40. B cells (5×10^4/well) were immediately stained for CD38 expression or were stimulated with SAC (1:60,000) and IL-2 (20 U/ml) in the presence or absence of membranes (2 μl) of Sf9 cells infected with either recombinant human CD40 ligand or wild-type baculovirus. In some experiments, membranes (2 μl) were again added to the culture wells on day 4. Cells were harvested after culture for various lengths of time as indicated and stained with FITC-conjugated anti-CD38 mAb or FITC-conjugated control mAb, and CD38 expression was analyzed by flow cytometry. Numbers in each histogram indicate the ΔMFI for staining with anti-CD38 mAb calculated by subtracting the MFI of staining with the control mAb.

The temporally limited expression of CD40 ligand by activated T cells suggests that prolonged engagement of B cell CD40 might negatively influence B cell responses. Previous studies had indicated that engagement of CD40 with mAb or a soluble trimeric CD40 ligand construct could inhibit specific Ab responses by tonsillar memory B cells (11, 17, 18). Inhibition appeared to require simultaneous engagement of surface Ig and limited specific Ab production, but not proliferation or expansion of tonsillar B cells with a memory cell phenotype ($CD38^-IgD^-$). As specific Ab production originated from memory B cells only (17, 18), effects of CD40 ligation on naive B cells could not be estimated. Moreover, since specific Ag or engagement of surface Ig was required to elicit Ab production (17, 18), the effect of CD40 ligation alone could not be estimated. Finally, differential effects of CD40 ligation dependent on the stage of B cell activation were not assessed. This is of particular relevance because it has been shown that both Ag and CD40 ligation are necessary for secondary Ab responses of peripheral blood B cells from normal volunteers immunized with the

