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Soluble CD8 in patients with rheumatic diseases

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SUMMARY

An ELISA was used to measure soluble CD8 (sCD8) in the sera and synovial fluids (SF) of patients with rheumatic diseases. Patients with rheumatoid arthritis (RA) had raised levels of sCD8 both in their sera and in their SF compared with patients with osteoarthritis and age-matched healthy controls. In individual RA patients, serial serum sCD8 levels initially fell and then rose preceding clinical improvement. In four patients where serum sCD8 levels rose and clinical improvement occurred, subsequent spontaneous decreases of serum sCD8 level preceded increased clinical disease activity by up to 2 weeks. In general, RA SF mononuclear cells (SFMNC) spontaneously produced high levels of sCD8. In contrast, autologous peripheral blood MNC only produced comparable levels after mitogenic stimulation. Incubation of SFMNC with increasing concentrations of human recombinant tumour necrosis factor alpha resulted in a dose-dependent potentiation of sCD8 release into the supernatant. There was an inverse relationship between the ability of SFMNC to release sCD8 and soluble interleukin-2 receptor, indicating that the $CD8^+$ T cell population may play an important immunoregulatory role in RA.

Keywords soluble CD8 rheumatic diseases soluble interleukin-2 receptor tumour necrosis factor

The CD8 and CD4 glycoproteins are non-polymorphic members of the immunoglobulin gene superfamily expressed on the surface of distinct populations of T lymphocytes. Initially it was thought that CD8 and CD4 were markers of phenotypic function, CD8 being the suppressor/cytotoxic subset and CD4 cells being the helper subset. However, while most cytotoxic T cells express CD8, many cells with a helper function also express CD8 (Swain, 1981). Conversely $CD4^+$ T cells can exhibit suppressor and cytotoxic functions (Krensky *et al.*, 1982). It is now known that expression of CD8 and CD4 is associated weakly with T cell function but strongly with MHC restriction. T cells expressing CD8 recognize antigen presented by MHC class I molecules whereas $CD4^+$ T cells recognize antigen in the context of class II MHC (Swain, 1983). The detailed role of CD8 and CD4 in T cell function is at present unclear. They may serve to increase the energy of binding between the antigen presenting cell and the T cell (Norment *et al.*, 1988). However, recent findings indicate that these accessory molecules may also deliver regulatory signals possibly via a specific tyrosine protein kinase (Barber *et al.*, 1989).

Membrane-associated human CD8 protein is composed of two chains—alpha and beta. The CD8 complex is expressed either as an alpha/beta heterodimer or as an alpha homodimer. It appears that expression of the alpha chain is required for cell surface expression of the beta chain (Di Santo, Knowles & Flomenberg, 1988; Shiue, Gorman & Parnes, 1988). In addition to expressing the 34 kD alpha chain on their surface, activated T lymphocytes also release a soluble form of this protein (sCD8) (Fujimoto, Levy & Levy, 1983). The release of this molecule from T cells may occur by two different mechanisms. A labelled 27-kD form of CD8 is released from T cells following cell surface iodination and probably reflects proteolytic cleavage at the cell surface (Fujimoto, Stewart & Levy, 1984). Additionally, alternative splicing of the primary CD8 transcript excluding nucleotide sequences from exon 4, results in the synthesis of a 30-kD protein that has no transmembrane domain and is, therefore, secreted (Giblin, Ledbetter & Kavathas, 1989). This transcript represents approximately 15% of the total CD8 alpha mRNA in normal human tissues (Norment *et al.*, 1989).

Extensive analysis of T cell subsets has been performed in active rheumatoid arthritis (RA) (Duke *et al.*, 1983; Throen *et al.*, 1989). While immunophenotyping and immunohistology of cell subsets can give valuable information regarding numbers and distribution in pathological tissue it does not provide functional information about cellular subsets. The level of sCD8 has been shown to correlate with $CD8^+$ T cell activation both *in vitro* and *in vivo* (Tomkinson *et al.*, 1989). Therefore, as the serum and synovial fluid levels of sCD8 probably reflect the

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activity of this T cell subset *in vivo*, we have measured sCD8 levels in cross-sectional and longitudinal studies to assess $CD8^+$ T cell activity during the course of active RA.

