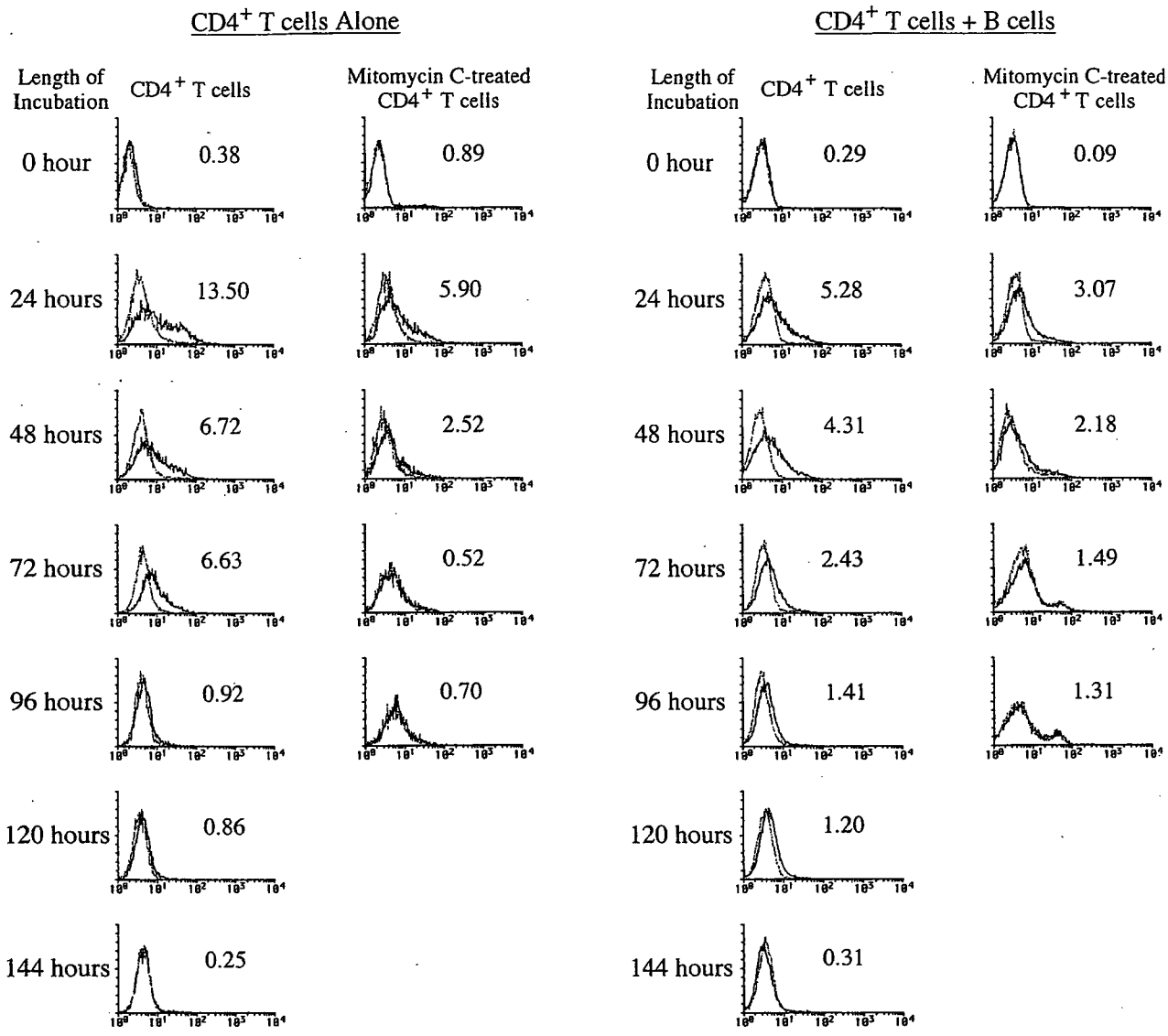

FIGURE 1. Kinetics of CD40 ligand expression by anti-CD3-stimulated $CD4^+$ T cells cultured with or without B cells. Either control $CD4^+$ T cells or those treated with mitomycin C before stimulation (1×10^5/well) were incubated with or without B cells (2.5×10^4/well) in anti-CD3 (200 ng/well)-coated 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.

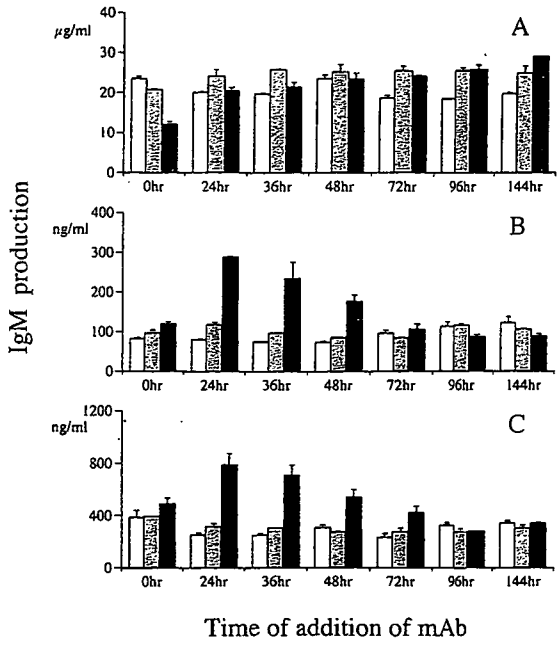

FIGURE 2. The role of CD40-CD40 ligand interaction in T cell-dependent Ig secretion by B cells. B cells (2.5×10^4/well) were cultured with (A) anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells, (B) anti-CD3 (200 ng/well)-stimulated control $CD4^+$ T cells, or (C) a 1/1 mixture of 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.

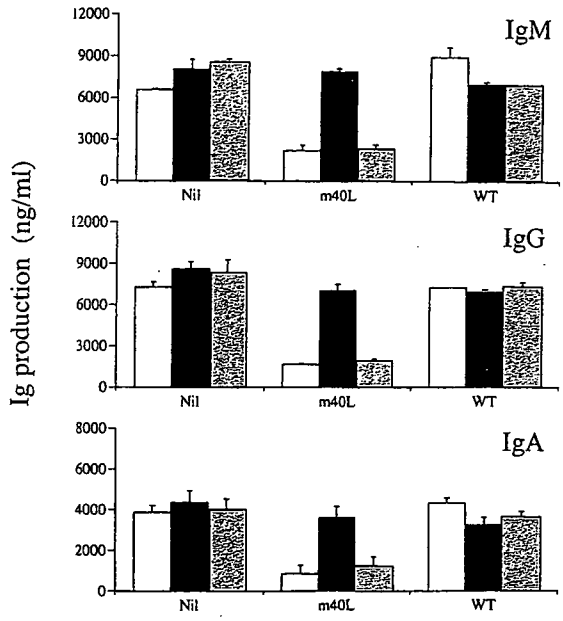

FIGURE 4. mAb to murine CD40 ligand (MR1) completely blocks the suppressive effect of recombinant murine CD40 ligand on Ig secretion by B cells. B cells (2.5×10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells (1×10^5/well) in the presence of 6 μl of membranes 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.

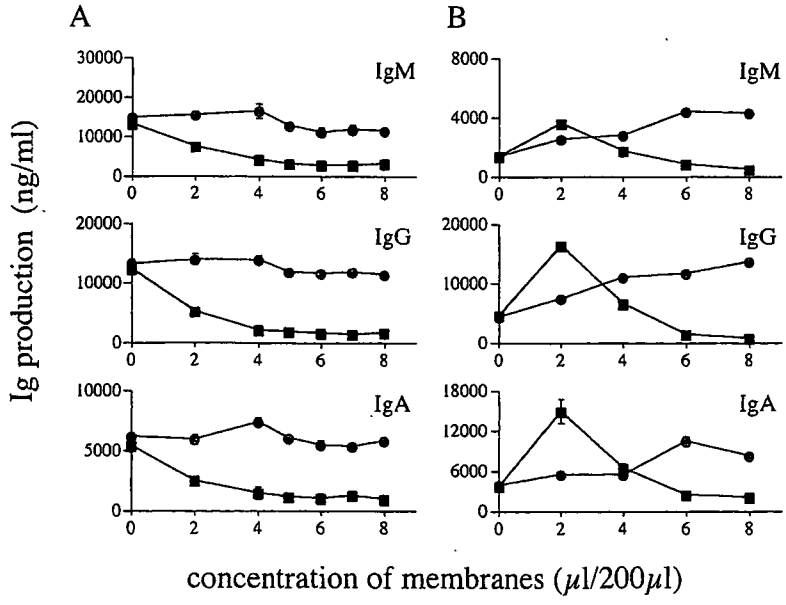

FIGURE 3. Supplemental CD40 ligand suppresses Ig secretion by B cells in a dose-dependent manner. B cells (2.5×10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells (2×10^5/well) (*A*) or with SAC + IL-2 (*B*) in the presence of various concentrations of 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

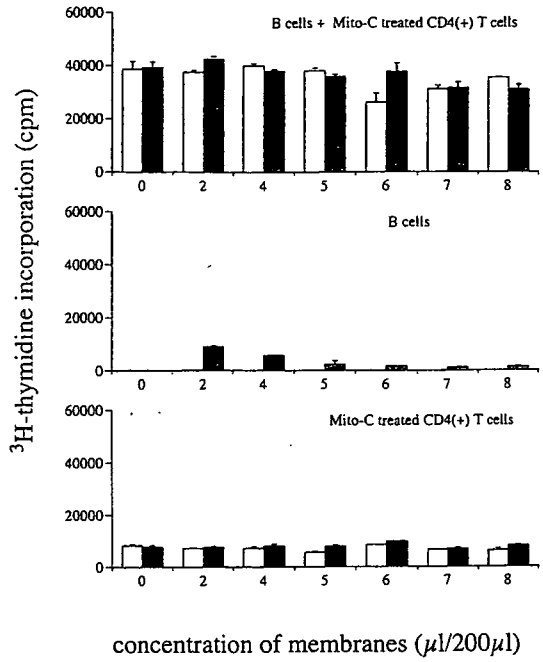

FIGURE 5. The effect of supplemental CD40 ligand on DNA synthesis of B cells cultured with anti-CD3 activated CD4$^+$T cells. B cells (2.5×10^4/well) were cultured alone or with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated CD4$^+$ T cells (1×10^5/well). Various concentrations of membranes from 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.