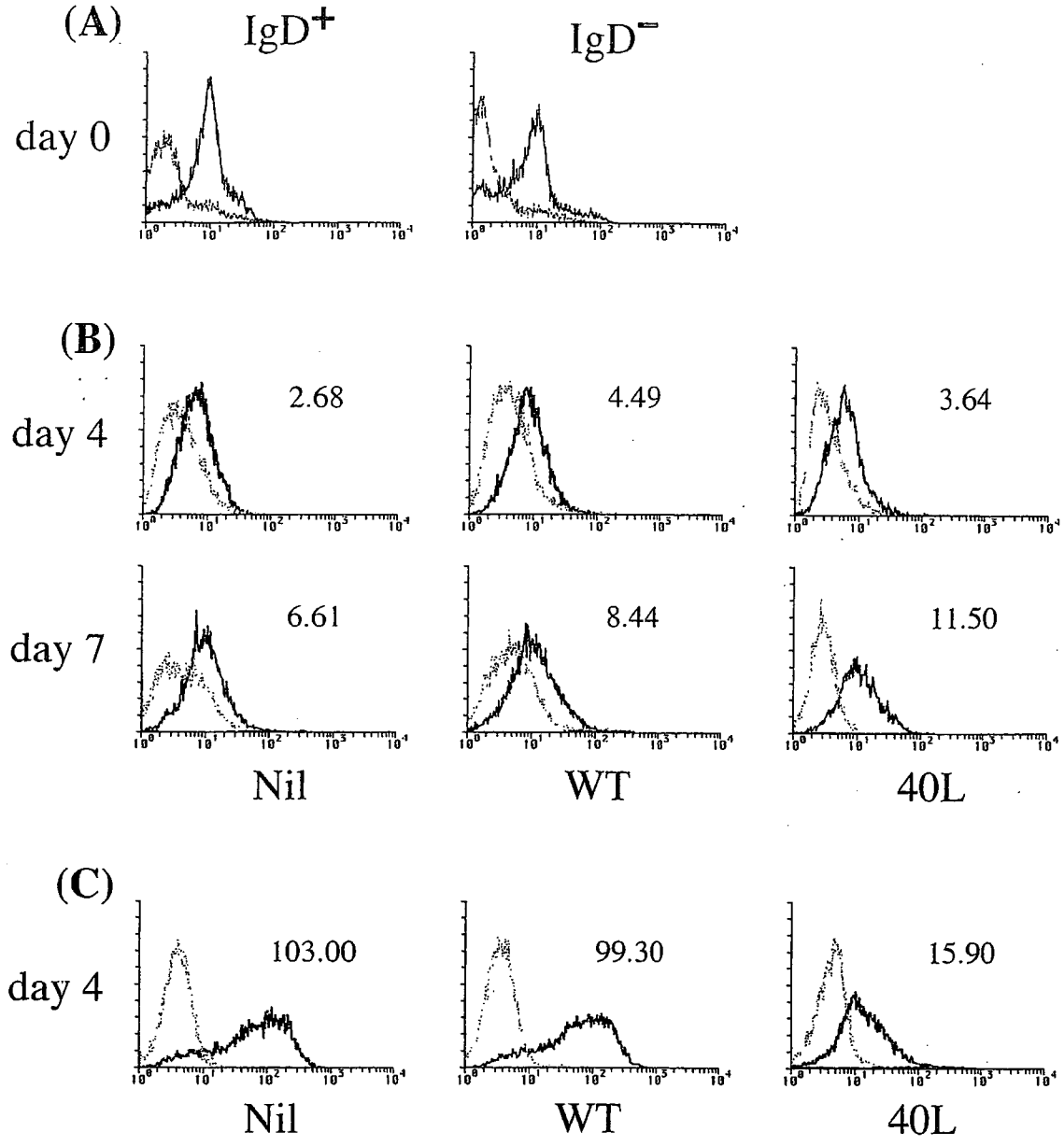


FIGURE 11. The effect of CD40 ligation on CD38 expression by IgD^+ or IgD^- B cell populations. (*A*) IgD^+ or IgD^- B cells were immediately analyzed for CD38 expression. IgD^+ (*B*) or IgD^- (*C*) B cells (5×10^4/well) were cultured with SAC (1:60,000) and IL-2 (20 U/ml) in the absence or presence of membranes (2 μl) of Sf9 cells infected with either recombinant human CD40 ligand or wild-type baculovirus. Cells were harvested after culture for various lengths of time as indicated and stained with FITC-conjugated anti-CD38 mAb or FITC-conjugated control mAb, and CD38 expression was analyzed by flow cytometry. Numbers in each histogram indicate the ΔMFI for staining with anti-CD38 mAb calculated by subtracting the MFI of staining with the control mAb.

virus ΦX174 (33). One possible explanation for this apparent discrepancy could relate to differential sensitivity of B cells at various stages of maturation.

The current data provide information that addresses some of these issues. First, it is apparent that CD40 ligation can stimulate Ab production from both naive IgD^+ and memory IgD^- B cells. As previously shown (10), the stimulatory signal is provided during the initial few hours of culture. The major difference between IgD^+ naive and IgD^- memory B cells appears to relate to the sensitivity of the latter to CD40 signaling, with very low concentrations of CD40 ligand needed for induction of Ab production by IgD^- memory B cells, whereas higher concentrations became rapidly suppressive. This result is different from a previous report claiming that tonsillar memory B cells were unresponsive to CD40 ligation (18). The discrepancy may relate to unique properties of tonsillar memory B cells or, more likely, to the utilization in the previous report of a single anti-CD40 mAb to modulate responses (18). The current results show that IgD^- memory B cells are uniquely sensitive to both the positive and negative influences of CD40 ligation, and therefore, the degree of cross-linking caused by the anti-CD40 mAb was likely to inhibit responses of tonsillar B cells. The current results are consistent with previous findings that physiologic ligation of CD40 is necessary for the induction of peripheral memory B cell responses (33).

The unique sensitivity of IgD^- memory B cells to signaling via CD40 does not appear to relate to the density of CD40 expressed by naive and memory B cells, as these populations did not differ

