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Production of IL-6 by T cells from the femoral head of patients with rapidly destructive coxopathy (RDC)

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SUMMARY

RDC is a syndrome with unknown etiology that causes rapid destruction of a hip joint. We have investigated the production of osteoclast-activating cytokines (IL-6, IL-1α and tumour necrosis factor-alpha (TNF-α)), interferon-gamma (IFN-γ) and IL-8 by T cells in the affected joint. The level of IL-6 produced by the T cell lines (TCL) established from the femoral head was significantly higher than that from patients' or healthy donors' peripheral blood mononuclear cells (PBMC). IL-6 production by the TCL from synovial membrane or from patients' PBMC was also significantly higher than that from healthy donors' PBMC. IL-1α production by the TCL from the femoral head was significantly higher than any of the other groups when all the TCL were used for the analysis. TNF-α production was highest in the TCL from patients' PBMC. The levels of IFN-γ or IL-8 were not significantly different among these four groups. The plasma levels of all these cytokines except for IFN-γ, that was rather lower, in RDC patients were not significantly different from those in osteoarthrosis or trauma patients, or healthy donors. These results suggest that T cells at the affected femoral head, and also synovial membrane to some extent, are involved in bone resorption through the production of IL-6 and probably IL-1α in patients with RDC.

Keywords rapidly destructive coxopathy T cell IL-6 osteoclast-activating factor

INTRODUCTION

RDC is a syndrome that causes rapid destruction of the hip joint, not only of the femoral head but also the acetabulum in mostly middle-aged and elderly women [1,2]. RDC is apparently different from the other diseases of the hip joint, including rheumatoid arthritis, aseptic necrosis, and Charcot's joint, with the following characteristics: (i) the average age at onset is greater than that of the other diseases; (ii) most of the patients have a unilateral lesion; (iii) the condition occurs mostly in a normal hip followed by severe joint destruction within 3–12 months [1,2]. Total hip replacement is presently the only effective modality for treatment [1,2]. Neither the etiology nor the immunopathology of RDC is understood, although autoimmune reactions to the articular cartilage are suggested to be responsible for RDC in animal models [3–6]. This obscurity is due in part to the difficulty in obtaining appropriate samples for the immunological analysis from the affected femoral head.

In various orthopaedic diseases, cytokines generated in the bone environment seem to be responsible for stimulating osteoclastogenic bone resorption. IL-6 has been reported to induce bone resorption by activating osteoclasts [7–11]. Similarly, both IL-1α and IL-1β function to activate osteoclasts in conjunction with IL-6 [10–13]. Tumour necrosis factor-alpha (TNF-α) is also a known participant in bone resorption [10]. Furthermore, IL-8 may be involved in the joint destruction as well [14], but interferon-gamma (IFN-γ) inhibits IL-1-mediated bone resorption [15]. We have investigated the production of osteoclast-activating cytokines (IL-6, IL-1α and TNF-α) by T cells at the disease site in order to understand the immunopathology of RDC.

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MATERIALS AND METHODS

Subjects

All 11 RDC patients investigated in this study were women with a mean age of 70·0 ± 9·3 years who were negative for histories of smoking, alcohol consumption, steroid treatment or apparent allergies. These patients were recruited from August 1993 to July 1994. Diagnosis was based on the following criteria: (i) progressive destruction of an anatomically normal hip joint over a short period of time (within 1 year) evaluated by both physical examination and x-ray photographs; and (ii) exclusion

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of metabolic disorders, such as gout, and infectious diseases (syphilis, type B hepatitis, type C hepatitis, and adult T cell leukaemia). No anti-inflammatory drugs were administered to any of these RDC patients before surgery.

The femoral head and synovial membrane were extracted from five of 11 RDC patients at the time of the total hip replacement operation. The femoral head was sectioned at the femoral neck and bisected longitudinally. One piece was prepared for regular pathological analysis, while the other was used to isolate infiltrating lymphocytes. One half of the femoral head or a portion of the synovial membrane was crushed or minced into small pieces and washed in PBS. Then the cells infiltrating into these tissues were collected. These samples were not available in the other six patients. Heparinized peripheral blood samples were obtained from all 11 RDC patients. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Conray gradient centrifugation from heparinized peripheral blood following plasma separation. Plasma was also obtained from the following age- and sex-matched groups: 11 patients with rheumatoid arthritis (RA; 68·9 ± 3·3 years, all women), four patients with osteoarthrosis (66·5 ± 7·6 years, all women), 10 patients with bone and soft-tissue trauma (63·4 ± 7·3 years, all women), and 13 healthy donors (67·0 ± 3·5 years, all women). The plasma samples were aliquotted and cryopreserved at −80°C until use.

