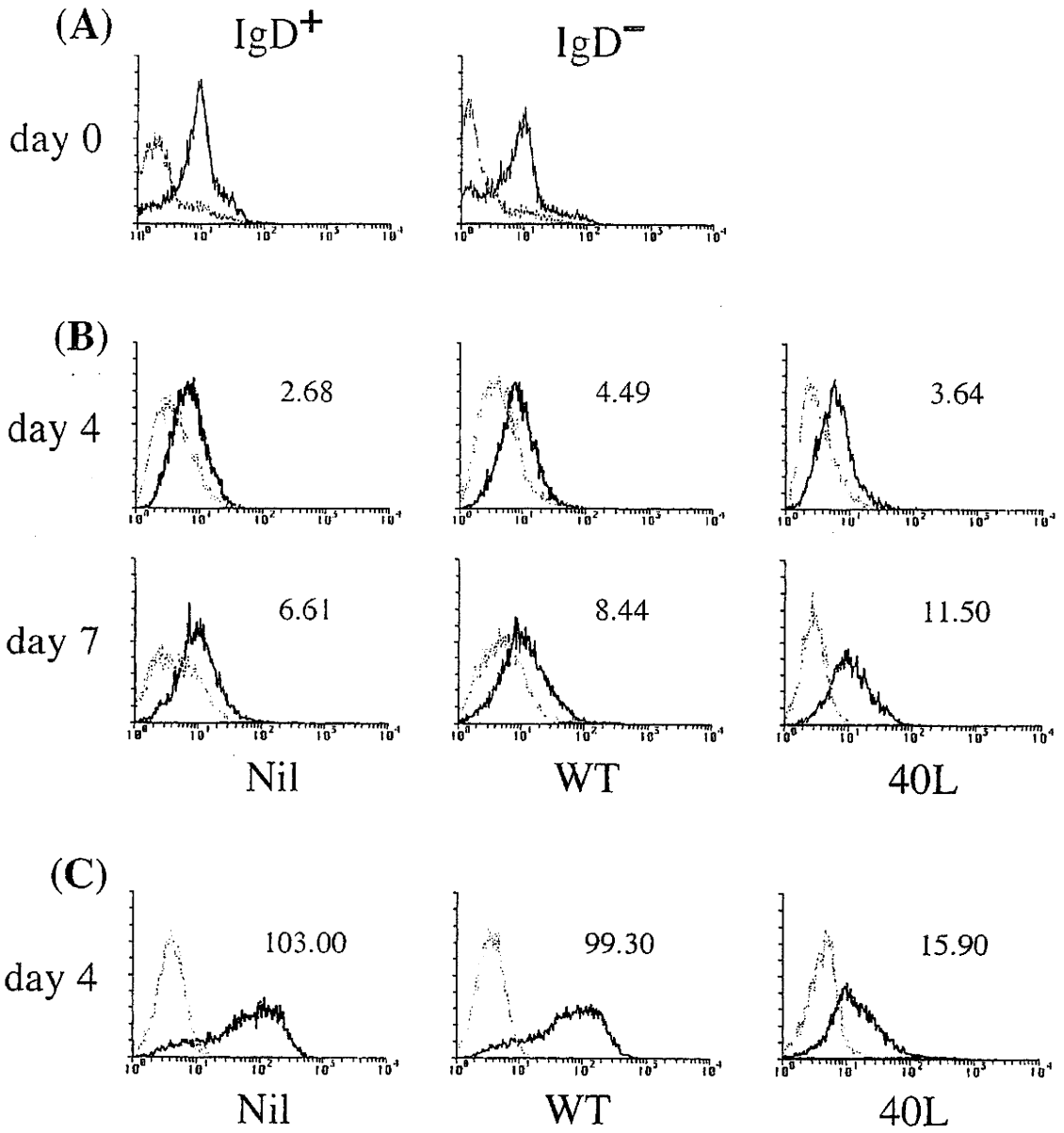

FIGURE 11. The effect of CD40 ligation on CD38 expression by IgD^+ or IgD^- B cell populations. (*A*) IgD^+ or IgD^- B cells were immediately analyzed for CD38 expression. IgD^+ (*B*) or IgD^- (*C*) B cells (5×10^4/well) were cultured with SAC (1:60,000) and IL-2 (20 U/ml) in the absence or presence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