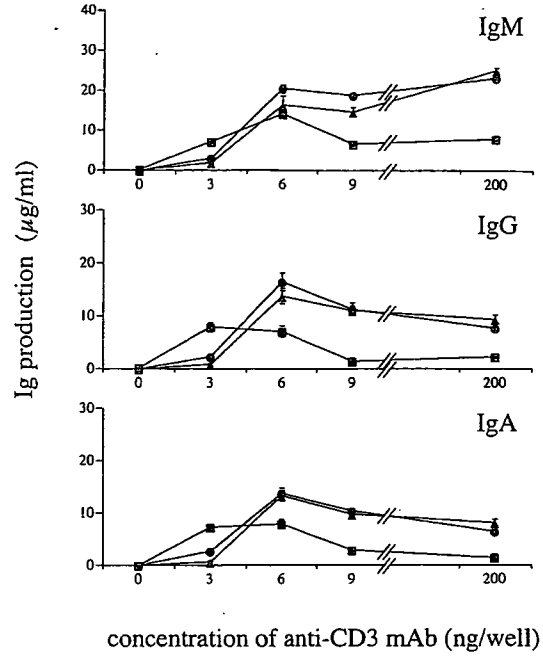

FIGURE 6. The impact of recombinant CD40 ligand on Ig secretion by B cells depends on the density of CD40 ligand expressed by activated T cells. B cells (2.5×10^4/well) were cultured with mitomycin C-treated CD4$^+$ T cells (7.5×10^4/well) stimulated with various concentrations of anti-CD3. Membranes (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).

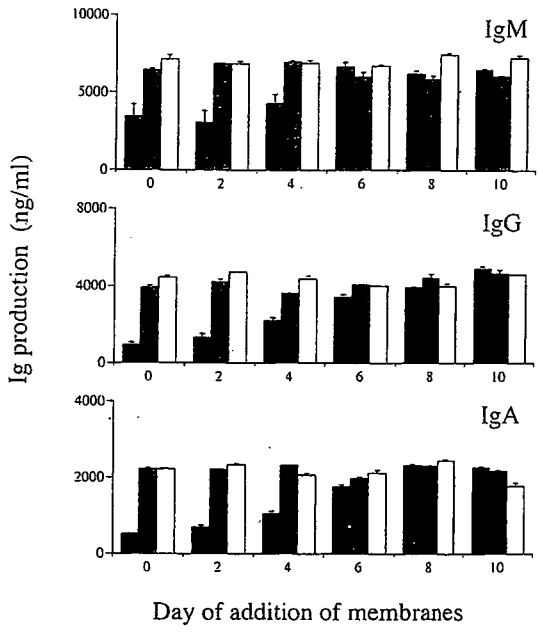

FIGURE 7. Kinetics of the suppression of B cell responses by CD40 ligand. B cells (2.5 × 10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated $CD4^+$ T cells (1 × 10^5/well). Membranes (6 μl) of Sf9 cells infected with either recombinant human CD40 ligand (black) or wild-type 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.

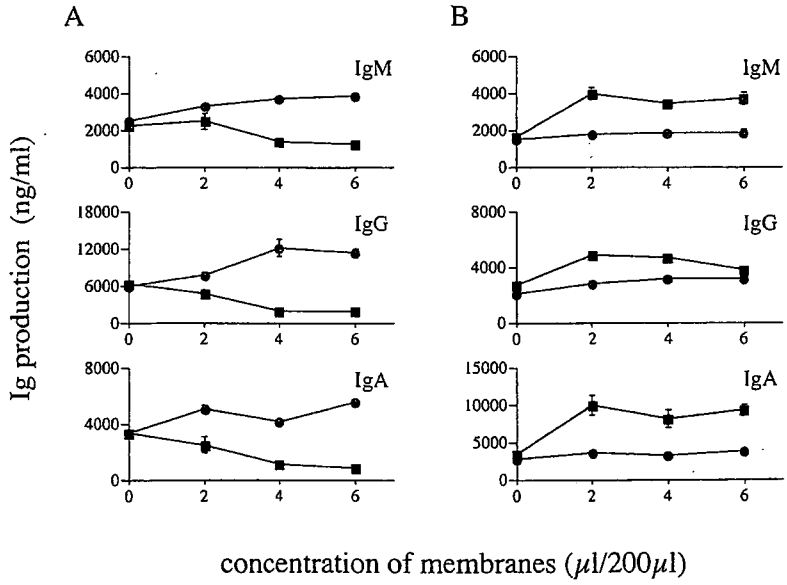

FIGURE 8. Sensitivity of B cells to suppression by CD40 ligand is dependent on the stage of B cell activation. B cells (5 × 10^4/well) were cultured with anti-CD3 (200 ng/ml)-stimulated mitomycin C-treated $CD4^+$ T cells (1 × 10^5/well) for either 48 h (*A*) or 96 h (*B*). Afterward, cells were harvested 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.

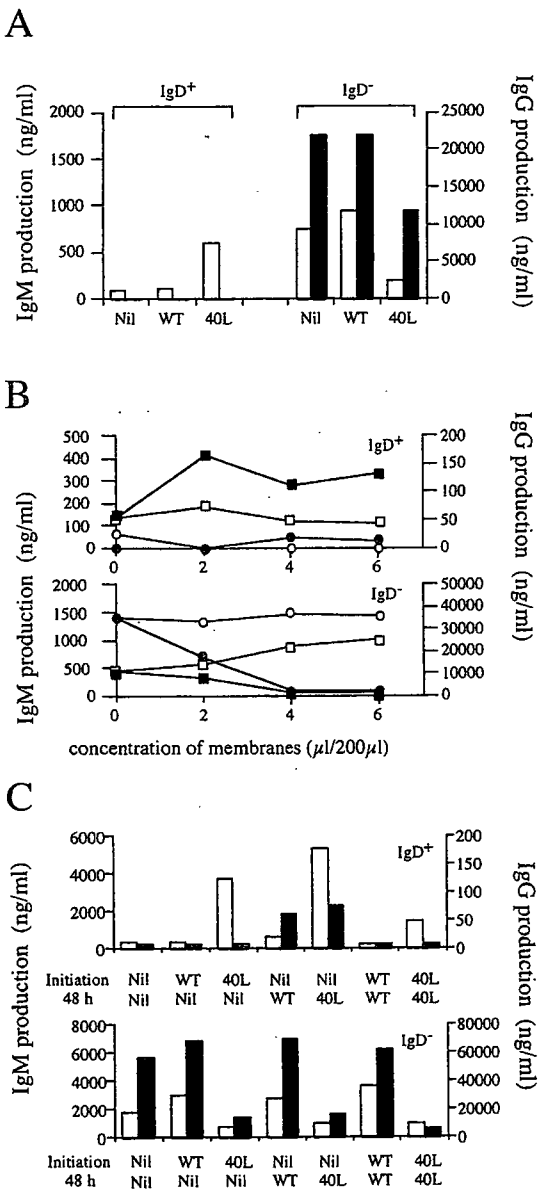

FIGURE 9. Difference of the effect of CD40 ligand on Ig secretion by IgD^+ or IgD^- B cell subpopulations. *A*, IgD^+ (*left* bars) or IgD^- (*right* bars) B cells (5×10^4/well) were cultured with SAC (1:60,000) and IL-2 (20 U/ml) in the absence or presence of membranes (2 μl) of Sf9 cells infected with 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.