Establishment of T cell lines

T cell lines (TCL) were established from the cells from the femoral head, synovial membrane and PBMC of five of 11 RDC patients, and the age- and sex-matched three healthy donors by limiting dilution method as reported previously [16,17]. Briefly, cells were plated at two, four and eight cells per well in 96-well round-bottomed plates (Falcon, Lincoln Park, NJ) with RPMI 1640 medium containing 10% fetal calf serum (FCS; Bioserum, Victoria, Australia), 50 Gy-irradiated allogeneic PBMC from healthy donors as feeder cells (2×10^5 cells/well), 10 μg/ml phytohaemagglutinin (PHA-P; Difco Labs, Detroit, MI) and 100 U/ml human recombinant IL-2 (rIL-2; Shionogi, Osaka, Japan). The cells were incubated in humidified 5% CO_2 in air at 37°C. RPMI 1640 medium containing 10% FCS and 100 U/ml rIL-2 was used for expansion of TCL. The feeder cells were added every 7 days. Surface marker analysis of the proliferating cells was carried out by direct immunofluorescence technique using FITC-conjugated anti-CD3, PE-conjugated anti-CD4, and FITC-conjugated anti-CD8 MoAbs (Nichirei, Tokyo, Japan). The stained cells were analysed under a fluorescence microscope. Cells displaying a uniform T cell phenotype (i.e. $CD3^+CD4^+CD8^-$ or $CD3^+CD4^-CD8^+$) were used in the studies. Mean numbers of wells containing proliferating cells per plate (96 wells) were five, 11, or 28 when two, four, or eight cells per well were plated in the case of the femoral head. Those were 0, 0·5, 8 in synovial membrane, and 10 (11), 35 (33), 80 (83) in patients' PBMC (or healthy donors' PBMC), respectively. Therefore, cloning efficiency was lowest in cells of synovial membrane, lower in cells of femoral head, and high in PBMC. Among 53 TCL of the femoral head used for the study, 22, 10, and 21 were from wells containing two, four, and eight plated cells per well, respectively. All 29 TCL of synovial membrane were from wells containing eight cells per well. All TCL from PBMC were from wells containing two cells per well.

Preparation of culture supernatants

TCL were cultured with RPMI 1640 medium, 10% FCS and 100 U/ml rIL-2 without the feeder cells for 7 days before use. These TCL were then washed three times and resuspended in medium containing 10% FCS alone at 5×10^5 cells/ml and cultured in the wells of 48-well flat-bottomed plates (Falcon) for 18 h in the absence of any stimuli or in the presence of 1 ng/ml phorbol myristate acetate (PMA; Sigma Chemical Co., St Louis, MO). Thereafter cell-free supernatants were harvested, aliquotted and cryopreserved at −80°C until use. For kinetic study, frozen TCL were thawed in the morning of experiments in the presence of feeder cells, and were incubated with RPMI 1640 medium, 10% FCS and 100 U/ml rIL-2 for up to 30 days without additional stimuli by feeder cells. These TCL were harvested at day 8, day 15 and day 22 of culture, washed three times, and were cultured for 18 h at 5×10^5 cells/ml with RPMI 1640 medium plus 10% FCS alone, with PMA (1 ng/ml), with PMA and PHA (10 μg/ml), or with PMA and ionomycin (0·5 μg/ml) (Sigma) in the wells of 48-well flat-bottomed plates (Falcon). Kinetic study later than 30 days was not applicable since most TCL died within 30 days under these culture conditions.

Cytokine assays

Cytokine concentration in the culture supernatants of TCL or plasma was measured by ELISA kits according to the manufacturer's instructions: IL-6 kit (sensitivity >5 pg/ml) (Immunotech International, Marseilles, France), IL-1α kit (>1·5 pg/ml) (Cayman Chemical Co., Ann Arbor, MI), IL-1β kit (>1·5 pg/ml) (Immunotech International), TNF-α kit (>5 pg/ml) (Immunotech International) and IFN-γ kit (>5 pg/ml) (Endogen, Boston, MA). IL-8 was also measured by a kit kindly provided by Dr N. Mukaida (Kanazawa University, Kanazawa, Japan) (>16 pg/ml) [17].

Histological and statistical analyses

Specimens of the femoral head from five patients with RDC used to establish the TCL were stained by standard haematoxylin–eosin (H–E) staining. Wilcoxon signed-rank test was used for statistical analysis.

RESULTS

Histological analysis

The pronounced infiltration of mononuclear cells and osteoclast-like multinuclear cells was observed at the site of destruction of the femoral head in all five RDC patients whose T cells were tested for cytokine production. Representative results are shown (Fig. 1). Empty lacunae in the trabeculae, which indicate the death of bone cells, were observed at the centre of destruction of the femoral head (Fig. 1a,b). Normal trabeculae were usually observed in the periphery of the destruction of the femoral head in all cases (Fig. 1c,d). Many osteoclast-like multinuclear cells (shown by the arrows in Fig. 1b,d) were observed along the margin of the trabeculae.