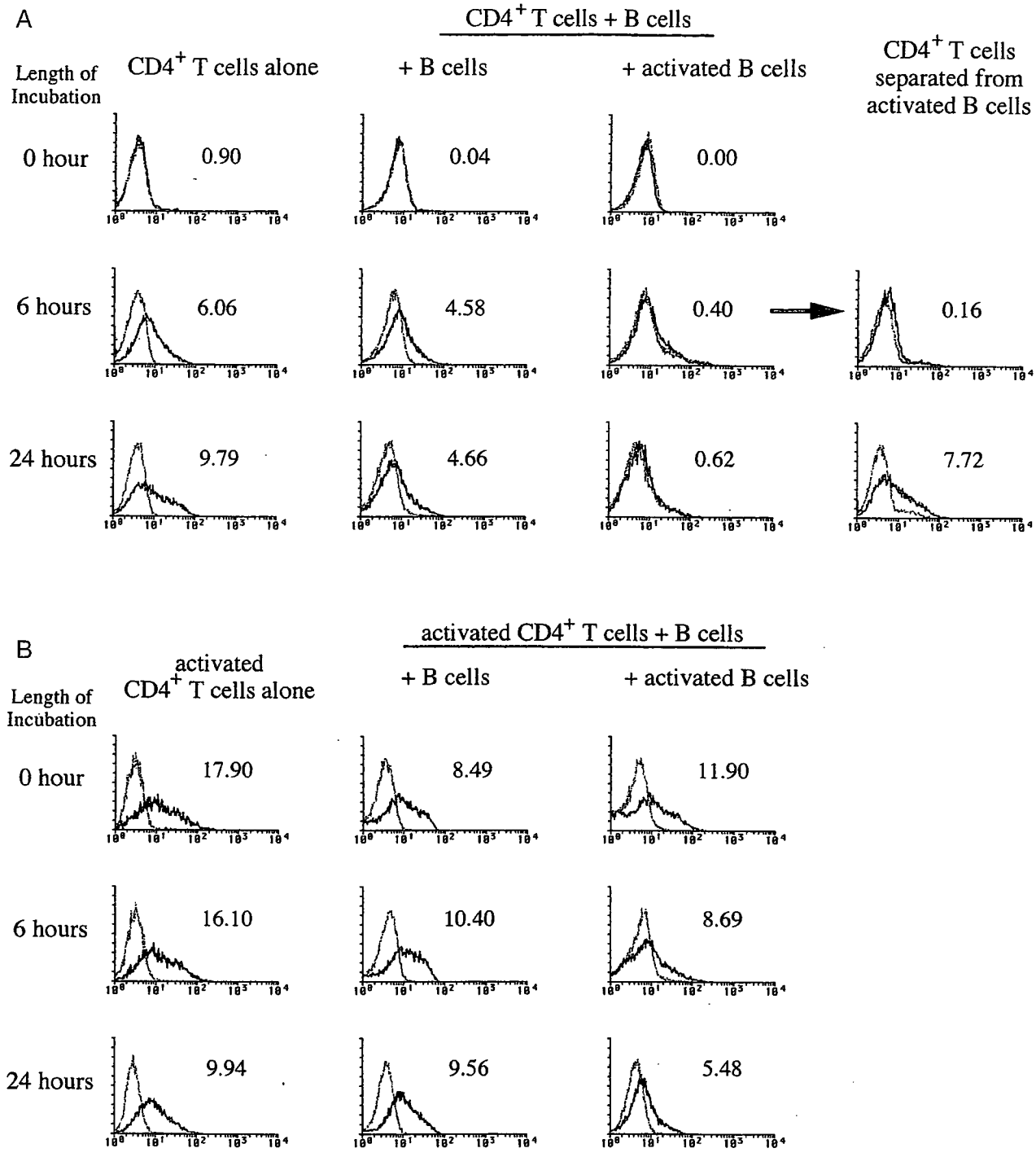


FIGURE 12. Activated B cells strongly inhibit CD40 ligand expression by $CD4^+$ T cells. B cells (5×10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated control $CD4^+$ T cells (1×10^5/well) for 48 h. Afterward, cells were harvested from the wells, and activated B cells were separated from T cells by rosetting with neuraminidase-treated SRBC. *A*, Either resting B cells or activated B cells (2.5×10^4/well) were cultured with resting $CD4^+$ T cells (1×10^5/well) in anti-CD3 (200 ng/well)-coated wells for the length of time indicated. Afterward, cells were stained with a CD19-PE mAb and either 4D9-8 or a control mAb followed by anti-mouse IgG1-FITC, and CD40 ligand expression by CD19-negative cells was analyzed by flow cytometry. Where indicated, B cells were removed and $CD4^+$ T cells were assessed for CD40 ligand expression immediately or after an 18-h incubation with anti-CD3. *B*, Similar experiments were undertaken in which either resting B cells or activated B cells were cultured with $CD4^+$ T cells that had been previously activated without B cells in anti-CD3 (200 ng/well)-coated wells for 48 h. Numbers in each histogram indicate the mean fluorescence for staining with mAb to CD40 ligand calculated by subtracting the MFI for staining with the control mAb.

significantly (data not shown). It is possible, however, that expression of CD40-associated signaling molecules may differ as B cells differentiate to a memory phenotype. Stimulation of Ig production via CD40 appears to involve members of the CD40-associated TRAF family of signaling molecule, such as CRAF-1/TRAF-3, or perhaps others (34–37). Differences in the density and/or availability of these

various CD40-associated signaling molecules may occur with B cell differentiation and may account for changes in the sensitivity to signaling by ligation of CD40.

The second feature of the regulation of B cell responses by CD40 ligation is the rapid induction of suppressive potential following activation. IgD^+ naive B cells and IgD^- memory B cells differed in the time required for suppressive activity to become manifest and also in the intensity of CD40 ligation for suppression to be apparent. Memory B cells became rapidly responsive to the inhibitory influences of CD40 ligation, whereas naive B cells required more prolonged activation. However, after 48 h of activation, the effect of CD40 ligation was uniformly inhibitory. The change in sensitivity to CD40 ligation did not reflect changes in density of surface CD40, as this did not vary significantly after 48 h of activation with SAC and IL-2 (data not shown). Of note, the sensitivity to CD40-mediated inhibition itself was transient, lasting only about 24 to 48 h. Afterward, B cells again became responsive to the positive effects of CD40 ligation. As the initial round of B cell division occurs in this time frame (38, 39), these results are consistent with the conclusion that activated B cells become sensitive to inhibition by CD40 ligation, whereas daughter cells generated as a result of cell division may become resistant to inhibition but responsive to the positive influences of CD40 ligation. These findings suggest that the signaling potential of CD40 ligation may be tightly regulated as B cells progress through the cell cycle and are consistent with the possibility that the availability or activity of CD40-associated signaling molecules that govern responsiveness may vary in a cell cycle-dependent manner.