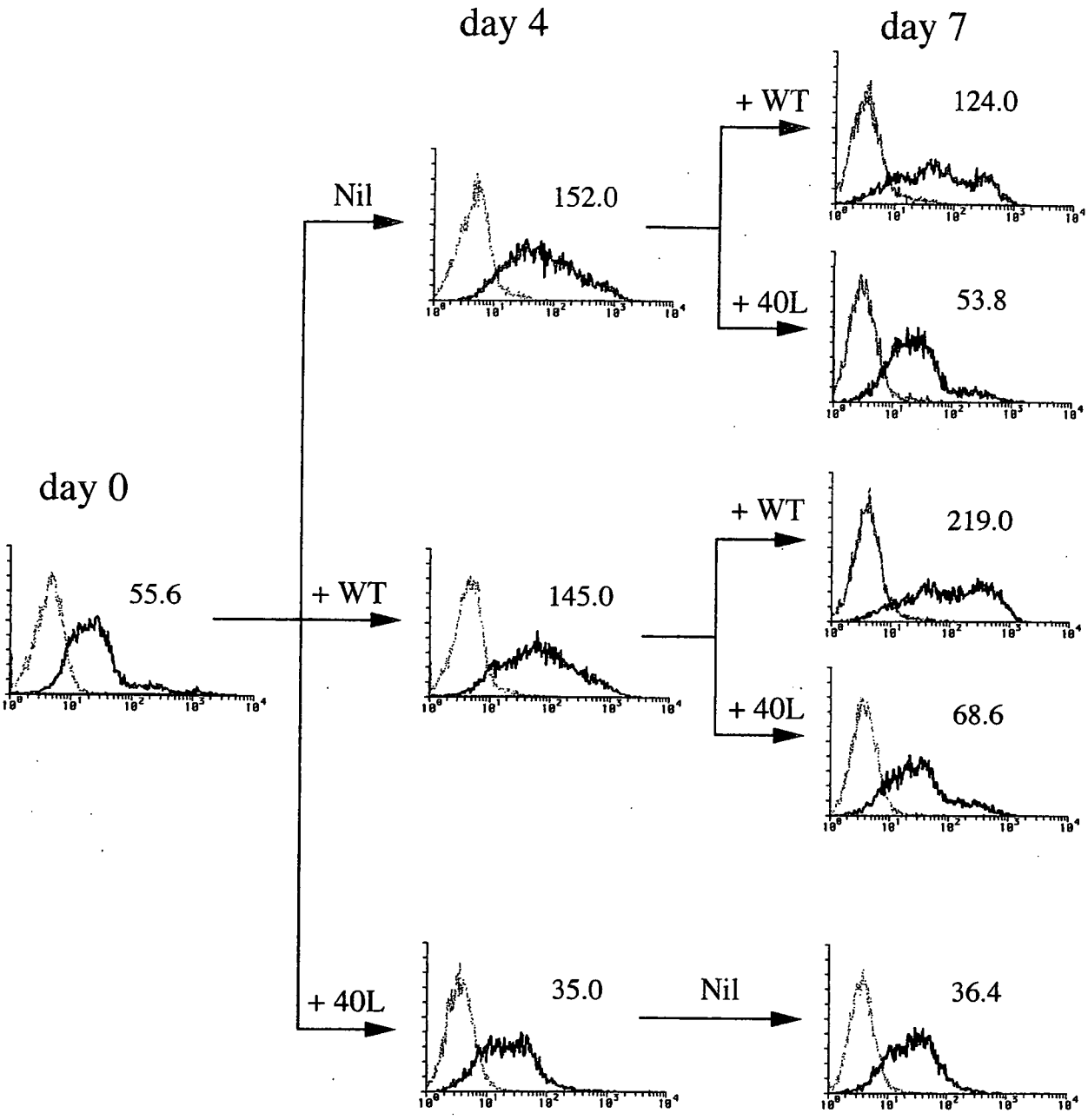

FIGURE 10. CD38 expression is decreased by engagement of CD40. B cells (5×10^4/well) were immediately stained for CD38 expression or were stimulated with SAC (1:60,000) and IL-2 (20 U/ml) in the presence or absence of membranes (2 μl) of Sf9 cells infected with either recombinant human CD40 ligand or 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

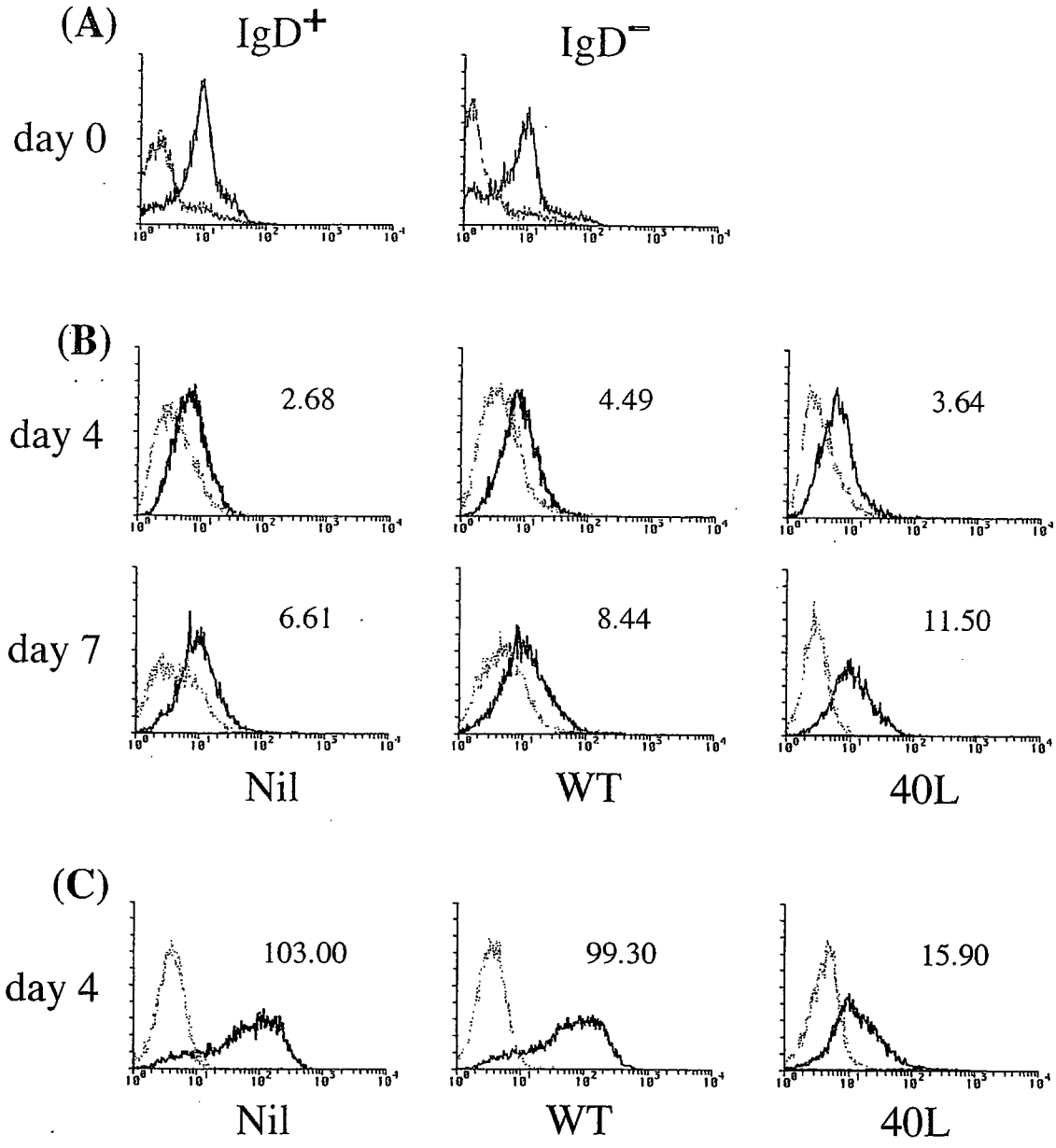

FIGURE 11. The effect of CD40 ligation on CD38 expression by IgD^+ or IgD^- B cell populations. (*A*) IgD^+ or IgD^- B cells were immediately analyzed for CD38 expression. IgD^+ (*B*) or IgD^- (*C*) B cells (5×10^4/well) were cultured with SAC (1:60,000) and IL-2 (20 U/ml) in the absence or presence 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