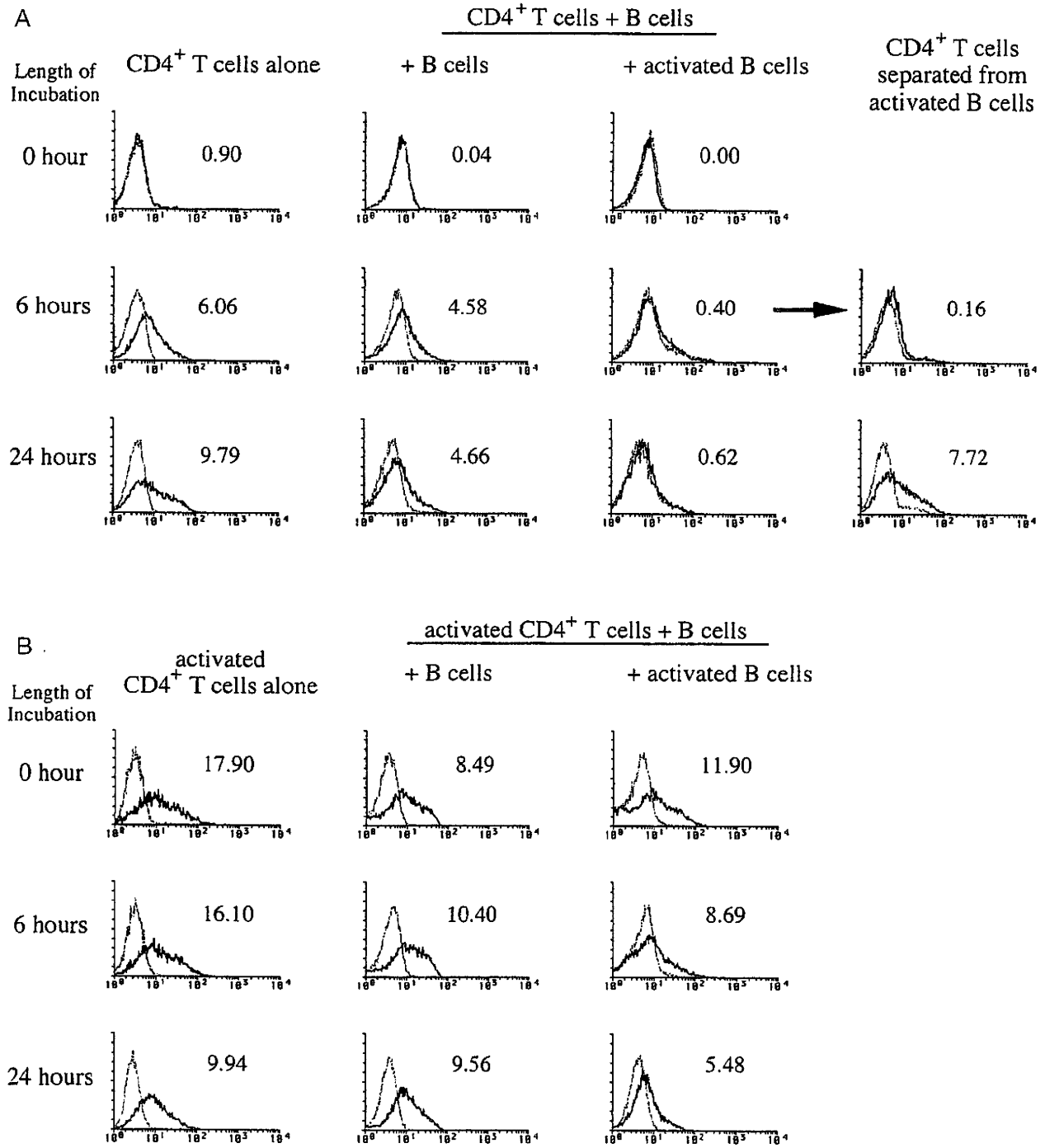

FIGURE 12. Activated B cells strongly inhibit CD40 ligand expression by $CD4^+$ T cells. B cells (5×10^4/well) were cultured with anti-CD3 (200 ng/well)-stimulated control $CD4^+$ T cells (1×10^5/well) for 48 h. Afterward, cells were harvested from the wells, and activated B cells were separated from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

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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; $p<0.05$).

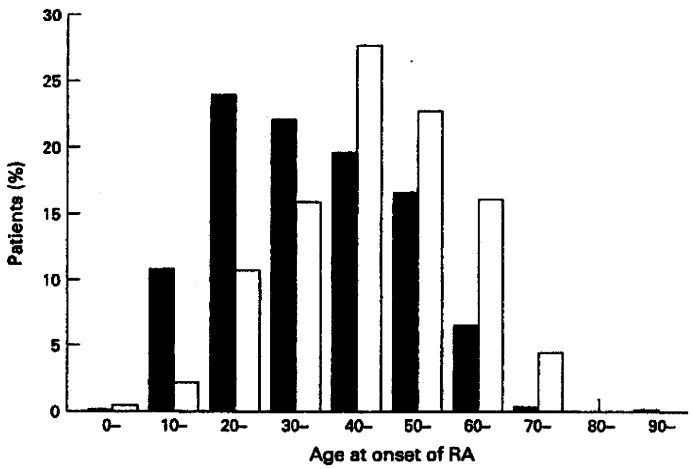

Figure 1 Distribution of age at onset. ■ : Group I patients who visited Osaka University Hospital from 1960 to 1965, □: group II patients who attended Shichikawa Arthritis Research Centre from 1985 to 1990.

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.

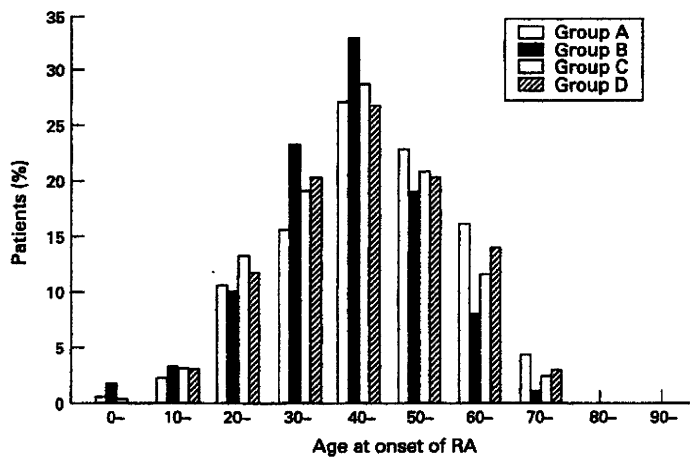

Figure 2 Distribution of age at onset. Group A: 366 patients at Shichikawa Arthritis Research Centre between 1985 and 1990, Group B: 286 patients at Osaka University Hospital between 1985 and 1990, Group C: 233 patients at Yukioka Hospital between 1985 and 1990, Group D: 370 patients at Osaka-Minami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

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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].