The inhibitory potential of CD40 ligation was different from that previously suggested (17, 18) in that simultaneous ligation of surface Ig was not required for CD40-mediated inhibition to be apparent. Thus, CD40-mediated inhibition was noted when B cells were stimulated either with activated T cells or with SAC + IL-2 that could cross-link VH3 containing surface IgM as well as surface IgG (40, 41). No consistent difference in the degree of inhibition was noted for memory or naive B cells activated in either manner. Of interest, activated naive B cells were most suppressed by CD40 ligand when they had been initially stimulated in a manner involving ligation of CD40. The signaling pathways involved in this phenomenon remain to be elucidated, but the data suggest the possibility that signaling through CD40 in naive cells alters the subsequent functional outcome of CD40 engagement. As IgD^- memory B cells presumably have previously been stimulated in secondary lymphoid organs by CD40 ligation (42), the alteration in CD40 response coupling of memory B cells may reflect a persistent change resulting from antecedent CD40 ligation.

The inhibitory effect of CD40 ligation on activated B cells specifically involved inhibition of Ab production and not proliferation. Thus, initial DNA synthesis in T cell-dependent cultures was unimpaired by concentrations of CD40 ligand that inhibited Ig production, and CD40 ligation of SAC- and IL-2-stimulated B cells was found to enhance the number of viable cells in longer cultures in a manner that was independent of whether Ig secretion was inhibited or enhanced. Previous experiments have yielded similar results from anti-Ig-stimulated tonsillar memory B cells in which proliferation was costimulated, whereas specific Ab production was inhibited (17, 18). These results have been interpreted as indicating that CD40 ligation may preferentially induce differentiation of memory B cells and limit development of plasma cells (11). This interpretation is based on the impact of CD40 ligation on CD38 expression by germinal center B cells. Thus, CD40 ligand appears to inhibit the generation of $CD38^{bright}$ plasma cells and enhance the differentiation of $CD38^-CD20^+$ memory cells from $CD38^+$ tonsillar germinal center cells (11). The current results confirm that CD40 ligand inhibited CD38 expression, but this effect was predominantly limited to the IgD^- memory B cell population. In contrast, CD40 ligation modestly up-regulated CD38 expression by activated IgD^+ naive B cells. As many B cells entering germinal centers are IgD^+ naive cells (42), the current result appears to conflict with the aforementioned data. Moreover, it is unlikely that CD40 ligation inhibited Ig production by limiting plasma cell generation, because SAC + IL-2 rarely induces the differentiation of plasma cells from either IgD^+ or IgD^- B cells but, rather, stimulates Ig production from rapidly dividing B lymphoblasts (43, 44). In addition, the number of Ig-secreting cells stimulated by SAC + IL-2 is relatively small (38, 43) compared with the number of memory B cells induced to up-regulate CD38. Therefore, the possibility that CD40 ligation of peripheral B cells specifically limits the differentiation of plasma cells seems unlikely. Rather, it seems more likely that CD40 ligation may have a direct effect on CD38 expression, independent of the implication of that expression on the stage of maturation or functional differentiation of the B cell. This interpretation is supported by the observation that double staining for intracytoplasmic Ig and surface CD38 has shown that only the $CD38^{bright}$ B cells are producers of Ig (data not shown). Although CD40 ligation inhibited the generation of $CD38^{bright}$ Ig-producing cells, it also diminished expression of CD38 by $CD38^{dim}$ B cells that do not produce Ig. Moreover, the late addition of CD40 ligand caused rapid down-modulation of CD38 expression induced by initial stimulation with SAC and IL-2. These effects are more consistent with the conclusion that CD40 ligation directly regulates CD38 expression and has a separate action on the differentiation of B cells into Ig secreting cells.

The final experiments examined the capacity of B cells to down-regulate CD40 ligand expression by activated T cells. Some studies have shown that activated B cells can down-regulate T cell expression of CD40 ligand by a CD40-CD40 ligand dependent mechanism (30, 45), whereas others have reported that CD40 stabilizes CD40 ligand expression by T cells (46). Of note, the studies showing down-modulation of T cell CD40 ligand expression have usually employed B or T cell lines or clones and high ratios of B cells to T cells. In the current studies, down-modulation of T cell CD40 ligand expression was demonstrated using fresh peripheral blood B and T cell and at ratios that were comparable to those used to examine Ig production. The striking observation was the markedly enhanced capacity of activated compared with resting B cells to down-regulate CD40 ligand expression by activated T cells. These results are consistent with the conclusion that as B cells are activated they become more sensitive to the inhibitory effects of CD40 ligand and also develop an enhanced capacity to down-regulate CD40 ligand expression.