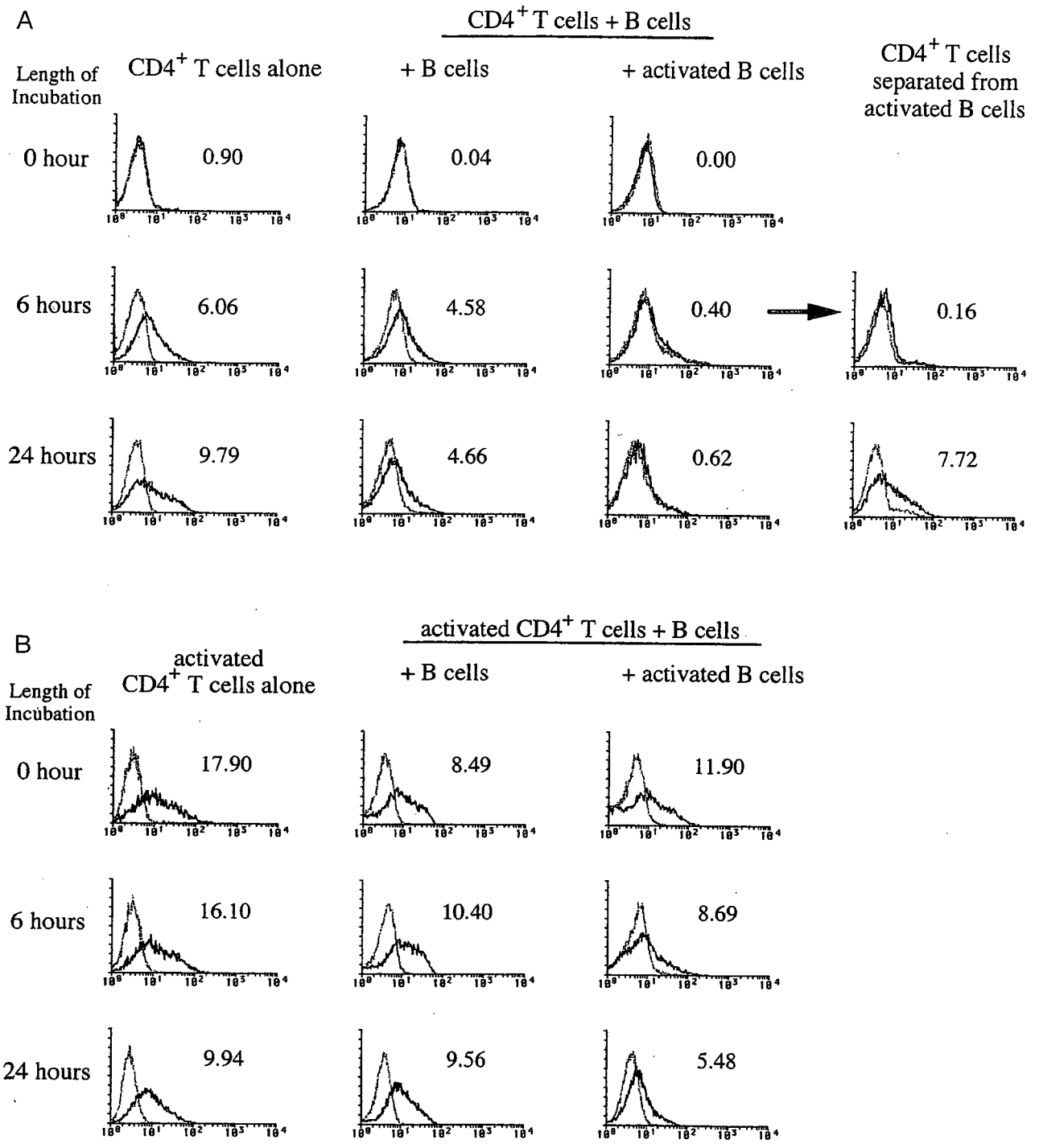

FIGURE 12. Activated B cells strongly inhibit CD40 ligand expression by $CD4^+$ T cells. B cells (5×10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated control $CD4^+$ T cells (1×10^5/well) for 48 h. Afterward, cells were harvested from the wells, and activated B cells were separated from 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.

References

1. Noelle, R. J., J. A. Ledbetter, and A. Aruffo. 1992. CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. *Immunol. Today 13:431.*
2. Foy, T. M., D. M. Shepherd, F. H. Durie, A. Aruffo, J. A. Ledbetter, and R. J. Noelle. 1993. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J. Exp. Med. 178:1567.*
3. Grewal, I. S., J. Xu, and R. A. Flavell. 1995. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature 378:617.*
4. Foy, T. M., J. D. Laman, J. A. Ledbetter, A. Aruffo, E. Claassen, and R. J. Noelle. 1994. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J. Exp. Med. 180:157.*
5. Gray, D., P. Dullforce, and S. Jainandunsing. 1994. Memory B cell development but not germinal center formation is impaired by in vivo blockade of CD40-CD40 ligand interaction. *J. Exp. Med. 180:141.*
6. Van Essen, D., H. Kikutani, and D. Gray. 1995. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. *Nature 378: 620.*
7. Banchereau, J., P. De Paoli, A. Vallé, E. Garcia, and F. Rousset. 1991. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science 251:70.*
8. Banchereau, J., and F. Rousset. 1991. Growing human B lymphocytes in the CD40 system. *Nature 353:678.*
9. Jabara, H. H., S. M. Fu, R. S. Geha, and D. Vercelli. 1990. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med. 172: 1861.*
10. Nishioka, Y., and P. E. Lipsky. 1994. The role of CD40-CD40 ligand interaction in human T cell-B cell collaboration. *J. Immunol. 153:1027.*
11. Arpin, C., J. Déchanet, C. van Kooten, P. Merville, G. Grouard, F. Brière, J. Banchereau, and Y.-J. Liu. 1995. Generation of memory B cells and plasma cells in vitro. *Science 268:720.*
12. Heath, A. W., R. Chang, N. Harada, L. Santos-Argumedo, J. Gordon, C. Hannum, D. Campbell, A. B. Shanafelt, E. A. Clark, R. Torres, and M. Howard. 1993. Antibodies to murine CD40 stimulate normal B lymphocytes but inhibit proliferation of B lymphoma cells. *Cell. Immunol. 152:468.*
13. Fluckiger, A. C., J. F. Rossi, A. Bussel, P. Bryon, J. Banchereau, and T. Defrance. 1992. Responsiveness of chronic lymphocytic leukemia B cells activated via surface Igs or CD40 to B-cell tropic factors. *Blood 180:3173.*
14. Funakoshi, S., D. L. Longo, M. Beckwith, D. K. Conley, G. Tsarfaty, I. Tsarfaty, R. J. Armitage, W. C. Fanslow, M. K. Spriggs, and W. J. Murphy. 1994. Inhibition of human B-cell lymphoma growth by CD40 stimulation. *Blood. 83:2787.*
15. Hess, S., and H. Engelmann. 1996. A novel function of CD40: induction of cell death in transformed cells. *J. Exp. Med. 183:159.*
16. Bergman, M. C., J. F. Attrep, A. C. Grammer, and P. E. Lipsky. 1996. Ligation of CD40 influences the function of human Ig-secreting B cell hybridomas both positively and negatively. *J. Immunol. 156:3118.*
17. Callard, R. E., J. Herbert, S. H. Smith, R. J. Armitage, and K. E. Costelloe. 1995. CD40 cross-linking inhibits specific antibody production by human B cells. *Int. Immunol. 7:1809.*
18. Silvy, A., C. Lagresle, C. Bella, and T. Defrance. 1996. The differentiation of human memory B cells into specific antibody-secreting cells is CD40 independent. *Eur. J. Immunol. 26:517.*
19. Kung, P. C., G. Goldstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science 206: 347.*
20. Jumper, M. D., Y. Nishioka, L. S. Davis, P. E. Lipsky, and K. Meek. 1995. Regulation of human B cell function by recombinant CD40 ligand and other TNF related ligands. *J. Immunol. 155:2369.*
21. Lipsky, P. E., J. F. Attrep, A. C. Grammer, M. J. McIlraith and Y. Nishioka. 1997. Analysis of CD40-CD40 ligand interactions in the regulation of human B cell function. *Ann. NY Acad. Sci. In press.*
22. Rosenberg, S. A., and P. E. Lipsky. 1979. Monocyte dependence of pokeweed mitogen-induced differentiation of immunoglobulin-secreting cells from human peripheral blood mononuclear cells. *J. Immunol. 122:926.*
23. Thiele, D. L., M. Kurosaka, and P. E. Lipsky. 1983. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotrophic agent L-leucine methyl ester. *J. Immunol. 131:2282.*
24. Galili, U., and M. Schlesinger. 1974. The formation of stable E rosettes after neuraminidase treatment of either human peripheral blood lymphocytes or of sheep red blood cells. *J. Immunol. 112:1628.*
25. Wysocki, L. J., and V. L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA 75:2844.*
26. Hirohata, S., D. F. Jelinek, and P. E. Lipsky. 1988. T cell-dependent activation of B cell proliferation and differentiation by immobilized monoclonal antibodies to CD3. *J. Immunol. 140:3736.*
27. Geppert, T. D., and P. E. Lipsky. 1987. Accessory cell independent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3. *J. Immunol. 138:1660.*
28. Tohma, S., and P. E. Lipsky. 1991. Analysis of the mechanisms of T cell-dependent polyclonal activation of human B cells. Induction of human B cell responses by fixed activated T cells. *J. Immunol. 146:2544.*
29. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65: 55.*
30. Yellin, M. J., K. Sippel, G. Inghirami, L. R. Covey, J. J. Lee, J. Sinning, E. A. Clark, L. Chess, and S. Lederman. 1994. CD40 molecules induce down-modulation and endocytosis of T cell surface T cell-B cell activating molecule/ CD40-L. Potential role in regulating helper effector function. *J. Immunol. 152: 598.*
31. Hirohata, S., L. S. Davis, and P. E. Lipsky. 1989. Role of IL-2 in the generation of $CD4^+$ suppressors of human B cell responsiveness. *J. Immunol. 142:3104.*
32. Jelinek, D. F., J. B. Splawski, and P. E. Lipsky. 1986. Human peripheral blood B lymphocyte subpopulations: functional and phenotypic analysis of surface IgD positive and negative subsets. *J. Immunol. 136:83.*
33. Nonoyama, S., D. Hollenbaugh, A. Aruffo, J. A. Ledbetter, and H. D. Ochs. 1993. B cell activation via CD40 is required for specific antibody production by antigen-stimulated human B cells. *J. Exp. Med. 178:1097.*
34. Cheng, G., A. M. Cleary, Z. Ye, D. I. Hong, S. Lederman, and D. Baltimore. 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science 267:1494.*
35. Hostager, B. S., Y. Hsing, D. E. Harms, and G. A. Bishop. 1996. Different CD40-mediated signaling events require distinct CD40 structural features. *J. Immunol. 157:1047.*
36. Rothe, M., V. Sarma, V. M. Dixit, and D. V. Goeddel. 1995. TRAF2-mediated activation of NFκB by TNF receptor 2 and CD40. *Science 269:1424.*
37. Ishida, T., T. Tojo, T. Aoki, N. Kobayashi, T. Ohishi, T. Watanabe, T. Yamamoto, and J.-I. Inoue. 1996. TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proc. Natl. Acad. Sci. USA 93:9437.*