Cytokine production by TCL

One hundred and fifty TCL were established from five of 11 RDC patients (53 from femoral head, 29 from synovial membrane, and 46 from PBMC) and the PBMC of three healthy donors (22 TCL) (Table 1). Most TCL in each group were

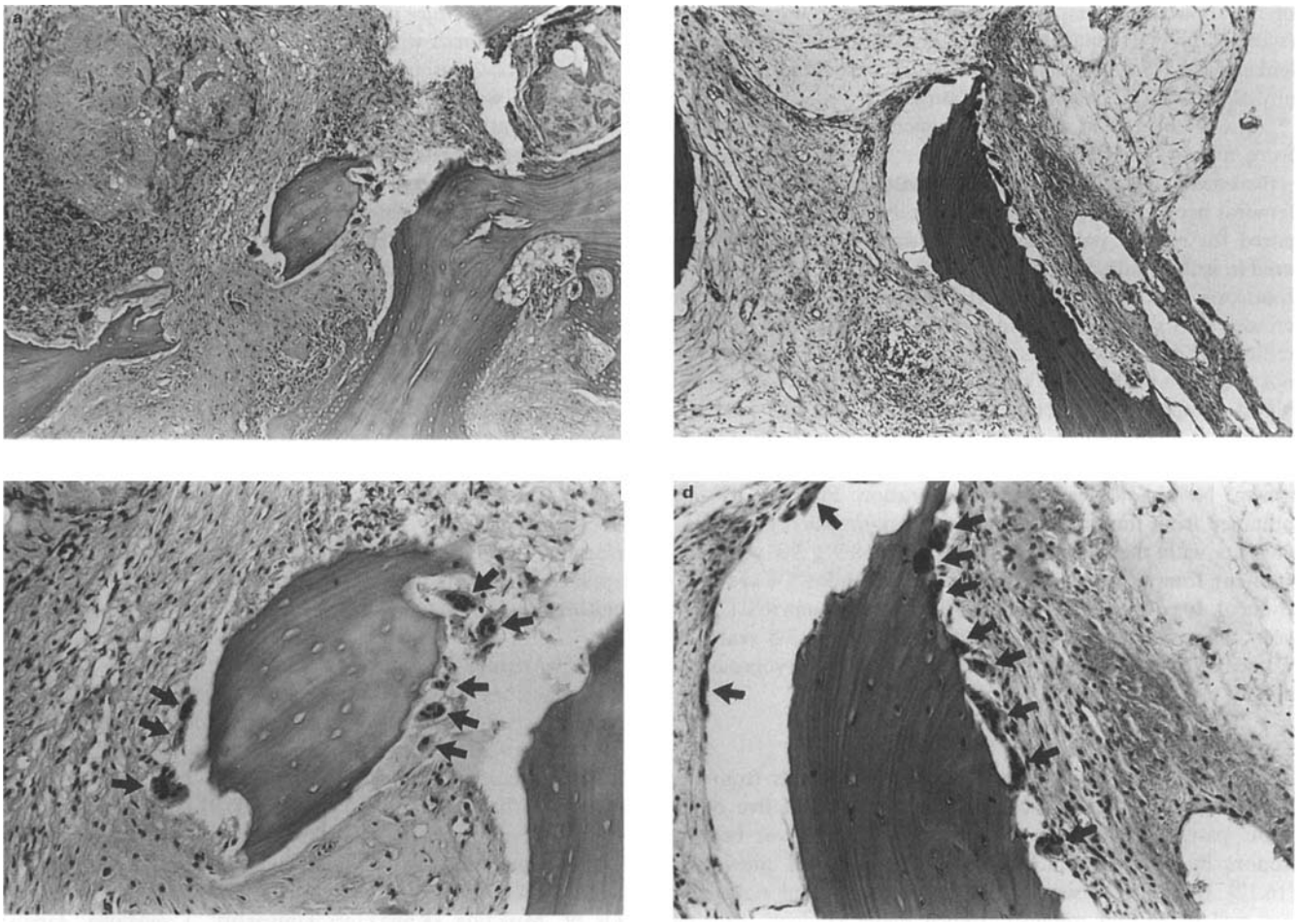

Fig. 1. Histology of the femoral head extracted from patients with RDC. (a,b) Granulation, pronounced infiltration of mononuclear cells, empty lacunae in the trabeculae and many osteoclast-like multinuclear cells were observed at the centre of destruction by haematoxylin–eosin staining. (a) Mag. ×20, (b) mag. ×50. (c,d) Although empty lacunae were not observed in the trabeculae, both osteoclast-like multinuclear cells and mononuclear cells were also observed in the periphery of destruction. ((c) Mag. ×20, (d) mag. ×50). The osteoclast-like multinuclear cells are indicated by the arrows (b and d).

$CD3^+CD4^+CD8^-$, and the relative numbers of $CD4^+$ and $CD8^+$ TCL were not largely different in the four groups. The means ± s.d. of cytokine concentration in the culture supernatants from all the TCL (both cytokine-secreting and -non-secreting) are shown in Table 1. TCL from the femoral head produced a significantly larger amount of IL-6 than that from patients' or healthy donors' PBMC, IL-1α than that from any of the other groups, and TNF-α than that from healthy donors' PBMC in the absence of PMA. The level of IFN-γ or IL-8 produced by the TCL from the femoral head was not significantly different from that produced by any of the other groups, although it was higher than the others.