Three general mechanisms of rapidly down-regulating of CD40 ligand expression have been identified. These include capping and endocytosis of CD40 ligand following interaction with CD40 (30), blocking of the CD40 ligand binding site by CD40 released by B cells (45), and the induction of cleavage and shedding of soluble CD40 ligand after binding CD40 (47). It is unlikely that blocking of CD40 ligand by shed CD40 plays a major role in the current observations, as acid washing to remove bound CD40 (48) revealed only a modestly greater density of CD40 ligand expressed by activated T cells (data not

shown). Rather, endocytosis or proteolytic cleavage and shedding of CD40 ligand triggered by engagement of B cell CD40 appears to be a more likely explanation. The explanation for the greater efficiency of activated compared with resting B cells to down-modulate CD40 ligand expression by activated T cells has not been delineated. However, the difference cannot be explained by an enhanced expression of CD40 by SAC + IL-2-activated B cells, as their CD40 density is not increased significantly (data not shown). Rather, activated B cells appear to have developed the capacity to down-modulate CD40 ligand on activated T cells more effectively than resting B cells by a mechanism independent of changes in density of CD40 expression. Such a mechanism may also explain the enhanced capacity of transformed B cell lines compared with fresh B cells to down-modulate CD40 ligand expression by activated T cells (30).

The results of these studies have several implications. It is clear that CD40-CD40 ligand interactions during B cell-T cell collaboration subserve much more complex biologic functions than previously anticipated. After B cells are initially activated by ligation of CD40, they become much more vulnerable to inhibition by subsequent CD40 engagement. This may explain the finding that partial blocking of CD40-CD40 ligand interactions in vivo with a CD40-IgG1 construct was found to enhance production of IgM Abs, whereas germinal center formation was intact (5). The segregation of activated B cells away from T cells in the dark zones of germinal centers (42) may be an important mechanism to protect B cells from inhibition of differentiation by ongoing CD40 ligation. Alternatively, recurrent CD40-CD40 ligand interactions in the germinal centers between activated B cells expressing CD40 and those expressing low levels of CD40 ligand (48) may be important in promoting clonal expansion in the dark zones of germinal centers without premature differentiation to plasma cells. Activated B cells themselves become more able to regulate the expression of T cell CD40 ligand and, therefore, may direct the intensity of the signals provided via CD40. These complex bidirectional interactions appear to be essential in propagating normal Ab responses.

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Increase in age at onset of rheumatoid arthritis in Japan over a 30 year period

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Abstract

***Objectives*—To determine changes in demographic variables and severity of rheumatoid arthritis (RA) that may have occurred during the 30 year period from 1960 to 1990 in Japan.**

***Methods*—Using records of patients diagnosed with RA from two hospitals, demographic and clinical features at initial visit were compared between two groups, one from 1960 to 1965 (group I) and the other from 1985 to 1990 (group II).**

***Results*—Mean age at the time of onset of the disease increased significantly from 37.5 years in group I to 46.9 in group II. The peak age at onset of RA shifted from the third to the fifth decade between group I and group II. There was no obvious change in morbidity as determined by seropositivity, rheumatoid nodules, and assessments of hip involvement.**

***Conclusion*—The age at onset of RA was delayed during a recent 30 year period in Japan. This increase in age at onset might result from environmental changes that occurred in Japan or may reflect a birth cohort phenomenon. Improvement of severity of disease was not found in this study.**

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In the second half of the 20th century, there have been significant changes in socioeconomic status, personal life styles, and public health measures throughout much of the world and especially in Japan. Therapeutic management of disease has also undergone considerable changes. In this context, if the clinical features of rheumatoid arthritis (RA) are affected by environmental factors, these features, too, may have changed. In fact, some authors have presented the hypothesis that RA is becoming less severe and the incidence of the disease is declining.[1–9] We performed a retrospective cross sectional study, reviewing records of patients diagnosed with RA from two hospitals (one set of records obtained from 1960 to 1965 and the other from 1985 to 1990), to determine possible changes during this period in demographic and clinical features of Japanese patients with RA.