MATERIALS AND METHODS

Patients

Synovial fluid from knee joints (therapeutic aspirations) and simultaneous peripheral blood samples were obtained from 67 outpatients (aged 50·3 ± 4·6 years; mean ± s.e.m.) with classical or definite RA according to the American Rheumatism Association criteria (Ropes *et al.*, 1958) or from 16 patients with osteoarthritis (OA) (mean age 63·8 ± 6·1 years). All patients were receiving non-steroidal anti-inflammatory drugs (NSAIDs). In addition, one patient received prednisolone (5 mg/day), one received penicillamine, four received sulphasalazine, and two patients received hydroxychloroquine. The control population consisted of age- and sex-matched healthy volunteers (mean age 48·2 ± 3·5 years).

Longitudinal studies were performed on eight patients admitted to hospital with active RA. Clinical assessment of disease activity was performed by the same physician at 3-day intervals. The following standardized measures were used: Ritchie articular index; duration of morning stiffness (EMS); patients' pain score; and physician's global assessment of disease activity. Concurrent laboratory assessment was performed by haemoglobin concentration, total leucocyte count, platelet count and erythrocyte sedimentation rate (ESR). 'Onset of clinical improvement' was defined as the day when Ritchie articular index was below 10; ESR < 20 mm/h; and EMS < 30 min (Wood, Symons & Duff, 1988).

Synovial fluid and serum

Synovial fluid effusions were taken into EDTA tubes and blood into dry glass tubes for serum collection. To remove cells from the synovial fluid and sera, samples were centrifuged at 10 000 ***g***, aliquoted, and stored at −20°C until used.

Isolation of peripheral blood mononuclear cells (PBMNC) and synovial fluid MNC

These were isolated as previously described (Symons *et al.*, 1989). The cells were resuspended at 2×10^6/ml in serum-free RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM: Gibco, Paisley, UK). The cells were then distributed in 200-μl aliquots in 96-well microtitre plates (Costar; Northumbria Biologicals, UK). PBMNC were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere for 24, 48 and 72 h with or without a previously determined optimal concentration of concanavalin A (Con A) (2·5 μg/ml; Sigma). Synovial fluid MNC were maintained for the same time periods in the absence of mitogen. At the end of the culture, the medium was aspirated, centrifuged at 10 000 ***g***, and stored at −20°C until tested. To assess the effect of TNF alpha on the release of CD8 antigen from synovial fluid MNC, cells were cultured for 72 h either in media alone or with human recombinant (Hr) tumour necrosis factor-alpha (TNF-α) (up to 10 ng/ml; a generous gift of Dr G. Adolf, Boehringer Institute, Vienna, Austria). At the end of the culture, cell-free supernatants were obtained by centrifugation at 10 000 ***g*** and stored at −20°C until used.