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,

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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

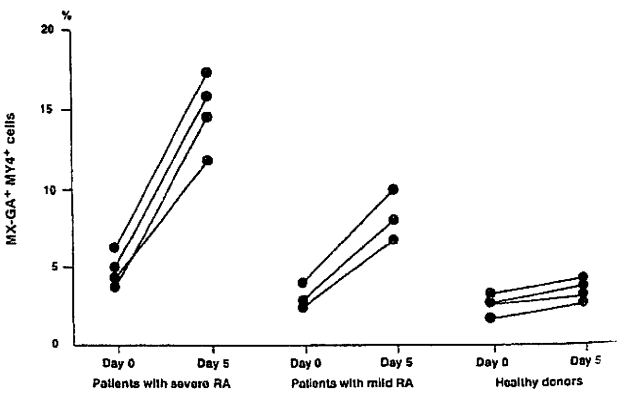

Figure 1. CD14 antigen expression on CD15+ cells. MNC from iliac bone marrow aspirates of patients with severe or mild RA or healthy donors were cultured with auto-iliac serum for 5 days.

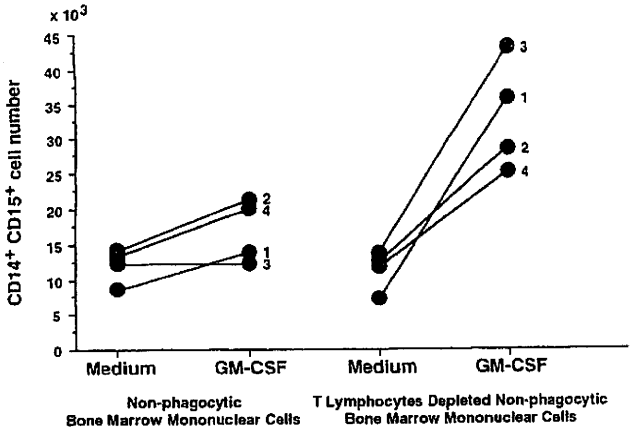

Figure 2. Effect of T lymphocytes on CD14 antigen expression on CD15+ cells from patients with severe RA. Nonphagocytic bone marrow mononuclear cells or T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured in the presence of rhGMCSF (50 U/ml) or without GMCSF for 5 days. Numbers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

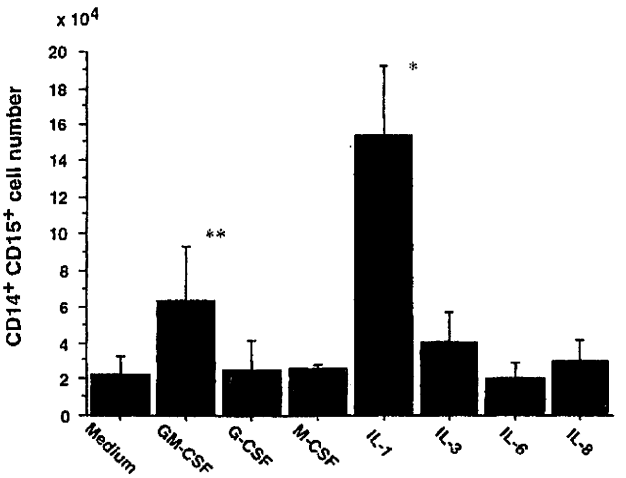

Figure 3. Effect of cytokines on CD14 antigen expression on CD15+ cells from patients with RA. T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured in the presence of cytokines (100 U/ml) for 5 days. *p < 0.0001 and **p = 0.0037 compared with culture with medium only.

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 ($p < 0.01$).

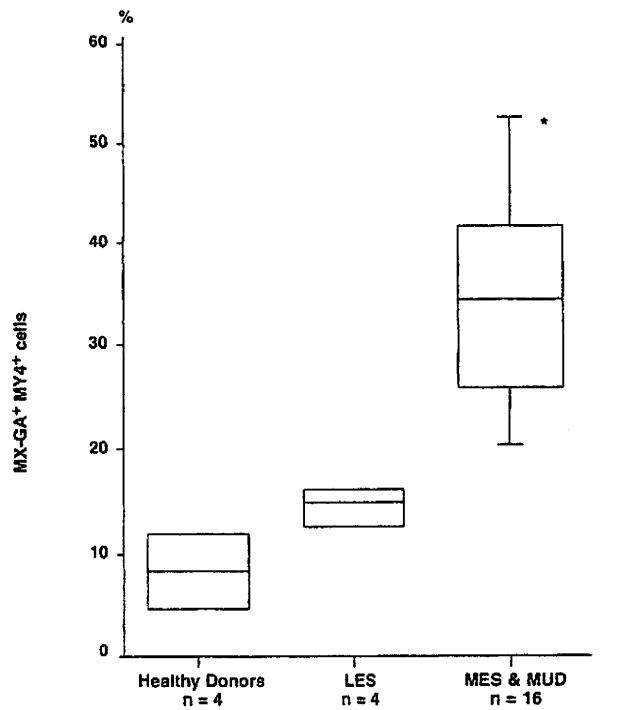

Figure 4. Effect of IL-1ß on CD14 antigen expression on CD15+ cells from patients with RA and healthy donors. T lymphocyte depleted nonphagocytic bone marrow mononuclear cells were cultured 5 days in the presence of IL-1ß (100 U/ml). The line in middle of the box represents the median. The top of the 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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Phenotypic Characteristics of Bone Marrow Cells in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Our previous study showed the presence of abnormal myeloid lineage cells in the epiphyseal bone marrow adjacent to joints affected with severe rheumatoid arthritis (RA). Now, we investigated whether there were any changes of other marrow cell populations related to RA, and whether there were any pathologically characteristic changes in the iliac bone marrow, which is one of the major systemic hematopoietic organs.

Methods. 2-Color flow cytometry was carried out to analyze the phenotypes of mononuclear cells (MNC) fractions in bone marrow aspirates and venous blood from 56 patients with RA and 7 non-RA controls.

Results. The absolute number of MNC in the iliac bone marrow was increased by 3-fold in the RA patients compared with the non-RA controls. In contrast, no significant increase of MNC was observed in the tibial epiphyseal bone marrow or peripheral blood. The ratio of each MNC fraction in the iliac bone marrow did not differ significantly between the RA patients and the non-RA controls. In lymphocyte subsets, the percentage of HLA-DR+CD8+ cells to all CD8 cells in the iliac bone marrow increased significantly in the RA patients compared with the non-RA controls. Abnormal myeloid cells (MX-GA+MY4+ cells), specific to severe RA, were found to be more concentrated in the iliac bone marrow than in the tibial epiphyseal bone marrow.