Methods

Records of 566 patients (125 males, 441 females) who visited the Department of Orthopaedic Surgery at Osaka University Hospital from 1960 to 1965 (group I), and 366 patients (67 males, 299 females) who visited the Shichikawa Arthritis Research Centre between 1985 and 1990 (group II) were examined. The first hospital is located in an urban area and the second in a rural area. All patients were diagnosed as having definite or classic RA according to diagnostic criteria of the American Rheumatism Association.[10] This study comprised records for all consecutive patients, who visited the hospital for the first time during those periods. At both institutes, one of the study's authors (K S) was working as the chief of rheumatologists for each chronological period studied, and the same data items could be obtained. Data were gathered and recorded for each patient upon the initial visit, including sex, age, disease duration, age at onset, seropositivity, presence of rheumatoid nodules, and hip involvement. Data for both groups were then compared.

Clinical examinations of both groups of patients were performed by experienced rheumatologists. Seventy nine per cent of patients in group I and 73% of those in group II were examined by one of the study's authors (K S). Statistical analysis was performed using the χ^2 test and Student's t test.

Results

Table 1 shows a basic comparison of clinical data between groups I and II. Group I consisted of 125 males and 441 females (total 566), and group II of 67 males and 299 females (total 366). The male to female ratio was 1:3.5 in group I and 1:4.5 in group II, with no significant difference. The mean age at initial visit was 42.6 years (range 7-96) in group I and 54.5 (range 11-82) in group II. This difference was statistically significant ($p<0.001$). The mean duration of the disease was 4.9 years (range 0.5-44) in group I and 7.5 (range 0.5-40) in group II, showing statistical significance ($p<0.001$). This significant difference in the mean disease duration was the case for female patients (4.9 years versus 8.1 years for group I and group II, respectively; $p<0.001$), although there was no significant difference in male patients (4.9 years versus 4.8 years). The mean age at onset of 46.9 years (range 9-76) in group II was significantly higher compared with 37.5 (range 7-96) in group I ($p<0.001$), and this was the case for both sexes (41.3 versus 54.0 for male patients and 36.5 versus 45.3 for female patients). There was no significant difference in seropositivity between the two groups (69.5% versus 75.1%), and this was the case regardless of the specific 10 year range of age at onset (data not shown). Similarly, percentages of patients with rheumatoid nodules in group I and group II (9.9% versus 12.3%) and those

Table 1 Comparison of clinical characteristics between groups I and II

	Group I	Group II
Initial visit (year range)	1960–1965	1985–1990
Institution	Osaka University Hospital	Shichikawa Arthritis Research Centre
Number of patients total	566	366
Male	125	67
Female	441	299
Ratio of males to females	1:3.5	1:4.5
Age at initial visit (y) : (mean (SD))	42.6 (14.3)	54.5 (11.7)*
Disease duration (y) total : (mean (SD))	4.9 (6.1)	7.5 (8.2)*
Male : (mean (SD))	4.9 (6.8)	4.8 (4.5)
Female : (mean (SD))	4.9 (5.9)	8.1 (8.7)*
Age at onset (y) total : (mean (SD))	37.5 (14.1)	46.9 (13.9)*
Male : (mean (SD))	41.3 (15.3)	54.0 (12.1)*
Female : (mean (SD))	36.5 (13.6)	45.3 (13.8)*
Seropositivity (%)	69.5	75.1
Rheumatoid nodule positivity (%)	9.9	12.3
Rate of hip involvement (%)	14.9	16.0

*Significant difference ($p<0.001$) was observed between these two groups.

with hip joint involvement (14.9% versus 16.0%) did not differ greatly.

To adjust for disease duration and compare patients with similar duration, each group was divided into three subgroups according to duration: less than five years (subgroup a), five years or more but less than 10 years (subgroup b), and 10 years or more (subgroup c).

Table 2 shows a comparison between the subgroups of group I and those of group II. With regard to the male to female ratio, statistical difference was observed only in subgroups c (1:3.8 in group I and 1:9.5 in group II; $p<0.05$). Higher mean age at initial visit was evident in each subgroup of group II (40.8 in subgroup Ia versus 55.0 in IIa, 43.0 in Ib versus 52.3 in IIb, and 48.4 in Ic versus 55.3 in IIc; $p<0.05$). There was no statistical difference in mean disease duration in any of the subgroups. Mean age at onset was significantly higher in group II within any subgroup (39.2 in Ia versus 53.1 in IIa, 36.6 in Ib versus 45.5 in IIb, and 32.9 in Ic versus 37.4 in IIc; $p<0.05$ respectively). There was no notable difference in seropositivity and rheumatoid nodule positivity in any subgroup, whereas the rate of hip involvement in group II was significantly lower in subgroup a (15.4% in Ia versus 7.2% in IIa; $p<0.05$).