38. Jelinek, D. F., and P. E. Lipsky. 1987. Regulation of human B lymphocyte activation, proliferation and differentiation. *Adv. Immunol. 40:1.*
39. Simkin, N. J., D. F. Jelinek, and P. E. Lipsky. 1987. Inhibition of human B cell responsiveness by prostaglandin E_2. *J. Immunol. 138:1074.*
40. Kristiansen, S. V., V. Pascual, and P. E. Lipsky. 1994. Staphylococcal protein A induces biased production of immunoglobulin by V_H3 expressing B lymphocytes. *J. Immunol. 153:2974.*
41. Sasso, E. H., J. G. Silverman, and M. Mannik. 1988. Human IgM molecules that bind staphylococcal protein A contain V_HIII H chains. *J. Immunol. 142: 2778.*
42. Liu, Y.-J., and J. Banchereau. 1996. The paths and molecular controls of peripheral B-cell development. *Immunologist 4:55.*
43. Vernino, L., L. M. McAnally, J. Ramberg, and P. E. Lipsky. 1992. Generation of nondividing high rate immunoglobulin secreting plasma cells in cultures of human B cells stimulated with anti-CD3 activated T cells. *J. Immunol. 148: 404.*
44. Jelinek, D. F., and P. E. Lipsky. 1983. The role of B cell proliferation in the generation of immunoglobulin secreting cells in man. *J. Immunol. 130:2597.*
45. van Kooten, C., C. Gaillard, J.-P. Galizzi, P. Hermann., F. Fossiez, J. Banchereau, and D. Blanchard. 1994. B cells regulate expression of CD40 ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. *Eur. J. Immunol. 24:787.*
46. Castle, B. E., K. Kishimoto, C. Stearns, M. L. Brown, and M. R. Kehry. 1993. Regulation of expression of the ligand for CD40 on T helper lymphocytes. *J. Immunol. 151:1777.*
47. Ludewig, B., V. Henn, J. M. Schroder, D. Graf, and R. A. Kroczek. 1996. Induction, regulation, and function of soluble TRAP (CD40 ligand) during interaction of primary $CD4^+$ $CD45RA^+$ T cells with dendritic cells. *Eur. J. Immunol. 26:3137.*
48. Grammer, A. C., M. C. Bergman, Y. Miura, K. Fujita, L. S. Davis, and P. E. Lipsky. 1995. The CD40 ligand expressed by human B cells costimulates B cell responses. *J. Immunol. 154:4996.*

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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; $p<0.05$).

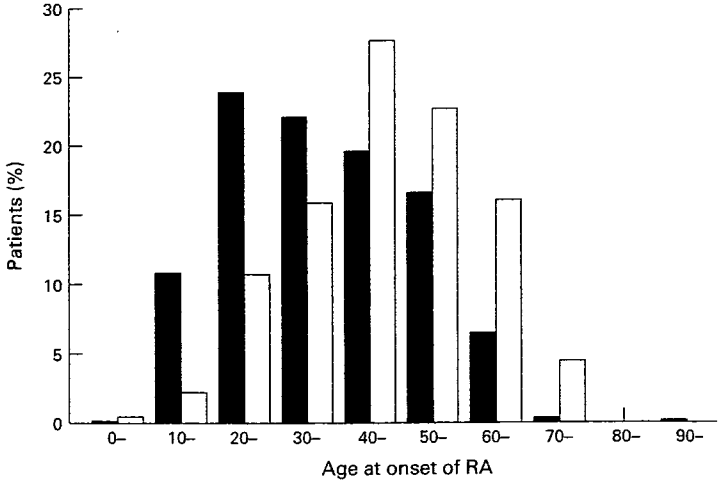

Figure 1 Distribution of age at onset. ■ : Group I patients who visited Osaka University Hospital from 1960 to 1965, □: group II patients who attended Shichikawa Arthritis Research Centre from 1985 to 1990.

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.

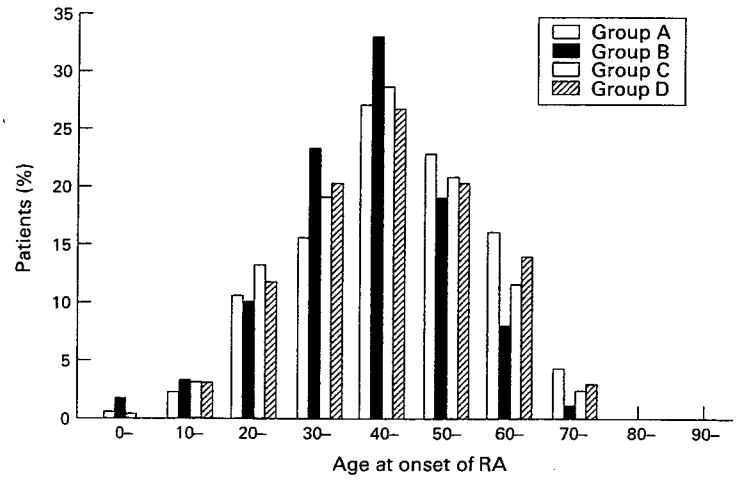

Figure 2 Distribution of age at onset. Group A: 366 patients at Shichikawa Arthritis Research Centre between 1985 and 1990, Group B: 286 patients at Osaka University Hospital between 1985 and 1990, Group C: 233 patients at Yukioka Hospital between 1985 and 1990, Group D: 370 patients at Osaka-Minami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

upon their time, making it more difficult for them to visit a major hospital.

A decline in the severity of RA has been pointed out in Europe and Australia.[1 5 7-9 13] This decline, indicated by seropositivity, radiographic erosions, and rheumatoid nodule positivity has been described by Silman *et al.*[5 7-9] A decline in radiographic severity has also been noted by Heikkilä *et al.*[1] Because, in our study, the patients in group II have a longer disease duration, it could be reasonably assumed that more severe and more patients with longstanding disease were recruited for group II. However, a comparison of data, based on patients with similar disease duration, did not show any significant change over a 30 year period in seropositivity, rheumatoid nodule positivity, and rate of hip involvement, although a decreased rate of hip involvement was observed in group II patients with a disease duration of less than five years.

With regard to the severity of RA, a decrease during the last 30 years was not evident in this limited study. To answer definitely the question of whether the severity of RA has been declining in the Japanese population, it will be necessary to gather and compare additional data in a more extensive, systematic, epidemiological study.

1 Heikkilä S, Isomäki H. Long-term outcome of rheumatoid arthritis has improved. Scand J Rheumatol 1994;23:13-5.

2 Hochberg MC. Changes in the incidence and prevalence of rheumatoid arthritis in England and Wales: 1970-1982. Semin Arthritis Rheum 1990;19:294-302.

3 Jacobsson LTH, Hanson RL, Knowler WC, *et al.* Decreasing incidence and prevalence of rheumatoid arthritis in Pima Indians over a twenty-five-year period. Arthritis Rheum 1994;37:1158-65.

4 Linos A, Worthington JW, O'Fallon WM, Kurland LT. The epidemiology of rheumatoid arthritis in Rochester, Minnesota: a study of incidence, prevalence and mortality. Am J Epidemiol 1980;111:87-98.

5 Silman A, Davies P, Currey HLF, Evans SJW. Is rheumatoid arthritis becoming less severe? J Chron Dis 1983; 36:891-7.

6 Silman AJ. Has the incidence of rheumatoid arthritis declined in the United Kingdom? Br J Rheumatol 1988;27:77-9.

7 Silman AJ. Are there secular trends in the occurrence and severity of rheumatoid arthritis? Scand J Rheumatol 1989; (suppl 79):25-30.

8 Silman AJ. Recent trends in rheumatoid arthritis (letter). Br J Rheumatol 1992;31:326-30.

9 Silman AJ. Trends in the incidence and severity of rheumatoid arthritis. J Rheumatol 1992;(suppl 32)19:71-3.

10 Popes MW, Bennett GA, Cobb S, Jacox R, Jessar RA. 1958 revision of diagnostic criteria for rheumatoid arthritis. Bull Rheum Dis 1958;9:175-6.

11 Nobunaga M. Does the therapy of RA really progress? Ryumachi 1986;26:35-40.

12 Statistics Bureau, Management and Coordination Agency. *Population by age group and indexes of age structure (1920-1989).* Japan Statistical Association, 1990; Printing Bureau, Ministry of Finance publication no. (ISBN)4-620-80010-4. (Japan Statistical Yearbook, 40th ed).