Conclusion. Characteristic pathologic changes of the iliac bone marrow suggest an important role of systemic bone marrow in the progression of RA. (*J Rheumatol 1994;***21:1608–14**)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
MYELOID LINEAGE CELLS
BONE MARROW

Preceding the induction of polyarthritis, maturation and proliferation of bone marrow cells were observed, and interleukin 1 (IL-1) and IL-6 levels in bone marrow serum were elevated in collagen induced arthritis and adjuvant arthritis in rats[1-3]. These pathological changes were maintained while arthritis continued. The results suggested that bone marrow plays an important role in inducing polyarthritis.

Our studies have demonstrated the existence of abnormal myeloid cells, which strongly express the difucosyl or trifucosyl type 2 chain (dimetric or trimetric Le^x, a specific marker of human undifferentiated cells[4,5]), in the epiphyseal bone marrow adjacent to joints affected with active severe subset of RA[6]. These myeloid lineage cells, which accumulate in the epiphyseal bone marrow in various stages of maturity, were found to exist in severe and active subset of RA. However, these cells were not found in the epiphyseal bone marrow of normal controls, nor in patients with mild RA, osteoarthritis or infectious arthritis. Thus, these myeloid lineage cells appear to be specific for severe RA. Maintaining these cells *in vitro* could not be achieved using commercially available culture medium, but was possible when epiphyseal bone marrow serum from patients with severe RA was added to the medium[7]. These results show the characteristic changes in epiphyseal bone marrow in RA. Recently other investigators have also reported abnormalities in bone marrow in patients with RA[8-10].

These results left us with 2 major questions to be answered: (1) are there any changes in other bone marrow cell populations related to RA and (2) are there any characteristic pathological changes of the iliac bone marrow, which is one of the major sites of systemic hematopoietic organs.

To answer these questions, we studied bone marrow blood cells from a large number of patients with RA. Our test subjects were obtained from the iliac crest and the tibial proxymal epiphysis at the time of surgical procedures.

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MATERIALS AND METHODS

Subjects. Bone marrow blood samples and peripheral blood samples were obtained from 56 patients with RA (51 women, 5 men) who met American College of Rheumatology criteria[11], and 7 healthy volunteers (non-RA controls) (4 women, 3 men) after informed consent was obtained. All the patients were operated on at Osaka University Hospital or the related facilities from 1990 to 1993. The average age of the RA patients and the non-RA controls was 55 and 40 years, respectively (range: 30–75, and 26–55, respectively). The average duration of the disease was 9.3 years (range: 3–30). According to our reported criteria[12,13], we classified patients with RA into 3 disease subsets; the subset with least erosive disease (LES), the subset with more erosive disease (MES) and the subset with mutilating disease (MUD). In the LES, erosive articular changes were primarily limited to the smaller peripheral joints. In the MES, the larger axial joints were also involved. In the most severely affected subset (MUD), which involves mutilating disease, almost all joints were extensively damaged. There were 17 patients in LES, 26 in MES, and 13 in MUD, respectively.

Cell preparations. At the studies of bone marrow cells, peripheral blood contamination of aspirates is thought to be inevitable[14,15], so a preliminary experiment was carried out. We collected iliac bone marrow aspirates in 5 ml fractions up to 30 ml and measured the number of myeloid cells to determine the level of contamination. When the volume aspirated from the iliac bone marrow exceeded 20 ml, the number of myeloid cells began to decrease and a significant difference was recognized due to contamination by peripheral blood (data not shown). Thus, the first 5 ml of bone marrow aspirate could be considered to most closely reflect the number of cells in the iliac bone marrow. So in our study, the first 5 ml of bone marrow aspirate was used for analysis.

Five ml of heparinized bone marrow aspirate was obtained from the iliac crest and the tibial proximal epiphysis by needle puncture at the time of operation. A 5 ml sample of heparinized venous blood was obtained simultaneously. MNC fraction, from bone marrow aspirate and venous blood, were separated by Ficoll-Hypaque density gradient (1.077 g/ml) centrifugation. In MNC fraction of the bone marrow aspirates, a lot of premature cells were contained depending on their density.

Cell staining. For FACS analysis, the cells were washed twice with phosphate buffered saline (PBS) and adjusted to 10^6/ml in RPMI 1640. Then 100 μl of this cell suspension was exposed to 5 μl of antibody (T4, T8, MY4, Leu11c, B1-RD1), or 10 μl of antibody (MX-GA, anti-HLA-DR) for 30 min at 4°C. The cells were then washed 3 times in PBS and fixed with 1% formaldehyde in PBS.

Monoclonal antibodies. The monoclonal antibodies (Mab) used were fluorescein-conjugated T4 (CD4), T8 (CD8), B1-RD1 (CD20) (all from Coulter Immunology Hialeah, FL) and MX-GA (CD15, clone HL5) (from Kyowa Medix, Japan), and phycoerythrin conjugated Leu11c (CD16), anti-HLA-DR (both from Becton Dickinson Mountain View, CA) and MY4 (CD14) (from Coulter Immunology Hialeah, FL).

2-Color flow cytometry. 2-Color flow cytometric analysis was performed using a FACScan (Becton Dickinson Mountain View, CA) equipped with an argon laser at 488 nm. All specimens were analyzed on the day of collection. To exclude debris or dead cells, the cells were gated on the basis of forward and right angle scatter. Each test employed 20,000 MNC and the number of positive cells was expressed as a percentage of the total cell count.

Morphologic studies. For light microscopy, sorted cells suspensions at 10^6/ml in RPMI 1640 were cytospun in 50 μl aliquots onto glass slides for 5 min at 6000 rpm (Shandon Cheshire, England) and stained by the May-Giemza, peroxidase and specific esterase method.