The levels of IL-6 and IL-1α produced by the TCL from synovial membrane without PMA were significantly higher than those from healthy donors' PBMC (Table 1). IL-6 produced by the TCL from patients' PBMC was significantly higher than that from healthy donors' PBMC (Table 1). TNF-α produced by the TCL from patients' PBMC was significantly higher than that from synovial membrane or healthy donors' PBMC.

The levels of IL-6 and IL-1α produced by the TCL from the femoral head in the presence of PMA were also significantly higher than those by the TCL from patients' or healthy donors' PBMC (Table 1). The level of IFN-γ produced by the TCL from synovial membrane in the presence of PMA was significantly higher than that from healthy donors' PBMC.

The frequency of the cytokine-secreting (secretor) TCL largely varied among the groups in each cytokine (Table 1). Therefore, the data were also analysed as the mean ± s.d. from only the secretor TCL, to understand more clearly the capability of cytokine production per TCL in these groups (Table 2). IL-6 produced by the TCL from the femoral head with or without PMA was significantly higher than that from patients' or healthy donors' PBMC. IL-6 produced by the TCL from synovial membrane or patients' PBMC without PMA was also significantly higher than that from healthy donors' PBMC. TCL from patients' PBMC produced the highest level of TNF-α in the absence of PMA.

Kinetic study was performed using four different secretor TCL to investigate possible non-specific effects of feeder cells for cytokine production (Table 3). These TCL were harvested at day 8, day 15 and day 22 of culture, washed three times and were cultured with or without PMA, PMA and PHA, or PMA and ionomycin. The levels of IL-6 or TNF-α produced by these

Table 1. Cytokine production by T cell lines (TCL)

Disease	Sites (total TCC)	IL-6		IL-1α		TNF-α		IFN-γ		IL-8	
		Medium	PMA	Medium	PMA	Medium	PMA	Medium	PMA	Medium	PMA
RDC	FH (53)	38 ± 109* [20/47:43%]	42 ± 76* [33/47:70%]	2·3 ± 6·1* [13/47:28%]	6·3 ± 32* [16/47:34%]	2·7 ± 5·2* [8/47:17%]	167 ± 431 [45/47:96%]	39 ± 122 [11/38:29%]	766 ± 1326 [33/38:87%]	397 ± 1584 [38/38:100%]	2116 ± 5504 [38/38:100%]
RDC	SM (29)	33 ± 93* [13/25:52%]	49 ± 122 [20/25:80%]	0·6 ± 1·2* [4/25:16%]	1·0 ± 1·4 [8/25:32%]	1·4 ± 2·0 [2/25:8%]	77 ± 125 [17/25:68%]	17 ± 28 [7/17:41%]	769 ± 582* [15/17:88%]	118 ± 127 [17/17:100%]	715 ± 665 [17/17:100%]
RDC	PBMC (46)	7·1 ± 9·4* [18/46:39%]	17 ± 21 [29/46:63%]	0·3 ± 1·1 [4/46:9%]	1·9 ± 8·0 [4/46:9%]	26 ± 77* [13/46:28%]	216 ± 227* [42/46:91%]	5·9 ± 19 [8/39:21%]	593 ± 817 [35/39:90%]	84 ± 111 [39/39:100%]	1253 ± 4916 [39/39:100%]
HD	PBMC (22)	4·1 ± 3·5 [5/22:23%]	21 ± 34 [17/22:77%]	0·0 ± 0·0 [0/22:0%]	1·0 ± 4·7 [1/22:6%]	0·7 ± 1·9 [2/22:9%]	290 ± 346* [22/22:100%]	2·2 ± 6·7 [2/22:9%]	320 ± 341 [17/22:77%]	121 ± 206 [22/22:100%]	2190 ± 2450* [22/22:100%]

T cells used for the study were 53, 29, 46, and 22 TCL from the femoral head (FH), synovial membrane (SM), patients' or healthy donor (HD)s' peripheral blood mononuclear cells (PBMC), respectively. The means ± s.d. (pg/ml) of the values from all the TCL (secretor and non-secretors) are shown. Values in the square parentheses represent the number of secretor TCL/total TCL (secretors and non-secretors), and their percentages. *$P <$ at least 0·05 compared with the other group as follows: the level of IL-6 produced by the TCL from FH without phorbol myristate acetate (PMA) *versus* that from patients' or HD's PBMC; IL-6 from SM without PMA *versus* that from HD's PBMC; IL-6 from patients' PBMC without PMA *versus* that from HDs' PBMC; IL-6 from FH with PMA *versus* that from patients' or HDs' PBMC; IL-1α from FH *versus* that from any other groups either with or without PMA; IL-1α from SM without PMA *versus* that from HDs' PBMC; tumour necrosis factor-alpha (TNF-α) from FH without PMA *versus* that from HDs' PBMC; TNF-α from patients' PBMC without PMA *versus* that from SM or HDs' PBMC; TNF-α from patients' or HDs' PBMC with PMA *versus* that from SM; IFN-γ from SM with PMA *versus* that from HDs' PBMC; IL-8 from HDs' PBMC with PMA *versus* that from SM.