13 Laurent R, Robinson RG, Beller EM, Buchanun WW. Incidence and severity of rheumatoid arthritis - the view from Australasia (letter). Br J Rheumatol 1989;28:360-1.

Enhanced Expression of CD14 Antigen on Myeloid Lineage Cells Derived from the Bone Marrow of Patients with Severe Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** We previously reported the accumulation of abnormal myeloid cell populations reacting with CD14 (MY4) monoclonal antibody in the iliac and epiphyseal bone marrow of patients with severe rheumatoid arthritis (RA). Therefore, we investigated *in vitro* production and modulation of CD14+ myeloid cells from iliac bone marrow cells.

Methods. Mononuclear cells were prepared from iliac bone marrow aspirates from patients with RA. The presence of unusual myeloid cells was assessed by 2 color flow cytometry of cells cultured under various conditions.

Results. Cultured iliac bone marrow cells of patients with severe RA produced 14.7% of CD14+CD15+ cells on average. Cultures derived from healthy donors and from patients with a milder form of RA produced fewer CD14+CD15+ cells (< 10%). The production of CD14+CD15+ cells was enhanced by granulocyte macrophage colony stimulating factor and interleukin 1ß, but inhibited by T lymphocytes.

Conclusion. Production and modulation of CD14+ myeloid cells were observed in iliac bone marrow of patients with severe RA. (*J Rheumatol 1997;***24:465–9**)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
MYELOID LINEAGE CELL
BONE MARROW
CD14 ANTIGEN

Abnormalities in bone marrow of patients with rheumatoid arthritis (RA) suggest that bone marrow might play a role in the pathogenesis of RA[1-5]. We reported the presence of an abnormal myeloid cell population in epiphyseal bone marrow adjacent to joints affected with severe RA[6]. Those abnormal myeloid cells were found to be supported by some soluble factor having myeloid growth activities[7]. These abnormal cells could be distinguished from normal myeloid cells by expression of the difucosyl type 2 chain structure (dimetric Le^x, a specific marker of human undifferentiated cells[8]) or CD14 antigen (MY4 antigen, expressed in mainly monocyte macrophages)[9].

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We investigated cellular changes in iliac bone marrow (one of the major sites of hematopoiesis in the adult) and tibial bone marrow[10]. In the iliac bone marrow of patients with severe RA, the absolute numbers of myeloid lineage cells were increased about 3 times relative to non-RA controls. Moreover, in this increased population of pan-myelocytes, a subset of abnormal myeloid cells (CD14+ myeloid cells) was discovered at higher incidence in iliac bone marrow than in tibial bone marrow in patients with severe RA. This abnormal myeloid cell subset could not be found in iliac and tibial bone marrow in non-RA controls and patients with mild RA. Based upon these findings *in vivo*, we report *in vitro* production and modulation of abnormal (CD14+) myeloid cells from iliac bone marrow of patients with RA.

MATERIALS AND METHODS

Patients. Bone marrow blood samples were obtained from 35 patients with RA (32 women, 3 men) who met the American College of Rheumatology criteria[11] and 8 healthy volunteers (6 women, 2 men), with informed consent. All patients had undergone reconstructive operations at Osaka University Hospital or the related facilities from 1990 to 1993. The average age of patients with RA and controls was 46.9 and 38.5 years, respectively (range 24–59 and 20–55, respectively). Disease activity of all patients was classified as the least erosive subset, the more erosive subset, or mutilating disease, as reported[12]. In this paper, we present "least erosive" as mild disease, and "more erosive" and "mutilating disease" as more severe manifestations of RA.

Cytokines. Recombinant human (rh) interleukin (IL)-1ß was a kind gift from Otuka Pharmaceutical Co. Ltd., Tokushima, Japan. RhIL-3, rhIL-6,

rhIL-8 (monocyte derived), rh monocyte colony stimulating factor (MCSF) and rh granulocyte macrophage colony stimulating factor (GMCSF) were purchased from Genzyme Corporation (Cambridge, MA, USA) and rh granulocyte colony stimulating factor (GCSF) was purchased from Amersham (Amersham, UK).

Monoclonal antibodies. The monoclonal antibodies (Mab) used were fluorescein isothiocyanate (FITC) conjugated CD15 (MX-GA, HL-5) (Kyowa Medix, Tokyo, Japan), phycoerythrin (PE) conjugated CD14 (MY4) (Coulter Immunology, Florida, USA). The CD15 (MX-GA) antibody detects a broad range of myeloid lineage cells from myeloblasts to polymorphonuclear cells[13].

Cell preparations. Heparinized bone marrow aspirate was obtained from anterior iliac crest by needle puncture at the time of operation. Mononuclear cells (MNC) from bone marrow aspirate were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient (1.077 g/ml) centrifugation (30 min, 400 × g). In some experiments, to deplete phagocytic cells, bone marrow aspirates were incubated at 37°C for 1 h with the addition of 1/10 volume of silica suspension (JIMRO, Takasaki, Japan) before Ficoll-Hypaque density gradient centrifugation. T lymphocytes were depleted by incubation with sheep red blood cells (SRBC) (JIMRO, Takasaki, Japan). The cells were then washed 3 times by phosphate buffered saline (PBS).

Cell cultures. Culture medium HL-1 (Ventrex, Portland, ME, USA) was used. Components of HL-1 include ultrapure, pyrogen-free water, a specialized, modified DME: F12 base, HEPES buffer, known amounts of insulin, transferrin, testosterone, sodium selenite, ethanolamine, and stabilizing proteins. Cells were resuspended in HL-1 supplemented with 5% heat inactivated fetal calf serum (Flow Laboratories, North Ryde, Australia) and cultured 5 days at 37°C in a humidified atmosphere of 5% CO_2 at cell densities 0.5–1.0 × 10^6/ml in the presence of iliac bone marrow serum from patients with RA or cytokines, as described.

2-color FACS analysis and cell sorting. The cultured cells were collected at Day 5 and washed 3 times in PBS. The cells were incubated with Mab of FITC conjugated CD15 (MX-GA) and PE conjugated CD14 (MY4) on ice for 30 min, then washed 3 times by PBS and fixed in 1% paraformaldehyde. 2-color flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA, USA) equipped with an argon laser at 488 nm. All specimens were analyzed on the day of collection. To omit debris, analysis was performed on cells selected (gated) by their forward and right angle scatter measurements. The data from 10,000 cells per test were collected and stored in list mode (Consort 30 Hewlett-Packard computer). The percentages of cells positive for either Mab staining were determined by markers formed around isotype and fluorescent matched control antibodies. The number of positive cells was expressed as a percentage of the total cell count. For cell sorting, cells were stained in the same way as for FACS analysis, then sorted on the FACStar Plus (Becton Dickinson). Reanalysis of the sorted cell population was performed and the efficiency of the sorting calculated. Sorting efficiencies exceeded 95% purity.

Statistical analysis. Results were expressed as the mean ± standard deviation. Data were analyzed by nonparametric Mann-Whitney U test or analysis of variance (ANOVA) with post hoc, where appropriate, p values < 0.05 were considered significant.

RESULTS

Production of CD14+ myeloid cells in the iliac bone marrow of patients with RA. To assess *in vitro* production of CD14+ myeloid cells, bone marrow MNC from various donors were incubated 5 days with 10% final concentration of autologous iliac bone marrow serum. In patients with more erosive and mutilating disease, the average ratio of CD14+CD15+ cells among whole MNC was 4.5% (3.3–5.9%) before incubation, but it increased significantly to 14.7% (11.9–17.1%) after a 5 day incubation ($p < 0.0001$). The values assessed from patients with mutilating disease were within the range of those assessed from patients with more erosive disease, and we assessed the values from patients with more erosive and mutilating disease together as more severe RA. In patients with the least erosive disease, the average ratio before incubation was 3.2% (2.8–4.3%), and it increased significantly to 8.1% (6.6–9.8%) after a 5 day incubation ($p < 0.05$). In healthy donors, the average ratio before incubation was 2.4% (1.4–2.8%) and after incubation 3.5% (2.5–3.9%). There was no statistical significance between the ratios before and after incubation in healthy donors (Figure 1).

Effect of T lymphocytes in producing CD14+ myeloid cells. To study the effect of T lymphocytes in producing CD14+CD15+ cells, we compared the ratios of CD14+CD15+ cells among nonphagocytic bone marrow MNC or T lymphocyte depleted nonphagocytic bone marrow MNC of patients with most erosive disease, after incubation for 5 days, in the presence of rhGMCSF (50 U/ml). The cell fractions were as follows: nonphagocytic bone marrow MNC: CD2 50%, CD14 < 3%, CD15 20%, CD34 5%; T lymphocyte depleted nonphagocytic bone marrow MNC: CD2 < 5%, CD14 < 3%, CD15 40%, CD34 15%. From the FACS analysis, the CD14+CD15+ cell numbers produced from 1.0 × 10^5 CD15+ cell were calculated. As shown in Figure 2, the numbers of CD14+CD15+ cells produced in the presence of T lymphocytes and without T lymphocytes were 17,000 ± 4,415 and 33,113 ± 8,162, respectively. In all 4 cases, CD14+CD15+ cells were more numerous cultured without T lymphocytes than in the presence of T lymphocytes ($p = 0.0133$). This result indicates the inhibitory effects of T lymphocytes on production of CD14+CD15+ cells.