Statistical analysis. Results are expressed as the mean ± standard error of the mean. Data were analyzed by the Mann-Whitney U test. P values less than 0.05 were considered significant.

RESULTS

Number of mononuclear cells. The numbers of MNC fraction were measured and the data are shown in Table 1. There was a marked increase in the absolute number of MNC in the iliac bone marrow from patients with RA compared with the non-RA controls. The means (± SEM) of the number of MNC were 3122 ± 225 for patients with RA and 1245 ± 311 for the non-RA controls ($p < 0.01$). The number of MNC was increased according to the severity of RA[12] ($p < 0.05$ for LES and $p < 0.01$ for MES and MUD vs non-RA controls) (Figure 1). In contrast, the absolute number of MNC in the tibial bone marrow and peripheral blood did not differ significantly between the patients with RA and the non-RA controls.

Lymphocyte subsets. We analyzed the ratio of the lymphocyte population by FACScan using CD4 and CD8 Mab as

Table 1. *Cell marker studies on bone marrow aspirates and peripheral blood from 56 patients with RA and 7 non-RA controls*

	Peripheral Blood		Iliac Bone Marrow		Tibial Bone Marrow	
	Controls	RA	Controls	RA	Controls	RA
No. of MNC/mm³	974±167	1401±162	1245±311	3122±225*	952±138	1625±212
Myeloid cells						
% CD15+CD16−	2.9±0.5	6.5±1.5	19.0±3.5	24.4±1.7	6.8±2.0	5.8±0.9
T cells						
% CD4	43.2±3.3	29.5±1.8**	17.4±3.3	17.6±1.1	15.7±6.9	28.0±1.7
% CD8	25.2±2.4	21.2±1.6	20.9±4.4	15.1±0.9	15.1±3.6	23.3±1.5
% DR+CD4+/CD4	10.9±1.8	14.1±1.5	12.7±3.4	18.7±1.6	15.4±2.1	15.3±1.4
% DR+CD8+/CD8	13.7±2.8	27.7±2.1**	14.1±4.1	33.2±2.3**	13.1±2.4	28.8±2.7**
CD4/CD8 ratio	1.84±0.34	1.71±0.17	0.91±0.1	1.2±0.1	0.95±0.20	1.30±0.08
B cells						
% CD20	10.4±2.7	10.3±1.0	10.8±2.4	9.8±0.8	9.3±3.2	13.4±1.8
NK cells						
%CD16	17.3±2.2	15.7±1.6	10.8±2.3	8.2±0.7	8.9±2.0	16.3±1.5

Results are expressed as mean ± SEM.

* $p < 0.01$ as compared with the non-RA controls.

** $p < 0.05$ as compared with the non-RA controls.

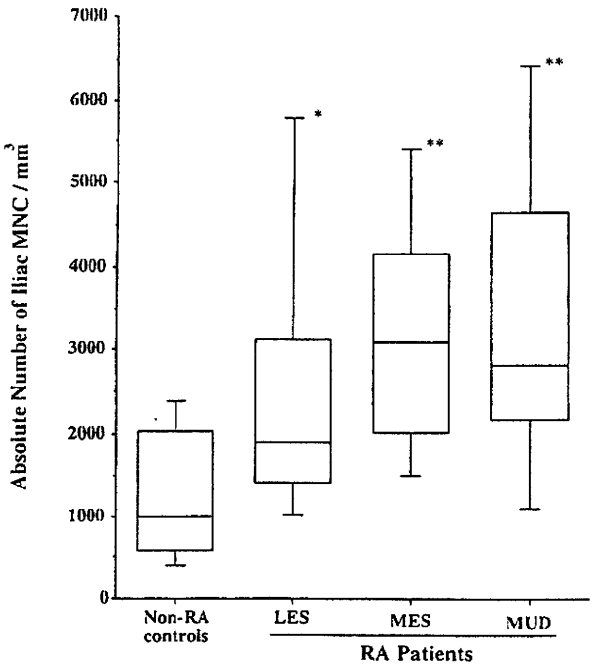

Fig. 1. The absolute number of iliac bone marrow mononuclear cells in non-RA controls and patients with RA. Patients with RA are classified into 3 disease subsets: the subset with least erosive disease (LES), the subset with more erosive disease (MES), and the subset with mutilating disease (MUD). The line in the middle of the box represents the median. The top of the box represents the 75th percentile, and the bottom of the box the 25th percentile. *p <0.05 and **p <0.01 as compared with the non-RA controls.

T cell markers, CD20 as a B cell marker and CD16 as a natural killer cell marker (Table 1). In the iliac bone marrow of patients with RA, the percentages of lymphocytes subsets did not differ significantly from the non-RA controls, but the number of MNC was increased 3-fold compared with the non-RA controls as mentioned above. The epiphyseal bone marrow, on the other hand, showed the similar number and percentage of lymphocytes subsets to those of peripheral blood, and are thought to reflect fundamentally the peripheral circulation. The peripheral blood of patients with RA demonstrated characteristic decrease of CD4 cells compared with the non-RA controls ($p < 0.05$). As to CD4/CD8 ratio there was no significant difference between patients with RA and the non-RA controls in the iliac bone marrow, the tibial bone marrow and the peripheral blood. In general, there were no significant differences in the percentage of lymphocytes in the iliac bone marrow between the patients with RA and the non-RA controls.