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Table 2. Cytokine production by the secretor T cell lines (TCL)

Disease	Sites (total TCC)	IL-6		IL-1α		TNF-α		IFN-γ		IL-8	
		Medium	PMA	Medium	PMA	Medium	PMA	Medium	PMA	Medium	PMA
RDC	FH (53)	80 ± 150* (20)	54 ± 82* (33)	8·1 ± 9·7 (13)	18 ± 54 (16)	9·8 ± 9·1 (8)	174 ± 439 (45)	134 ± 203 (11)	882 ± 1388 (33)	397 ± 1584 (38)	2116 ± 5504 (38)
RDC	SM (29)	58 ± 120* (13)	61 ± 134 (20)	3·0 ± 1·2 (4)	2·8 ± 0·9 (8)	5·3 ± 0·6 (2)	93 ± 132 (17)	42 ± 29 (7)	872 ± 539 (15)	118 ± 127 (17)	715 ± 665 (17)
RDC	PBMC (46)	14 ± 9·7* (18)	25 ± 22 (29)	3·5 ± 1·7 (4)	21 ± 21 (4)	84 ± 124* (13)	236 ± 228 (42)	29 ± 35 (8)	661 ± 836 (35)	84 ± 111 (39)	1253 ± 4916 (39)
HD	PBMC (22)	7·6 ± 3·5 (5)	25 ± 35 (17)	0·0 ± 0·0 (0)	22·0 (1)	5·3 ± 0·6 (2)	290 ± 346 (22)	15 ± 13 (2)	414 ± 334 (17)	121 ± 206 (22)	2190 ± 2450* (22)

The means ± s.d. (pg/mg) of the values of the cytokines by the secretor TCL in each group are shown. Values in parentheses represent the numbers of the secretor TCL. The level of IL-6 by the TCL of femoral head (FH) without ($P < 0·01$) or with ($P < 0·05$) phorbol myristate acetate (PMA) was significantly higher than that of patients' or healthy donor (HD)s' peripheral blood mononuclear cells (PBMC). That of synovial membrane (SM) ($P < 0·04$) or patients' PBMC ($P < 0·02$) without PMA was significantly higher than that of HDs' PBMC. The level of tumour necrosis factor-alpha (TFN-α) by the TCL of patients' PBMC without PMA was significantly higher than that of the other groups ($P < 0·05$). The level of IL-8 by the TCL of HDs' PBMC with PMA was significantly higher than that of SM ($P < 0·03$).

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Table 3. Kinetic study of IL-6 and tumour necrosis factor-alpha (TNF-α) production by the secretor T cell lines (TCL)*

	IL-6 (pg/ml)			TFN-α (pg/ml)		
Stimulus	Day 8	Day 15	Day 22	Day 8	Day 15	Day 22
Medium alone	5·6 ± 2·1 [32·8 ± 10·2]	9·8 ± 1·7	8·5 ± 4·6	7·2 ± 4·4 [8·2 ± 3·9]	11·8 ± 3·4	8·0 ± 2·6
PMA	75·8 ± 2·9 [73·6 ± 29·1]	41·0 ± 7·9	102 ± 61	704 ± 593 [508 ± 1100]	659 ± 617	533 ± 539
PMA plus PHA	87·9 ± 1·0	51·9 ± 1·2	114 ± 80	1787 ± 873	1534 ± 823	1170 ± 606
PMA plus ionomycin	87·1 ± 2·9	89·7 ± 3·9	159 ± 88	1184 ± 269	1525 ± 737	1643 ± 405

*Four different TCL derived from femoral head (FH 1–43, FH 1–58, FH 2–83, and FH 3–71) were thawed in the morning of experiments in the presence of feeder cells. These TCL had produced IL-6 and TNF-α before cryopreservation, and their mean values are shown in square parentheses. These TCL were incubated for up to 30 days in the medium plus IL-2 alone. TCL were harvested at day 8, day 15, and day 22, washed three times and were cultured with medium alone, with phorbol myristate acetate (PMA; 1 ng/ml), with PMA plus phytohaemagglutinin (PHA; 10 μg/ml), or with PMA plus ionomycin (0·5 μg/ml). After overnight incubation, cell-free supernatants were provided for measurement of IL-6 and TNF-α. Values represent mean ± s.d. of cytokine by these TCL. All the TCL died later than day 28 under these culture conditions, and therefore measurement was not carried out.

secretor TCL in medium alone were mostly consistent throughout experiments at different incubation periods. Those in the presence of PMA or the other stimuli were also mostly consistent throughout experiments, although the levels of IL-6 or TNF-α production with PMA plus PHA or PMA plus ionomycin were higher than those with PMA alone, which in turn were higher than those with medium alone. A non-secretor TCL (FH-2-62) failed to produce either IL-6 or TNF-α in medium alone at day 8 or day 15, while it produced a higher level of IL-6 or TNF-α in PMA, PMA and PHA or PMA and ionomycin (data not shown).