Effect of cytokines on production of CD14+ myeloid cells. To measure the effect of various cytokines on production of

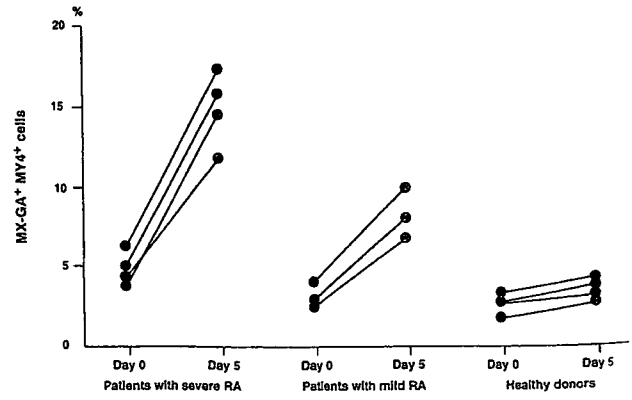

Figure 1. CD14 antigen expression on CD15+ cells. MNC from iliac bone marrow aspirates of patients with severe or mild RA or healthy donors were cultured with auto-iliac serum for 5 days.

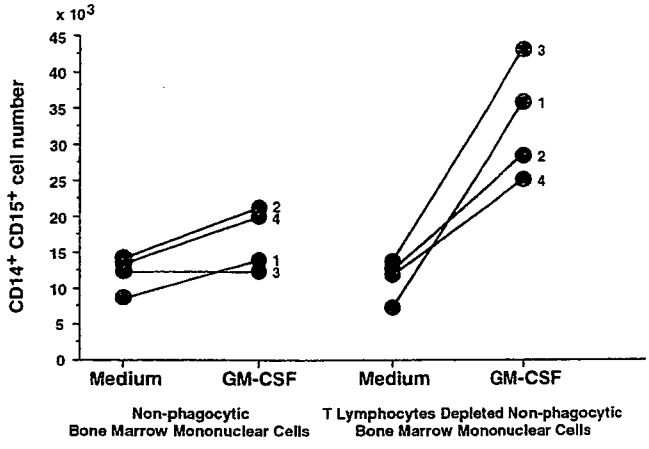

Figure 2. Effect of T lymphocytes on CD14 antigen expression on CD15+ cells from patients with severe RA. Nonphagocytic bone marrow mononuclear cells or T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured in the presence of rhGMCSF (50 U/ml) or without GMCSF for 5 days. Numbers indicate preparations from the same patients. Quantities of CD14+CD15+ cells produced from 1×10^5 CD14–CD15+ cells are shown.

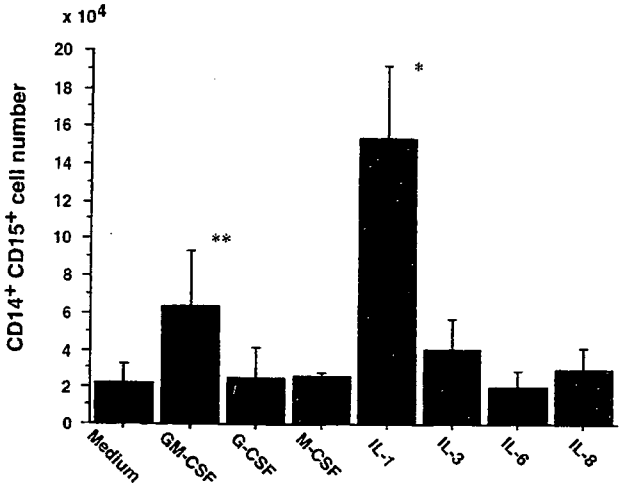

Figure 3. Effect of cytokines on CD14 antigen expression on CD15+ cells from patients with RA. T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured in the presence of cytokines (100 U/ml) for 5 days. *p < 0.0001 and **p = 0.0037 compared with culture with medium only.

CD14+CD15+ cells, T lymphocyte depleted nonphagocytic bone marrow MNC were prepared from a few patients with more erosive disease, and cultured in various concentrations of IL-1ß, IL-3, IL-6, IL-8, GCSF, MCSF, and GMCSF (final concentration of 1, 10, 50, 100, 200, 500 U/ml). With GMCSF and IL-1ß, CD14+CD15+ cells were produced in a dose dependent fashion. The optimal production was achieved with 50 U/ml of GMCSF and 100 U/ml of IL-1ß. So in the following experiments, all cytokines were used at a final concentration of 100 U/ml. T lymphocyte depleted nonphagocytic bone marrow MNC were prepared at cell density 0.5×10^6/ml from 5 patients with more erosive disease and cultured in IL-1ß, IL-3, IL-6, IL-8, GCSF, MCSF, and GMCSF (final concentration 100 U/ml). CD14+CD15+ cells were produced at significantly higher ratios by incubation with IL-1ß and GMCSF than without addition of cytokines ($p = 0.0037$ and $p = < 0.0001$, respectively). No significant enhancement was achieved by adding any other cytokines studied (Figure 3).

Reactivity of CD14– myeloid cells in the iliac bone marrow of patients with RA in the presence of IL-1ß. To compare the reactivity of CD14– myeloid cells in each disease subset, CD14–CD15+ cells obtained by sorting were incubated for 5 days in the presence of IL-1ß (100 U/ml). The average ratio of CD14+CD15+ cells was 8.0 ± 4.2% for healthy donors, 14.1 ± 3.2% for least erosive disease, and 35.6 ± 14.0% for more erosive and mutilating disease, respectively (Figure 4). Thus, the capacity of CD14–CD15+ cells to develop into CD14+CD15+ cells was significantly different between cells derived from more erosive and mutilating disease and from least erosive disease ($p < 0.01$).

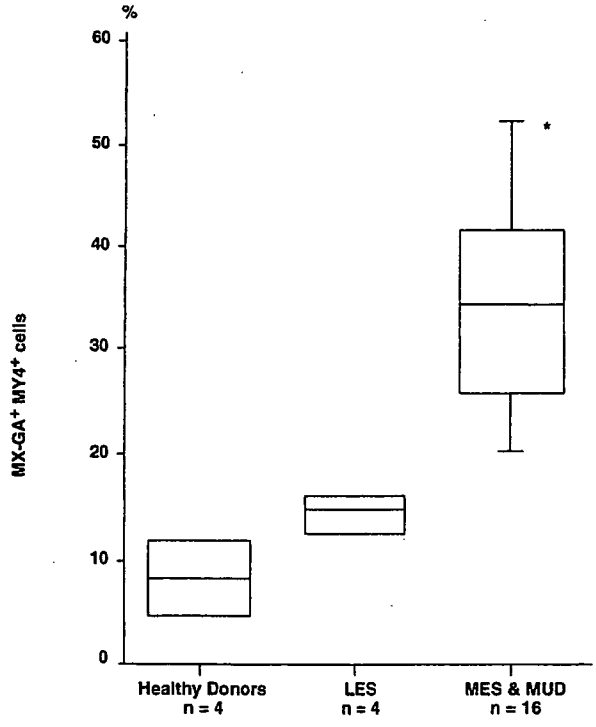

Figure 4. Effect of IL-1ß on CD14 antigen expression on CD15+ cells from patients with RA and healthy donors. T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured 5 days in the presence of IL-1ß (100 U/ml). The line in middle of the box represents the median. The top of the box represents the 75th percentile and the bottom the 25th percentile. *p < 0.01 compared with LES.

DISCUSSION

We previously reported the accumulation of unusual myeloid cell populations bearing oncofetal mono- or difucosylated type 2 chain in epiphyseal bone marrow adjacent to the affected joints in patients with severe RA[6]. These unusual myeloid cells were confirmed to react with CD14 and CD15 Mab[9]. In the present study, these Mab were used to detect unusual myeloid cells. Our aim was to determine the characteristics of these cells. CD14 surface antigen was once thought to be specific to monocyte macrophages and not be expressed on myeloid colony forming cells[14], but a subpopulation expressing low levels of CD14 together with CD16 (FcRIII) was recently identified. CD14 expression can also be observed on primary B cells harvested from blood[15–17]. By repeated morphological analyses of sorted CD14+CD15+ cells, we confirmed those cells to be myeloid cells[10]. Oncofetal membrane marker on the surface of these CD14+CD15+ cells might suggest hyperactivated metabolism in this lineage of cells, but the lineage of these unusual myeloid cells remains unknown.

What is the characteristic function of CD14+CD15+ cells in patients with severe RA? Precise and direct analyses have not yet been done because we have not succeeded in development of these abnormal myeloid cells into polymorphonuclear (PMN) cells (the developed functional cells of myeloid lineage) *in vitro*. We tried to determine whether PMN in the epiphyseal bone marrow of patients with severe RA had some special characteristics. Our previous studies revealed PMN cells with remarkably high levels of IL-1 in the same epiphyseal bone marrow as accumulations of CD14+CD15+ cells[18,19]. IL-1 is known to be a potent stimulator of the synthesis of matrix metalloproteinase by connective tissue cells of joints, such as the synovium, cartilage[20,21], and possibly bone and ligament. Thus, it is possible that highly activated myeloid lineage cells could induce diffuse and severe connective tissue destruction in severe active RA. Another question was how these CD14+CD15+ cells produced and accumulated in iliac bone marrow were transferred to the epiphyseal bone marrow. The peripheral blood circulation is the probable route for these cells to migrate from iliac to epiphyseal bone marrow, as reported by Santiago[22].