HLA-DR positive lymphocytes. We analyzed the percentage of HLA-DR+CD4 and CD8 cells among the total population of CD4 or CD8 cells (Table 1). The percentage of HLA-DR+CD4 cells with respect to all CD4 cells did not differ significantly between patients with RA and the non-RA controls in the iliac bone marrow and peripheral blood. In contrast, the percentage of HLA-DR+CD8 cells to all CD8 cells differed significantly between patients with RA and the non-RA controls in the iliac and tibial bone marrow and peripheral blood ($p < 0.05$). To investigate HLA-DR positive lymphocytes in more detail, we classified the patients with RA into 3 disease subsets[12] and compared each subset with the non-RA controls. The percentage of HLA-DR+CD8 cells to all CD8 cells in the iliac bone marrow was increased significantly in LES and MES when compared with the non-RA controls ($p < 0.05$), whereas the percentage of HLA-DR+CD4 cells to all CD4 cells did not differ significantly between each RA subset and the non-RA controls (Figure 2). Similar differences were observed in the peripheral blood and the tibial bone marrow. In the most severely affected

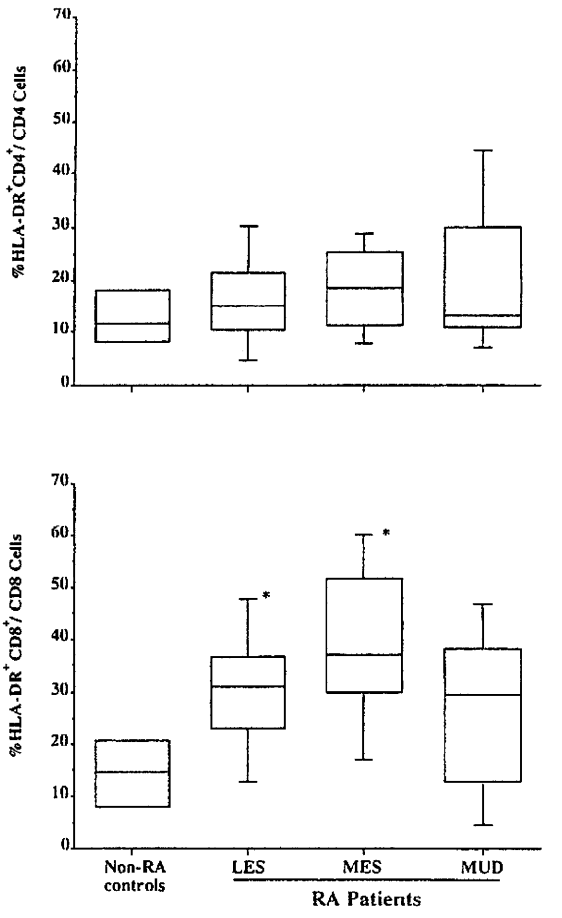

Fig. 2. HLA-DR antigen expression by CD4 and CD8 cells from the iliac bone marrow of the non-RA controls and patients with RA. Patients with RA were as classified in Figure 1. The line in the middle of the box represents the median. The top of the box represents the 75th percentile, and the bottom of the box the 25th percentile. *p <0.05 as compared with the non-RA controls.

patients, MUD, the percentage of HLA-DR+CD8 cells to all CD8 cells varied so greatly that no significant differences could be determined when compared with the non-RA controls.

Myeloid cell population. Myeloid cells were analyzed using the MX-GA (CD15) antibody which detects a broad range of myeloid lineage cells from myeloblasts to polymorphonuclear cells[16,17], and using CD16 which detects mature granulocytes (PMN) and natural killer (NK) cells. In short CD15+CD16− cells were composed of undifferentiated pregranulocyte cells. The CD15+CD16− cells were sorted and cytospun onto glass slides, and stained by the May-Giemza, peroxidase and specific esterase methods. Light microscopy showed that the CD15+CD16− cells were positive for peroxidase and specific esterase staining, and these cells were found to be promyelocytes and metamyelocytes (data not shown). Thus, CD15+CD16− cells were considered to be panmyelocytes.

In the iliac bone marrow, the percentage of myeloid cells among the total MNC fraction showed a clear tendency to increase in patients with RA (Table 1). In addition, the absolute number (/mm³) of myeloid cells in patients with RA in the 3 disease subsets, differed significantly from the number in the non-RA controls (LES: $p < 0.05$, MES and MUD: $p < 0.01$) (Figure 3).

Abnormal myeloid cells in bone marrow. We tried to analyze the systemic existence of the abnormal myeloid cells found in the involved epiphyseal bone marrow of RA[6]. A preliminary study employing the various monoclonal antibodies showed that abnormal and normal myeloid cells could be well separated using the MY4 (CD14) monoclonal antibody, which reacted with abnormal myeloid cells as well as monocyte-macrophages, but not with normal myeloid cells. In the tibial epiphyseal bone marrow of severe RA (MES and MUD), myeloid cells could be sometimes observed microscopically, and all these cells were recognized as abnormal MY4+ by FACS analysis. On the other hand, no myeloid cells could be found in the tibial epiphyseal bone marrow of the normal controls or LES. It was characteristic that myeloid cells in the iliac bone marrow could be separated into MX-GA+MY4− cells and MX-GA+MY4+ (abnormal) cells in severe RA (MES and MUD). But in the non-RA controls or LES, all myeloid cells in the iliac bone marrow were MX-GA+MY4− (Figure 4). To make clear the population of MX-GA+MY4+ cells in the iliac bone marrow, 2-color flow cytometric analysis was carried out. The purified population of MX-GA+MY4+ cells was selected by sorting, and was examined using cytospin preparations and histochemical stains. As shown in Figure 5, these cells were confirmed to be of myeloid lineage, but not monocyte lineage. In FACS analysis, contamination of counting error must be taken into consideration, and in this study the average background level was 4%. As shown in Table 2, in the tibial epiphyseal bone marrow, the mean percentage (± SEM) of abnormal myeloid cells (MX-GA+MY4+ cells) was almost the same as the background level. There was no significant difference in the patients with severe RA (MES and MUD) when compared with the non-RA controls, although several individual patients in MES and MUD had up to 11.8 and 13.7% of these cells, respectively. So in such cases the abnormal myeloid cell population was recognized. In the iliac bone marrow of patients with more severe RA (MES and MUD), this abnormal myeloid cell population was observed more clearly. In particular, the mean absolute number ± SEM (/mm³) of these cells in the iliac bone marrow showed a significant difference compared with the non-RA controls ($p < 0.05$). The results are summarized in Table 2.