Cytokine levels in plasma

The amounts of cytokines in the plasma of RDC patients were compared with those of patients with RA, osteoarthrosis (OA), trauma, and healthy donors (Table 4). Detectable levels of IL-6, TNF-α and IFN-γ were observed in the plasma from most RDC patients. IL-1α or IL-1β was detectable in plasma from five of 11 or one of eight patients with RDC. None of the cytokine levels in the plasma of RDC patients except IFN-γ was significantly different from those from patients with OA, trauma, or healthy donors in most cases. The level of IFN-γ in the plasma of RDC or trauma patients was somewhat lower than that of healthy donors.

In contrast to RDC, significantly higher levels of IL-6 ($P < 0{\cdot}02$ *versus* the other groups), TNF-α ($P < 0{\cdot}01$ *versus* trauma) and IL-8 ($P < 0{\cdot}01$ *versus* healthy donors) were observed in plasma from patients with RA. The level of IL-8 in the plasma of trauma patients was higher than that from healthy donors.

Table 4. Cytokines in plasma

Disease	IL-6	IL-1α	IL-1β	TNF-α	IFN-γ	IL-8
RDC	60 ± 128 [11/11]	11 ± 30 [5/11]	44 ± 123 [1/8]	106 ± 208 [8/11]	370 ± 242 [10/11]	443 ± 1256 [5/9]
RA	300 ± 325* [11/11]	6·7 ± 12 [5/11]	38 ± 57 [7/11]	749 ± 1048* [11/11]	1077 ± 1641 [10/11]	883 ± 835* [11/11]
OA	18 ± 30 [2/4]	5·0 ± 5·8 [2/4]	0·0 ± 0·0 [0/4]	47 ± 43 [3/4]	488 ± 636 [4/4]	153 ± 250 [3/3]
Trauma	65 ± 77 [8/9]	7·1 ± 12 [6/10]	30 ± 64 [6/10]	78 ± 74 [9/10]	282 ± 252 [10/10]	2144 ± 2371* [8/8]
HD	49 ± 85 [5/13]	72 ± 187 [8/13]	0·0 ± 0·0 [0/3]	130 ± 112 [12/13]	2197 ± 1967* [13/13]	66 ● 96 [8/13]

Plasma from 11 patients with RDC, 11 patients with rheumatoid arthritis (RA), four patients with osteoarthritis (OA), 10 patients with trauma, and 13 patients with healthy donors (HD). Values in square parentheses represent the number of positive samples/total samples. *$P <$ at least 0·03 *versus* the other group as follows: the level of IL-6 by RA *versus* that of any other group; tumour necrosis factor-alpha (TNF-α) by RA *versus* that of any other group; IFN-γ by HDs' *versus* that of RDC, OA, or trauma; IL-8 by trauma *versus* that of RDC, OA, or HD; IL-8 by RA *versus* that of HD.

DISCUSSION

This study demonstrates that TCL from the femoral head of RDC patients produced a significantly larger amount of IL-6 than that from patients' or healthy donors' PBMC. IL-1α production by the TCL of the femoral head was significantly higher than that of any of the other groups when only the secretor TCL were provided for analysis. These results suggest that the T cells at the affected femoral head are involved in bone resorption through production of IL-6, and probably IL-1α, that are capable of activating osteoclasts in patients with RDC.

TCL from synovial membrane also produced significantly higher amounts of IL-6 and IL-1α than those produced by the TCL from healthy donors' PBMC, suggesting that the T cells at the synovial membrane are also involved in bone resorption in patients with RDC. Furthermore, the levels of TNF-α and IL-6 produced by the TCL from patients' PBMC were also significantly higher than those from healthy donors' PBMC. The PBMC from patients may contain these IL-6-producing T cells that will either infiltrate into or come from the affected joint. Whether the higher level of TNF-α produced by the TCL from patients' PBMC is causative or resultant of T cell activation at the affected joint is presently unclear.

The level of IL-6 produced by TCL from the femoral head was significantly higher than that from patients' or healthy donors' PBMC in the analyses with both all the TCL and with only the secretors. IL-6 produced by the TCL from synovial membrane or patients' PBMC was also significantly higher than that from healthy donors' PBMC. Therefore, there may be two characteristics of the T cells of RDC patients: there are increased numbers of T cells producing IL-6, and these T cells produce higher amounts of IL-6 per cell compared with those of healthy donors' PBMC. In contrast, IL-1α produced by secretor TCL from the femoral head was significantly higher than that from the other sites in the analysis only with all the TCL, but not with the secretors alone. The numbers of TCL producing IL-1α from the femoral had were much larger than those from the other three groups. These IL-1α-secreting TCL were also found in synovial membrane and patients' PBMC, but not in healthy donors' PBMC. These results suggest an increase in the number of T cells capable of producing IL-1α in RDC patients in their affected femoral head.