ELISA for sCD8

To assay sCD8 in synovial fluid, sera and culture supernatants, an ELISA utilising two non-competing murine monoclonal antibodies (MoAbs) to the alpha chain of the CD8 protein was used (T Cell Sciences, Cambridge, MA, USA; Biological Industries, Cumbernauld, UK). Initially, 96-well microtitre plates (Nunc; Maxisorp; Paisley, UK) were coated for 48 h at 4°C with 100 μl of the first MoAb against the human CD8 alpha chain protein. The plates were then washed three times with PBS/0·2% (v/v) Tween 20, and non-specific binding sites were blocked by a 2-h incubation at 37°C with PBS/0·2% (v/v) Tween 20/1% (w/v) BSA. After washing, 100 μl of sample diluent (buffered-serum protein) was added to all wells except those used for blanking. Subsequently, 10 μl of freshly thawed sample were added to duplicate wells and incubated for 90 min at 37°C. Samples were then discarded, the plate washed, and then each well received 100 μl of horseradish peroxidase-conjugated MoAb that recognizes a different epitope on the CD8 alpha chain. After another 90 min at 37°C plates were washed and incubated with 100 μl of *O*-phenylenediamine (0·5 mg/ml) in citrate-phosphate buffer containing 0·01% (v/v) hydrogen peroxide. After a 30-min incubation at room temperature in the dark, colour was developed by the addition of 50 μl of 2N H_2SO_4 and the plates read immediately at 490 nm in a Dynatech MR700 microplate reader. Units of sCD8 were calculated from a standard curve constructed with serial dilutions of a supernatant from a $CD8^+$ human leukaemic T cell line (T Cell Sciences) designated 2000 U/ml. Samples were assayed on two separate occasions, interassay variation was 12 ± 4% (mean ± s.e.m.). To confirm that the ELISA was suitable for the detection of sCD8 in synovial fluid, serum, and culture supernatant samples were spiked with known amounts of standard sCD8 and assayed at various dilutions. Recovery was 82 ± 4% in synovial fluid, 95 ± 3% in serum, and 98 ± 2% in culture supernatant. All samples were coded and read blind in the assay. The ELISA was checked for reactivity with human anti-human IgG (IgM; rheumatoid factor). At 300 μg/ml (titre 1:2048; SRBC agglutination test) the rheumatoid factor preparation exhibited no reactivity in the ELISA.

ELISA for soluble interleukin-2 receptor (sIL-2R)

Mononuclear cell supernatants from synovial fluid cell cultures were also assayed for the presence of the sIL-2R (T Cell Sciences). This ELISA detects the soluble form of the p55, alpha chain or Tac protein of the two-chain human IL-2R. The ELISA was performed as previously described (Symons *et al.*, 1988).

RESULTS

Serum and synovial fluid sCD8 levels in patients and controls

Thirty-three patients with seropositive RA had a mean serum sCD8 of 534 ± 52 U/ml (mean ± s.e.m.) (Fig. 1). This was significantly higher than in a healthy age-matched control group (356 ± 49 U/ml; $P < 0.001$). In paired synovial fluid effusion samples from these patients the sCD8 levels were higher (677 ± 20 U/ml). A similar pattern was seen in a group of 29 seronegative (lacking serum anti-Ig) RA patients except that synovial fluid levels were similar to paired serum levels. In a group of 16 OA patients serum sCD8 levels were not significantly different from age-matched controls. However, OA synovial fluid levels were significantly lower (220 ± 41 U/ml;

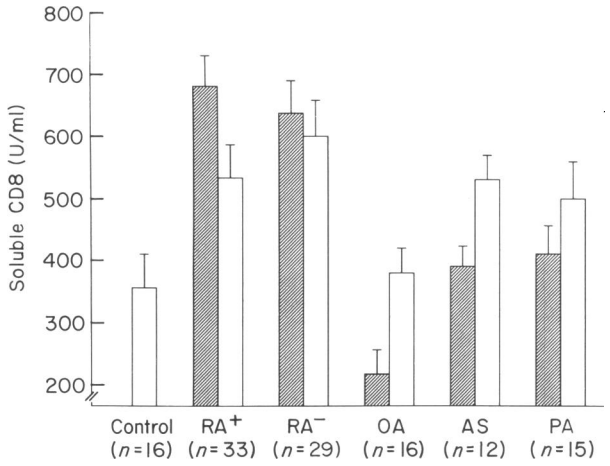

Fig. 1. Serum and synovial fluid sCD8 levels in patients and controls. Paired sera (open bars) and synovial fluid samples (hatched bars) obtained from patients with RA, osteoarthritis (OA), ankylosing spondylitis (AS) and psoriatic arthritis (PA) and control sera were assessed for sCD8 level by ELISA. Data are mean ± s.e.m.