The production of unusual (CD14+) myeloid cells from apparently normal (CD14–) myeloid cells was a novel finding. CD14–CD15+ cells in iliac bone marrow from normal donors showed no development to CD14+CD15+ cells even after incubation *in vitro* with high levels of IL-1ß or GMCSF. CD14–CD15+ cells derived from iliac bone marrow of patients with the least erosive disease showed some propensity to develop into CD14+CD15+ cells under the influence of high levels of IL-1ß, albeit at much lower final levels than those of patients with more erosive or mutilating disease. Reactivity of CD14–CD15+ cells in the hematopoietic iliac bone marrow was thought to be quite different relating to severity of RA. Because of the lower reactivity of CD14–CD15+ cells derived from patients with the least erosive disease, CD14+CD15+ cells could not be found in patients with milder RA *in vivo*.

Recently, tumor necrosis factor alpha, transforming growth factor beta, and interferon gamma have been investigated concerning the pathogenesis of RA[23–25], and may have strong effects on enhancing the development of CD14+CD15+ cells. Those factors were not evaluated in our study because we focused on the fundamental pathologic mechanism of development of CD14+CD15+ cells in iliac bone marrow of patients with RA. Among the effects of activating factors in this study, one interesting result is that IL-6 and IL-8 showed no enhancing effect in producing CD14+ myeloid cells. We previously reported elevations of IL-6 and IL-8 levels in iliac bone marrow serum of patients with RA closely related to the remarkable synovial proliferation[26]. It is difficult to draw conclusions from the estimation of cytokine levels in iliac bone marrow, but the mechanisms involved in production of CD14+ myeloid cells may be different from those promoting the proliferation of synovial tissue. Another indication of these differences is the function of T cells. Activation of T cells has been shown to be a fundamental mechanism in induction of synovitis in patients with RA[27], but in this study, T cells were proved to inhibit production of CD14+ myeloid cells. As T lymphocytes have many subpopulations, further work may elucidate the particular subsets of T cells that modulate growth of CD14+ myeloid cells. Studies of the pathophysiology of bone marrow in patients with RA will investigate the roles of various enhancing or inhibitory factors. However, our results suggest that the pathogenesis of RA may involve systemic hematopoietic organs such as iliac bone marrow.

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REFERENCES

1. Hamilton JA: Rheumatoid arthritis: Opposing actions of haemopoietic growth factors and slow-acting anti-rheumatic drugs. *Lancet 1993;*342:536–9.
2. Seitz M, Zwicker M, Pichler W, Gerber N: Activation and differentiation of myelomonocytic cells in rheumatoid arthritis and healthy individuals — Evidence for antagonistic *in vitro* regulation by interferon-gamma and tumor necrosis factor alpha, granulocyte monocyte colony stimulating factor and interleukin 1. *J Rheumatol 1992;*19:1038–44.
3. Kotake S, Higaki M, Sato K, *et al:* Detection of myeloid precursors (granulocyte macrophage colony forming units) in the bone marrow adjacent to rheumatoid arthritis. *J Rheumatol 1992;*19:1511–6.
4. Sohen S, Kita H, Tanaka S: Abnormalities in bone marrow mononuclear cells in patients with rheumatoid arthritis. *J Rheumatol 1993;*20:12–6.

5. Hirohata S, Yanagida T, Koda M, Koiwa M, Yoshino S, Ochi T: Selective induction of IgM rheumatoid factors by CD14+ monocytes-lineage cells generated from bone marrow of patients with rheumatoid arthritis. *Arthritis Rheum 1995;*38:384–8.
6. Ochi T, Hakomori S, Adachi M, *et al:* The presence of a myeloid cell population showing strong reactivity with monoclonal antibody directed to difucosyl type 2 chain in epiphyseal bone marrow adjacent to joints affected with rheumatoid arthritis (RA) and its absence in the corresponding normal and non-RA bone marrow. *J Rheumatol 1988;*15:1609–15.
7. Owaki H, Ochi T, Yamasaki K, *et al:* Elevated activity of myeloid growth factor in bone marrow adjacent to joints affected by rheumatoid arthritis. *J Rheumatol 1989;*16:572–7.
8. Hakomori S, Nudelman E, Levery SB, Kannagi R: Novel fucolipids accumulating in human adenocarcinoma: I. Glocolipids with di- or trifucosylated type 2 chain. *J Biol Chem 1984;*259:4672–80.
9. Ochi T, Tomita T, Shimaoka Y, *et al:* Production of unusual (di-Lex+, CD14+) myeloid cells in the bone marrow of patients with rheumatoid arthritis (abstr). *Arthritis Rheum 1994;*(suppl)37:S248.
10. Tomita T, Kashiwagi N, Shimaoka Y, *et al:* Phenotypic characteristics of bone marrow cells in patients with rheumatoid arthritis. *J Rheumatol 1994;*21:1608–14.
11. Arnett FC, Edworthy SM, Bloch DA, *et al:* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum 1988;*31:315–24.
12. Ochi T, Iwase R, Yonemasu K, *et al:* Natural course of joint destruction and fluctuation of serum Clq levels in patients with rheumatoid arthritis. *Arthritis Rheum 1988;*31:37–43.
13. Hamashima N, Ueda R, Takahashi T: Characterization of three monoclonal antibodies that react with high-molecular mass glycopeptides isolated from F9 mouse teratocarcinoma cells. *Differentiation 1986;*31:174–82.
14. Griffin JD, Mayer RJ, Weinstein HJ, *et al:* Surface marker analysis of acute myeloblastic leukemia: Identification of differentiation-associated phenotypes. *Blood 1983;*62:557–63.
15. Ziegler-Heitbrock HWL, Ulevitch RJ: CD14: Cell surface receptor and differentiation marker. *Immunology Today 1993;*14:121–5.
16. Morabito F, Prasthofer EF, Dunlap NE, Grossi CE, Tilden AB: Expression of myelomonocytic antigens on chronic lymphocytic leukemia B cells correlates with their ability to produce interleukin-1. *Blood 1987;*70:1750–7.
17. Ziegler-Heitbrock HWL, Passlick B, Flieger D: The monoclonal antimonocyte antibody My4 stains B lymphocytes and two distinct monocyte subsets in human peripheral blood. *Hybridoma 1988;*7:521–7.
18. Ochi T, Wakitani S, Shimaoka Y: Elevated activity of interleukin-1 epiphyseal bone marrow adjacent to affected joints in patients with rheumatoid arthritis (abstr). *Arthritis Rheum 1990;*(suppl)33:149.
19. Wakitani S, Ochi T, Ono K: Elevated interleukin-1 level in polymorphonuclear cells accumulating in bone marrow adjacent to the affected joints with severe rheumatoid arthritis. *Jpn J Rheumatol 1994;*5:243–50.
20. MacNaul KL, Chartrain N, Lark M, Tocci MJ, Hutchinson NI: Discoordinate expression of stromelysin, collagenase and tissue inhibitor of metalloproteinases-1 in rheumatoid synovial fibroblasts: Synergistic effects of interleukin-1 and tumor necrosis factor α on stromelysin expression. *J Biol Chem 1990;*265:17238–45.
21. Lefebvre V, Peeters-Joris C, Vaes G: Modulation by interleukin 1 and tumor necrosis factor α of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes. *Biochim Biophys Acta 1990;*1052:366–78.
22. Santiago-Schwarz F, Sullivan C, Carsons SE: Expression of CD33, CD34 antigens and colony forming capacity in RA peripheral blood (abstr). *Arthritis Rheum 1993;*(suppl)36:S157.
23. Alvaro-Gracia JM, Zvaifler NJ, Firestein GS: Cytokines in chronic inflammatory arthritis. V. Mutual antagonism between interferon-gamma and tumor necrosis factor-alpha on HLA-DR expression, proliferation, collagenase production and granulocyte macrophage colony-stimulating factor production by rheumatoid arthritis synoviocytes. *J Clin Invest 1990;*86:1790–8.
24. Butler DM, Picoli DS, Hart PH, Hamilton JA: Stimulation of human synovial fibroblast DNA synthesis by recombinant human cytokines. *J Rheumatol 1988;*15:1463–70.
25. Saxne T, Palladino MA Jr, Heinegard D, Talal N, Wollheim FA: Detection of tumor necrosis factor α but not tumor necrosis factor ß in rheumatoid arthritis synovial fluid and serum. *Arthritis Rheum 1988;*31:1041–5.
26. Tanabe M, Ochi T, Tomita T, *et al:* Remarkable elevation of interleukin 6 and interleukin 8 levels in the bone marrow serum of patients with rheumatoid arthritis. *J Rheumatol 1994;*21:830–5.
27. Panayi GS, Lanchburg JS, Kingsley GH: The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum 1992;*35:729–35.