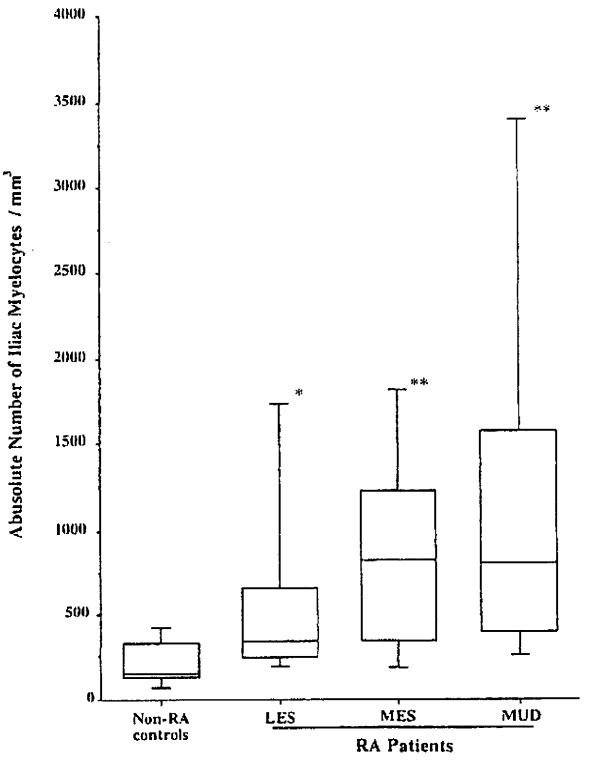

Fig. 3. The absolute number of iliac bone marrow myelocytes (CD15+CD16− cells) in the non-RA controls and patients with RA. Patients with RA were classified as in Figure 1. The line in the middle of the box represents the median. The top of the box represents the 75th percentile, and the bottom of the box the 25th percentile. *p <0.05 and **p <0.01 as compared with the non-RA controls.

DISCUSSION

Our first major question was whether there were any cellular changes in the iliac bone marrow which is one of the major systemic hematopoietic organs in adults. In infants and children, active hematopoiesis takes place in most of the marrow cavities, including the distal long bones. At 5–7 years of age, fat cells begin to replace the hematopoietic marrow in the extremities; in normal adults, hematopoiesis is con-

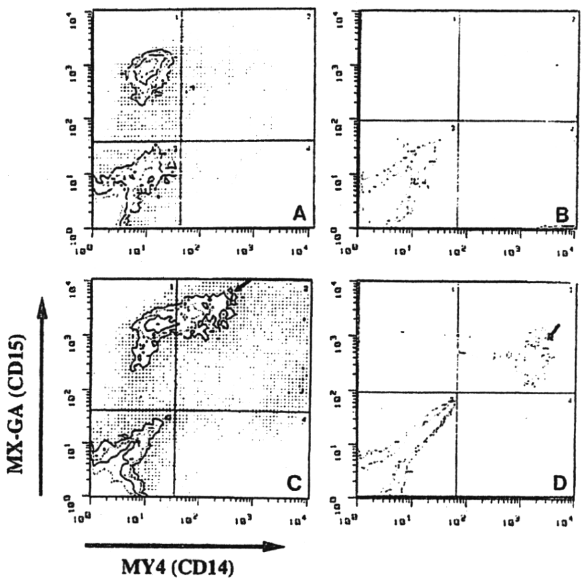

Fig. 4. 2-Color flow cytometric analysis of a normal donor and a patient with severe RA. Panel A: Cells in the iliac bone marrow of a normal donor. Panel B: Cells in the tibial epiphyseal bone marrow of a normal donor. Panel C: Cells in the iliac bone marrow of a patient with severe RA. Panel D: Cells in the tibial epiphyseal bone marrow of a patient with severe RA. In the iliac bone marrow of a normal donor, one myeloid cell population (MX-GA+MY4− cells) was recognized. In the tibial epiphyseal bone marrow, no myeloid cell population was recognized. In contrast, in the iliac bone marrow of a patient with severe RA, two myeloid cell populations (MX-GA+MY4− cells and MX-GA+MY4+ cells) were recognized. And in the tibial epiphyseal bone marrow, an abnormal cell population (MX-GA+MY4+ cells) was recognized. Arrows indicate the abnormal myeloid cell population (MX-GA+MY4+ cells).

fined to the vertebrae, ribs, sternum, pelvis, scapulae, skull, and the extreme proximal portions of the humeri and femora[18,19]. Thus, the tibial epiphyseal bone marrow is not hematopoietic in adults. We chose the iliac bone marrow to investigate hematopoietic marrow in the present study.

The absolute number of MNC (/mm³) in the iliac bone marrow of patients with RA was increased up to 3-fold compared with the non-RA controls, although the relative proportions of each MNC fraction in the iliac bone marrow was similar between patients with RA and non-RA controls. As there was no significant difference among 3 disease subsets, the increase of MNC was recognized as general to the iliac bone marrow of patients with RA, regardless of the severity of bone and joints destruction. Since the peripheral blood white cell count was not significantly increased in the patients with RA compared with the non-RA controls, the rapid turnover and functional enhancement of white blood cells in peripheral organs of patients with RA was suggested.