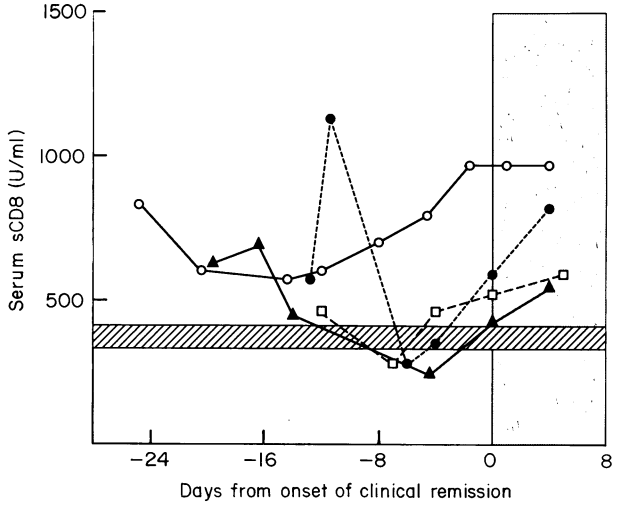

Fig. 2. Serial sCD8 serum levels in four patients (○, ▲, ● and □) with active rheumatoid arthritis. The first sCD8 measurement for each patient was on the day of admission to hospital. 'Onset of clinical improvement' is defined in methods section and is indicated by the shaded region. Mean (± s.e.m.) serum sCD8 concentration in 16 healthy age-matched controls is indicated by cross-hatched bar.

Table 1. Production of sCD8 by synovial fluid and peripheral blood mononuclear cells (PBMNC) from patients with rheumatoid arthritis

	sCD8 (U/ml)		
Patient no.	Synovial fluid	PBMNC	PBMNC + Con A
1	1960	380	1200
2	1400	325	930
3	425	150	400
4	1250	155	2200
5	940	355	1010
6	1880	145	1880
7	1250	250	730
8	250	250	750
Mean ± s.e.m.	1169 ± 219	251 ± 32	1137 ± 203

$P < 0{\cdot}02$) than the corresponding serum levels. In two inflammatory rheumatic diseases, ankylosing spondylitis and psoriatic arthritis serum levels of sCD8 were higher than the control population (527 ± 42 U/ml; $P < 0{\cdot}001$ and 504 ± 57 U/ml; $P < 0{\cdot}001$). Synovial fluid samples from these patients were, however, not significantly different from the serum level of the control population.

Cellular origin of synovial fluid and serum sCD8

MNC from the synovial fluid and peripheral blood of eight patients with RA were cultured for 48 h (previously determined as optimal) and the cell-free supernatants assayed for sCD8 content (Table 1). Synovial fluid MNC cultured for 48 h spontaneously produced high levels of sCD8. The autologous PBMNC spontaneously produced low levels of sCD8 (251 ± 39 U/ml) that did not increase with prolonged culture periods (up to 72 h, data not shown). In contrast, the same cells stimulated for 72 h in the presence of an optimal concentration of Con A, released levels of sCD8 comparable with the level of spontaneous release by synovial fluid MNC. Synovial fluid MNC from two patients (3 and 8) spontaneously produced relatively low levels of sCD8. PBMNC from these patients spontaneously produced low levels of sCD8 and in one case (patient 3) also responded poorly to Con A.

Serial sCD8 levels and clinical improvement

Initial serum sCD8 level in 10 patients requiring hospital admission with active RA was 730 ± 92 U/ml (mean ± s.e.m.). This was significantly higher than in 16 age-matched healthy controls ($P < 0{\cdot}001$). Figure 2 shows the serial serum sCD8 levels in four of the 10 patients. On admission, serum sCD8 levels were elevated compared with the control population. Following treatment, serum sCD8 levels in three of the four patients fell into the normal range. However, in all cases as the patients entered clinical remission the serum sCD8 levels began to rise, achieving similar levels to those seen on admission.

Serial sCD8 levels and clinical exacerbation

Figure 3 shows the serial serum sCD8 levels in four patients who following initial clinical improvement exhibited a subsequent clinical exacerbation of RA (defined as EMS > 30 min, ESR > 20 mm/h and Ritchie score > 10). In one patient serum sCD8 levels decreased over the first 11 days of treatment, and as the patient entered clinical remission serum sCD8 levels began to rise. In three other patients serum sCD8 levels began to rise after admission to hospital and continued to rise as the patients improved clinically. Subsequently, while the four patients were in clinical remission their serum sCD8 levels began to fall. This decline was followed by a marked exacerbation of disease activity as defined by Ritchie index, ESR and increased morning joint stiffness.