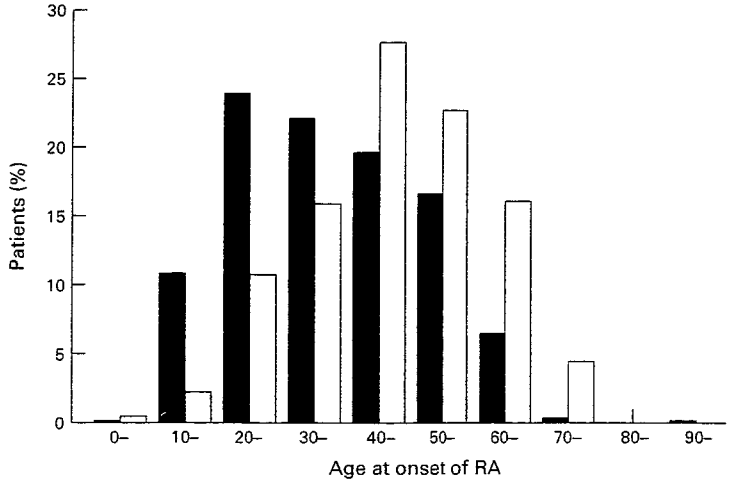

Figure 1 Distribution of age at onset. ■ : Group I patients who visited Osaka University Hospital from 1960 to 1965, □: group II patients who attended Shichikawa Arthritis Research Centre from 1985 to 1990.

Figure 1 shows the distribution of age at onset for groups I and II. A noticeable peak was observed in the 20s in group I and in the 40s in group II. Patterns of distribution of age at onset for female patients had a peak in the 20s in group I and in the 40s in group II, whereas for male patients the peak was in the 30s and 50s in group I and only in the 50s in group II. Similarly, the median age at onset was 36 in group I and 47 in group II. It was 35 for women in group I, 45 for women in group II, 41 for men in group I, and 53 for men in group II.

Discussion

Our study showed that the mean age at onset of group II patients was significantly higher than that of group I patients. A delay in the age at onset was a common feature of both men and women. This delay was still evident after adjusting for disease duration. Moreover, the peak age at onset shifted from the 20s to the 40s. This shift was the same with regard to female patients, while in male patients the peak age at onset shifted from the 30s and 50s to only the 50s. The higher mean age at initial visit in group II can be attributed to the higher age at onset.

Because this study was a comparison of two groups of RA patients recruited in two units, 30 years apart, the possible biases involved in this kind of study should be examined. We considered some possibilities of a selection bias in the study. Firstly, Osaka University Hospital, the source of patient records for group I, is located in an urban area, whereas Shichikawa Arthritis Research Centre, the source for those in group II, is in a rural area. Consequently, there was a possibility that the hospital for group I selectively attracted younger patients, or that the population served by an urban hospital was younger than the population served by a rural hospital. However, analysis of 286 patients with RA making a first visit between 1985 and 1990 at Osaka University Hospital, the same hospital that our group I patients visited, showed a peak age at onset in the 40s in both sexes, which correlates well with age at onset of the group II patients in this study (fig 2). For additional study, we obtained data from an urban hospital (Yukioka Hospital) where one of the authors (K S) has worked at an outpatient clinic, located near Osaka University Hospital, and other additional data from the Orthopaedic Department of Osaka-Minami National Hospital, situated in Kawachi-Nagano city, on the outskirts of Osaka (table 3). Analysis of records of 233 patients with RA between 1985 and 1990 in the former, and of 370 patients between 1991 and 1994 in the latter, showed a corresponding peak age at onset in the 40s with mean age at onset of 45.4 years and 44.8 years, respectively (fig 2).

These data showed a close resemblance with those of our group II patients, and the distribution pattern of age at onset of RA was the same, regardless of referral hospitals. Furthermore, Nobunaga has described the same phenomenon in Japanese literature[11]; specifically, mean age at onset of patients admitted with RA at the Department of Inter-

Table 2 Comparison of data from two sources for patients with similar disease duration

Disease duration	*<5 years*		*≥5 <10 years*		*≥10 years*	
Subgroup	a		b		c	
Group	I	II	I	II	I	II
Number of patients	353	178	112	83	101	105
Ratio of males to females	1:3.4	1:3.8	1:3.9	1:3.2	1:3.8	1:9.5*
Age at initial visit (y) (mean (SD))	40.8 (14.8)	55.0 (12.1)*	43.0 (13.7)	52.3 (12.3)*	48.4 (11.3)	55.3 (9.9)*
Disease duration (y) (mean (SD))	1.4 (1.3)	1.6 (1.4)	6.8 (1.3)	6.3 (1.3)	15.5 (6.5)	18.0 (7.8)
Age at onset (y) (mean (SD))	39.2 (14.6)	53.1 (12.3)*	36.6 (13.8)	45.5 (12.5)*	32.9 (11.6)	37.4 (11.9)*
Seropositivity (%)	70.4	72.4	64.5	76.3	72.8	79.4
Rheumatoid nodule positivity (%)	8.5	12.0	9.8	12.0	15.1	13.5
Rate of hip involvement (%)	15.4	7.2*	14.0	21.4	18.8	25.8

*Significant difference ($p < 0.05$) was observed between subgroups.