One of the characteristic findings in the iliac bone marrow of patients with RA was a significant enhancement of T cell activation. Although the percentage of CD8 cells among MNC was not statistically different between patients with RA and the non-RA controls, the percentage of HLA-DR+CD8 cells to all CD8 cells was significantly higher in both the iliac bone marrow and peripheral blood of patients with LES and MES, but not with MUD. The reason for this is unclear at present, but there could be some fundamental differences of the iliac bone marrow that are unique to MUD. Similar changes were also recognized in peripheral blood but were thought to reflect changes in the iliac bone marrow. The ratio of CD4 cells, on the other hand, was significantly decreased in the peripheral blood as also shown by

Fig. 5. Histochemical staining of purified MX-GA+MY4+ cells. MX-GA+MY4+ cells were stained by the esterase (A) and peroxidase (B) methods (magnification × 800). The morphological findings showed that these MX-GA+MY4+ cells were of the myeloid lineage.

Table 2. *Abnormal myelocytes (MX-GA+MY4+ myelocytes) in iliac and tibial bone marrow*

	n	Percentage in Tibial BM	Percentage in Iliac BM	Absolute Number in Tibial BM (/mm³)	Absolute Number in Iliac BM (/mm³)
Non-RA controls	7	3.7±0.9 (1.5-7.0)	4.4±0.6 (1.8-7.3)	41 ± 13	86 ± 26
LES	17	2.7±0.5 (0.6-7.0)	3.6±0.5 (0.4-8.0)	33 ± 3	96 ± 18
MES	26	4.0±0.6 (0.9-11.8)	6.4±1.3 (1.5-26.0)	102 ± 37	246 ± 79*
MUD	13	4.3±2.0 (1.0-13.7)	7.9±2.3 (1.8-29.5)	94 ± 74	219 ± 65**

Values are the mean ± SEM. Values in parentheses indicate the range. BM: bone marrow.
* $p < 0.05$ vs MES and the non-RA controls. ** $p < 0.05$ vs MUD and the non-RA controls.

prior studies[20,21]. However, the ratio of these cells in the iliac bone marrow was not significantly different to that in the non-RA controls. This could be a result of rapid turnover, and is probably related to the activation of CD4 cells in the peripheral blood. Although the precise pathological mechanisms are not yet known, the activation of T cell subsets could induce various immunologic enhancements such as the elevation of cytokines in the iliac bone marrow. For example, the elevated levels of IL-6 and IL-8 in the iliac bone marrow serum may be related to the remarkable synovial proliferation in multiple joints which is frequently seen in LES and MES[22].

Another characteristic finding in the iliac bone marrow of patients with RA was the induction of abnormal myeloid cells, which were previously reported in epiphyseal bone marrow[6]. In our preliminary study, these cells were clearly distinguished from normal myeloid cells by their antigenic reactivities (MX-GA+MY4+ as well as mono-Lex+di-Lex+ as reported[6]). Because of availability of Mab, in our study these abnormal myeloid cells were defined by staining with MX-GA and MY4. MX-GA+MY4+ cells showed a significant increase in the iliac bone marrow of patients in MES and MUD compared with baseline value found in the non-RA controls. In the tibial epiphyseal bone marrow, there was no significant difference in the percentage or cell number of MX-GA+MY4+ cells between severe RA and the normal controls. This is because the number of these abnormal cells in the epiphyseal bone marrow were reduced during the remission period of RA. In several active cases, however, abnormal myeloid cells were present at up to 11.8% in MES, and 13.7% in MUD, respectively. Thus, abnormal myeloid cells occurred to a greater extent in the iliac bone marrow than in the tibial epiphyseal bone marrow, and was a characteristic feature of MES and MUD. Although the precise physiology and mechanisms involved are still under study, we have reported that polymorphonuclear neutrophils (PMN; the final differentiation stage of myeloids) with a special activity for tissue injury such as PMN factor activity[23,24] and high IL-1 levels[25] were detected in the bone marrow of patients with severe RA (MES and MUD) where those abnormal myeloid cells accumulated.

We have previously analyzed the natural course of RA based on the extent of joint destruction, and reported the existence of 3 disease subsets of RA; LES, MES and MUD[12,13]. We classified these disease subsets according to the number of joints with erosion, serum Clq levels and the annual reduction of the ratio of carpal height[26]. In rough analysis, we found inflammatory variables [C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF)] were persistently remarkably high for at least 5 years after the onset of disease in MES and MUD. Among these inflammatory parameters, CRP levels were persistently above 4 mg/dl within 5 years of onset in MES and MUD, but were usually less than 2 mg/dl in LES. The present study suggested that these clinically defined 3 disease subsets showed significantly different tendencies in the iliac bone marrow cells. That is to say, the increase of HLA-DR+CD8+ T cells in the iliac bone marrow occurs in LES and MES, while the increase of MX-GA+MY4+ cells is characteristic of more severe disease subsets of RA (MES or MUD) (Figure 6). These results suggest that differences at the cellular level affect the natural courses of RA.

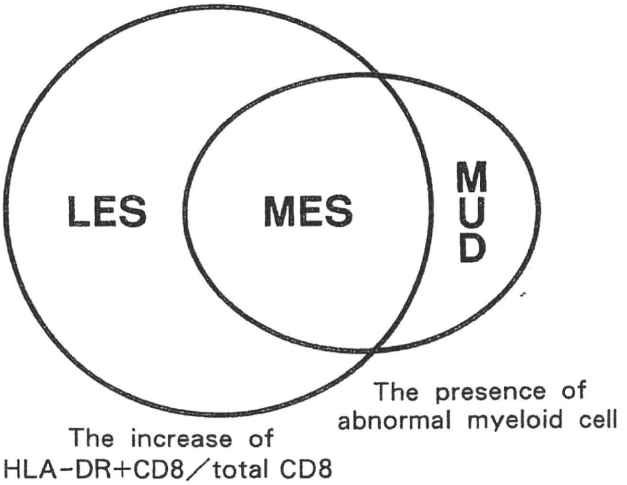

Fig. 6. Characteristic changes of iliac bone marrow cells in each RA disease subset. The increase of the percentage of HLA-DR+CD8 cells to all CD8 cells was recognized in LES and MES, and the presence of abnormal myeloid cells was characteristic to MES and MUD.

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