The mechanisms associated with the fact that the RDC affects mostly middle-aged and elderly women are not understood. IL-6 is suggested to be a crucial cytokine for the regulation of bone remodelling, primarily through osteoclast development and their activation [8,10]. IL-6 seems to be involved in the increased osteoclastogenesis associated with post-memopausal eostrogen deficiency [18]. Unlike other cytokines, large amounts of IL-6 appear to be produced by bone cells in bone organ culture in response to osteotropic hormones such as parathyroid hormone, IL-1 and 1α, 25-dihydroxy-vitamin D_3 [10]. Although this study demonstrates IL-6 production by T cells at the affected femoral head, it does not exclude either the production of a large amount of IL-6 by bone cells, or their involvement in osteoclast activation. Instead, bone cells may produce a large amount of IL-6 partly in response to IL-1α produced by the T cells at the disease site. None the less, these results suggest that IL-6 is one of the crucial cytokines in the development of RDC in middle-aged and elderly women.

IL-1 was the first cytokine identified for its effect on osteoclastic bone resorption [11]. It is a very powerful bone resorption stimulator [10]. IL-6 at the level of 10–100 pg/ml could significantly increase the formation of osteoclast-like multinuclear cells *in vitro* [7,9]. On the other hand, the bone-resorbing activity of IL-1α at the same concentration was 200 times greater than that of IL-6 [8]. Therefore, the levels of both IL-6 and IL-1α produced by the TCL of the femoral head may be sufficient for the activation of osteoclasts. IL-1α and IL-1β have an identical effect on bone resorption [19]. IL-1 stimulates IL-6 production by monocytes, fibroblasts, T cells [9], and osteoblasts [8,10]. Furthermore, IL-1 belongs to a group of cytokines (IL-6, IL-1 and TNF-α) with overlapping biologic properties, including endogenous pyrogenicity, induction of acute-phase proteins and T and B cell activation [11]. These findings suggest that IL-1 is another crucial cytokine in the development of RDC. TNF-α is also a known participant in bone resorption as an osteoclast-activating factor (OAF) [10].

The roles of IFN-γ on the regulation of bone remodelling are not yet understood. Although IFN-γ is a crucial cytokine for activation of T cells, natural killer (NK) cells and macrophages [20], it inhibits IL-1-stimulated bone resorption [15]. Therefore, the observed lower levels of IFN-γ in the circulation may facilitate IL-1α-mediated bone resorption in patients with RDC. IL-8 may be involved in joint destruction, since it is an important chemoattractant for polymorphonuclear and mononuclear cells, and is involved in many inflammatory reactions, including RA [14]. PMA (1ng/ml), a potent stimulant for inducing production of various cytokines in most T cells [21,22], induced high amounts of IL-6, TNF-α IFN-γ and IL-8 (but not IL-1α) in the majority of TCL regardless of their different origins.

One might consider that functions of TCL established *in vitro* do not reflect those of T cells *in vivo*. However, the TCL established from tumour-infiltrating lymphocytes with the same methods employed in this study displayed MHC-restricted and tumour-specific cytotoxicity, as reported previously [16,23]. The TCL from tumour-infiltrating lymphocytes do not require *in vitro* stimulation with autologous tumour cells for exhibiting tumour-specific cytotoxicity, whereas T cells from PBMC do.

Kinetic study using frozen TCL of femoral head showed that IL-6 or TNF-α production by these secretor TCL was relatively consistent throughout experiments at different time periods after addition of feeder cells. The results suggest that addition of feeder cells did not largely affect the results of IL-6 or TNF-α production by these TCL. However, this result can not deny possible effects of feeder cells as a non-specific stimulus for cytokine production by these TCL. Indeed, most TCL died off within 30 days if feeder cells were not added to the culture, although there were no other available methods to expand TCL from femoral head. Other approaches such as establishment of TCL recognizing autologous bone or synovial cells in a MHC-restricted manner will be taken to resolve this issue. Maximum levels of IL-6 or TNF-α production were achieved by stimulation of TCL with PMA plus ionomycin, as expected.

Levels of IL-6, but not the other cytokines, produced by TCL of femoral head in the presence of PMA were lower than those in the absence of PMA. Although mechanisms involved in this phenomenon are presently unknown, some TCL of

femoral head might be producing a maximum level of IL-6, but not the other cytokines, without any stimuli. Addition of PMA might rather down-regulate IL-6 production. This assumption might be in part supported by the fact that IL-6 production by frozen TCL (5·6 pg/ml) was much lower than that by unfrozen TCL (32 pg/ml) (Table 3).

In contrast to T cell-mediated cytokine production, the plasma levels of all cytokines tested in RDC patients, except for IFN-γ, were not significantly different from those in patients with OA or trauma, or healthy donors. The IFN-γ level in plasma of RDC patients was somewhat lower than that of healthy donors. In contrast, the plasma levels of IL-6, TNF-α and IL-8 in patients with RA were significantly higher than those in patients with RDC or trauma, or healthy donors, in agreement with the results reported previously [14,24]. These results suggest that RDC is not a disease associated with apparent immune disorders in the circulation at the serum level.

The s.d. of some of the mean values in this study is relatively large, even when only the secretor TCL were provided for analysis. This primarily may be due to the fact that the levels of cytokines produced by each T cell largely vary, regardless of different sites in the patients' samples or different donors, including both patients and healthy donors. Different levels of activation may be responsible for this phenomenon.