Table 3 Comparison of data from five sources for consecutive patients with RA

Institution	*Period*	*City*	*Area*	*Patients (n)*	*Peak age at onset*	*Mean disease duration (y)*
Group I; Osaka University Hospital (Dept Orthop Surg)	1960–1965	Osaka	urban	566	20s	4.9
Group II; Shichikawa Arthritis Research Centre (Dept Rheumatol)	1985–1990	Hisai	rural	366	40s	7.5
Osaka University Hospital (Dept Orthop Surg)	1985–1990	Osaka	urban	286	40s	5.7
Yukioka Hospital (Dept Rheumatol)	1985–1990	Osaka	urban	233	40s	—
Osaka-Minami National Hospital (Dept Orthop Surg)	1991–1994	Kawachi-Nagano	suburban	370	40s	11.6

nal Medicine, Medical Institute of Bioregulation, Kyushu University was 37.3 years between 1950 and 1960, 40.5 between 1966 and 1971, and 44.1 between 1978 and 1983.

Secondly, the possibility of selection bias because of the significant difference of disease duration should be considered. The mean duration of the disease was 4.9 years in group I and 7.5 years in group II. We therefore obtained the data concerning disease duration of patients making a first visit at either of the two hospitals, Osaka University Hospital between 1985 and 1990 and Osaka-Minami National Hospital between 1991 and 1994. As table 3 shows, disease duration was 5.7 and 11.6 years, respectively. Despite the varied length of mean disease duration, the peak age at onset was essentially similar between these hospitals.

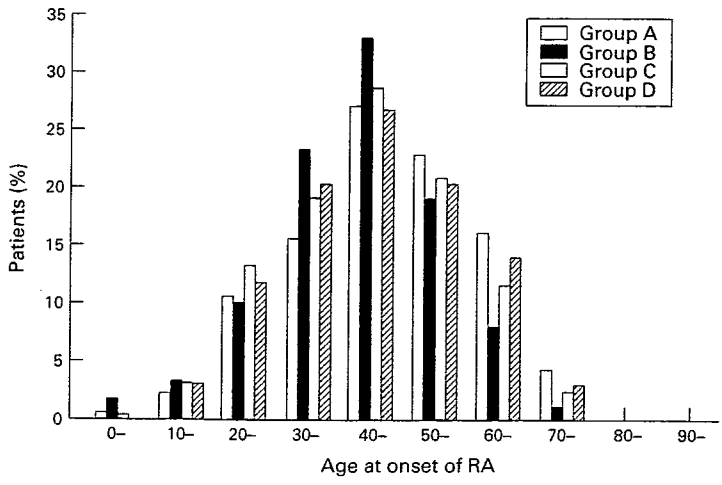

Figure 2 Distribution of age at onset. Group A: 366 patients at Shichikawa Arthritis Research Centre between 1985 and 1990, Group B: 286 patients at Osaka University Hospital between 1985 and 1990, Group C: 233 patients at Yukioka Hospital between 1985 and 1990, Group D: 370 patients at Osaka-Minami National Hospital between 1991 and 1994.

Thirdly, the relative increase of higher age at onset of RA may result from the increase in the average life span of the Japanese population in general. According to Japanese population statistics, the percentage of the population in their 20s was 17.7% in 1960 and 13.3% in 1985, whereas for those in their 40s it was 10.5% in 1960 and 14.3% in 1985.[12] These differences would not explain the considerable difference in distribution age at onset between two groups. Thus a delay in the age at onset seems to be a definite trend in Japan.

Numerous other demographic and environmental changes occurred in Japan during these years, such as increased female employment, reduction in the number of women having children, marrying later in life (especially in the case of women), amelioration of pollution in the environment, and reduction of microbiological infections. Although the effect of environmental changes on delay of age at onset of RA is beyond the scope of this study, some observable changes of this kind can be related to this delay.

The increased age at onset might reflect a birth cohort phenomenon. However, in any one time period, age at onset and year of birth are perfectly confounded, and in the comparison of two cross sectional surveys, such a hypothesis is impossible to test.

A significantly higher number of females was observed in group II when patients with long disease duration (≥10 years) were compared. These results showed that there is an increasing tendency for women with RA to be referred to a major hospital at a late stage in the disease. Although we cannot identify the reason for this phenomenon, one possible explanation is increased employment rates for women and a shift away from extended families living together, which in the past enabled grandparents to take care of children and household duties when necessary. As a result of such changes, women have greater constraints