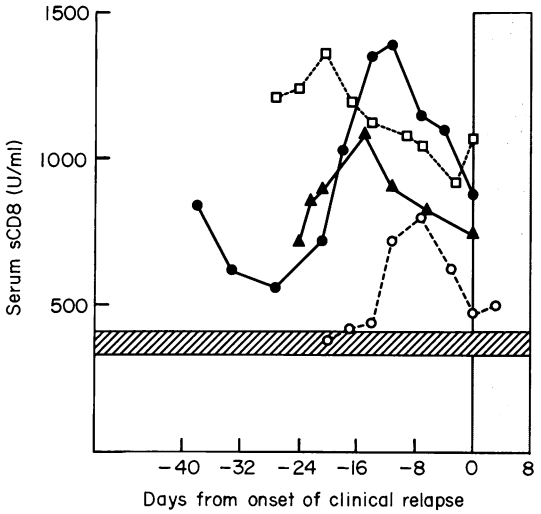

Fig. 3. Serial sCD8 serum levels in four patients who relapsed following a period of clinical improvement. Clinical relapse is defined by: increase in Ritchie index > 10; increase in ESR > 20 mm/h; increasing EMS > 30 min and indicated by the shaded region. Mean (±s.e.m.) serum sCD8 concentration in 16 healthy age-matched controls is indicated by cross-hatched bar.

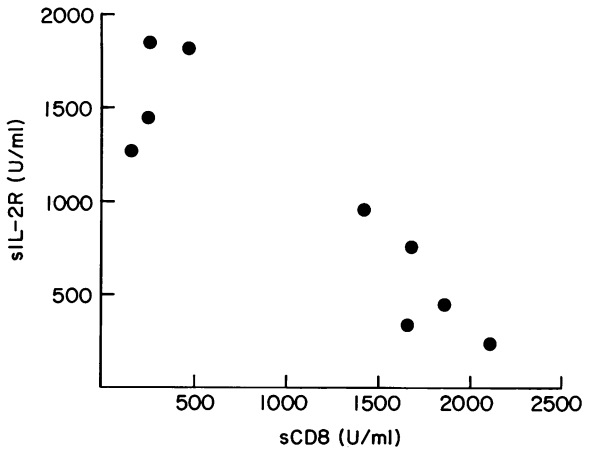

Fig. 4. Release of CD8 and interleukin-2 receptor (IL-2R) from synovial fluid MNC (SFMNC). SFMNC were isolated from patients with RA and cultured at 2×10^6/ml for 48 h in serum-free RPMI 1640. Cell-free supernatants were assayed for sCD8 and soluble IL-2R (sIL-2R) by ELISA.

Table 2. TNF-α stimulation of sCD8 release from synovial fluid mononuclear cells

Patient no.	sCD8 (U/ml) media	Increase in sCD8 release (%)* TNF-α (ng/ml) 10	1·0	0·1	0·01
1	2710	15	2	—	—
2	1620	63	42	36	ND
3	170	90	62	50	42
4	175	48	32	15	20

* Increase in sCD8 release over media control culture.
ND, not determined.

Induction of sCD8 release from synovial fluid MNC by TNF-α

Comparison of RA synovial fluid sCD8 levels with synovial fluid levels of the cytokines IL-1α, IL-1β, TNF-α, IL-2, sIL-2R and IL-6 using specific immunoassays revealed a weak correlation only with synovial fluid levels of TNF-α ($r = 0{\cdot}356$, $n = 27$, $P < 0{\cdot}1$, TNF-α RIA; IRE-Medgenix). As TNF-α potentiates $CD8^+$ T cell function (Ranges *et al.*, 1987) we incubated synovial fluid MNC from RA patients with increasing concentrations of human recombinant TNF-α for 48 h and measured the potentiation of CD8 release into the media. The results in Table 2 show that (as noted previously) MNC from synovial fluid of patients with RA spontaneously released measurable levels of CD8 into the media. As before, synovial fluid MNC either produced high levels (patients 1 and 2) or relatively low levels (patients 3 and 4). However, in all cases addition of increasing concentrations of human recombinant TNF-α caused a dose-dependent increase in the release of the CD8 antigen into the media.