In conclusion, this study demonstrates that T cells at the affected femoral head and synovial membrane may be involved to some extent in bone resorption through the production of IL-6 and probably IL-1α, and are capable of activating osteoclasts in patients with RDC. However, this hypothesis should be investigated further by examination of the effects of culture supernatants on bone resorption in an established bioassay system. Immunochemical staining of uncultured T cells with anti-IL-6 or -IL-1α antibody will also be necessary.

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REFERENCES

1 Postel M, Kerboull M. Total prosthetic replacement in rapidly destructive arthrosis of the hip joint. Clin Orthop 1970; **72**:138–44.
2 Komiya S, Inoue A, Sasaguri Y, Minamitani K, Morimatsu M. Rapidly destructive arthropathy of the hip: studies on bone resorptive factors in join fluid with a theory of pathogenesis. Clin Orthop Relat Res 1991; **284**:273–82.
3 Bobechko WP. Autoimmune reactions of articular cartilage. Excerpta Med 1973; **291**:18–26.
4 Sweet MBE. Experimental chondrolysis. S Afr Med J 1972; **46**: 465–7.
5 Yoshioka Y, Tsuru S, Yokoyama M, Nomoto K. Experimental study on immunological reactions against the articular cartilage. Int Archs Allergy Appl Immunol 1982; **68**:152–6.
6 Herman JH, Herzig EB, Crissman JD, Dennis MV, Hess EV. Idiopathic chondrolysis: an immunopathologic study. J Rheumatol 1980; **7**:694–705.
7 Roodman GD, Kurihara N, Ohsaki Y *et al.* Interleukin 6 a potential autocrine/paracrine factor in Paget's disease of bone. J Clin Invest 1992; **89**:46–52.
8 Ishimi Y, Miyaura C, Jin CH *et al.* IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990; **145**:3297–303.
9 Kurihara N, Bertolini D, Suda T, Akiyama Y, Roodman D. IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. J Immunol 1990; **144**:4226–30.
10 Mundy GR. Cytokines and growth factors in the regulation of bone remodeling. J Bone Miner Res 1993; **8**:505–10.
11 Dinarello CA. Interleukin-1 and interleukin-1 antagonism. Blood 1991; **77**:1627–52.
12 Gubler U, Chua AO, Stern AS *et al.* Recombinant human interleukin 1α: purification and biological characterization. J Immunol 1986; **136**:2492–7.
13 Helle M, Brakenhoff JPJ, Groot ERD, Aarden LA. Interleukin 6 is involved in interleukin 1-induced activities. Eur J Immunol 1988; **18**:957–9.
14 Endo H, Akahoshi T, Takagishi K, Kashiwazaki S, Matsushima K. Elevation of interleukin 8 levels in fluids of patients with rheumatoid arthritis and induction by IL8 of leukocyte infiltration and synovitis in rabbit joints. Lymph Cytokine Res 1991; **10**:245–52.
15 Gowen M, Mundy GR. Actions of recombinant interleukin 1, interleukin 2, and interferon-γ on bone resorption *in vitro*. J Immunol 1986; **136**:2478–82.
16 Itoh K, Platsoucas CD, Balch CM. Autologous tumor-specific cytotoxic T lymphocytes in the infiltrate of human metastatic melanomas: activation by interleukin 2 and autologous tumor cells, and involvement of the T cell receptor. J Exp Med 1988; **168**:1419–41.
17 Sagawa K, Mochizuki M, Masuoka K *et al.* Immunopathological mechanisms of human T cell lymphotropic virus type 1 (HTLV-1) uveitis. J Clin Invest 1995; **95**:852–8.
18 Jilka RL, Hangoc G, Girasole G *et al.* Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 1992; **257**:1992.
19 Dewhirst FE, Stashenko PP, Mole JE, Tsurumachi T. Purification and partial sequence of human osteoclast-activating factor: identity with interleukin 1β. J Immunol 1985; **135**:2562–8.
20 Robinson BWS, Mclemore TL, Crystal RG. Gamma interferon is spontaneously released by alveolar macrophages and lung T lymphocytes in patients with pulmonary sarcoidosis. J Clin Invest 1985; **75**:1488–95.
21 Patel SS, Duby AD, Thiele DL, Lipsky PE. Phenotypic and functional characterization of human T cell clones. J Immunol 1988; **141**:3726–36.
22 Rotteveel FTM, Kokkelink I, Lier RAWv *et al.* Clonal analysis of functionally distinct human $CD4^+$ T cell subsets. J Exp Med 1988; **168**:1659–73.
23 Itoh K, Salmeron MA, Morita T *et al.* Distribution of autologous tumor-specific cytotoxic T lymphocyte clones in human metastatic melanomas. Int J Cancer 1992; **52**:52–59.
24 Kahle P, Saal JG, Schaudt K, Zacher J, Fritz P, Pawelec G. Determination of cytokines in synovial fluids: correlation with diagnosis and histomorphological characteristics of synovial tissue. Ann Rheumat Dis 1992; **51**:731–4.