Relationship between release of CD8 and IL-2R from synovial fluid MNC isolated from patients with RA

Synovial fluid MNC isolated from nine patients with RA were cultured for 48 h and the spontaneously produced sCD8 and sIL-2R were assayed by ELISA. The results (Fig. 4) show that the MNC from synovial fluid of different patients behaved in one of two ways. One type of response produced high levels of sIL-2R in the supernatant (> 1200 U/ml) while the sCD8 level was relatively low (< 500 U/ml). In contrast, the second population produced low levels of sIL-2R (< 1000 U/ml) and high levels of sCD8 (> 1400 U/ml).

DISCUSSION

The presence of the soluble form of the CD8 alpha chain has been described previously in sera and also in culture medium from activated human T cells (Fujimoto *et al.*, 1983). In this paper we describe the retention of sCD8 in synovial exudate fluid and sera from patients with rheumatic diseases. Serum levels in RA patients were significantly raised compared with patients with non-inflammatory joint disease (OA) and age-matched, disease-free controls. Within the RA patients, synovial fluid levels in seropositive patients were significantly higher than serum levels. In seronegative RA patients synovial fluid levels were generally higher than corresponding serum levels, compatible with proliferating synovial tissue being a source of the sCD8 detected in the circulation. Consistent with this is the high spontaneous production of sCD8 in freshly isolated MNC from RA synovial exudate (Table 1). Similar levels could only be achieved by autologous blood MNC after stimulation with lectin.

We also studied the levels of sCD8 in three other arthritic conditions, OA, ankylosing spondylitis and psoriatic arthritis. Serum sCD8 levels in patients with OA were similar to the age-matched control population; interestingly, OA synovial fluid levels were significantly lower than the corresponding serum levels. Serum sCD8 levels in ankylosing spondylitis and psoria-

tic arthritis were also elevated compared with the control population. However, the synovial fluid levels were lower than the corresponding serum levels indicating that the inflamed knee joints that were aspirated may not have been the major source of the serum sCD8 protein.

In prospective sequential studies of individual patients with RA, serum levels of sCD8 on admission to hospital were elevated compared with healthy controls. Disease activity was monitored both by patients' symptoms and by objective clinical and laboratory measurements. As patients' disease activity diminished, so serum sCD8 levels fell into the normal range. However, as patients passed into clinical remission the sCD8 levels exhibited a secondary rise that was maintained until discharge from hospital. In a second group of patients who, following initial clinical improvement, exhibited a subsequent clinical relapse, serum sCD8 levels again showed an initial increase as the patients improved. However, in these patients serum sCD8 began to fall and in each case a subsequent clinical exacerbation occurred. In both groups the changes in serum sCD8 preceded the changes in clinical status suggesting that this was not a secondary event reflecting clinical disease activity but was more likely to be related to the activation of immunopathogenic mechanisms that produce inflammation. Although admission levels of sCD8 were high, in general a rising serum sCD8 was associated with onset of clinical remission whereas falling levels were associated with the onset of clinical exacerbation.

Different results were obtained when the same samples were tested for sIL-2R (Wood *et al.*, 1988) where sIL-2R levels fell before clinical remission and rose preceding exacerbation. Therefore, we examined the relationship between synovial fluid MNC production of sCD8 and sIL-2R. The results revealed two populations of patients, synovial fluid MNC from one population produced high levels of sCD8 and relatively low levels of sIL-2R, whereas the other population produced low levels of sCD8 and high levels of sIL-2R. Taken together, the data indicate that the size or activity of the $CD8^+$ T cell population in the rheumatoid synovium is inversely related to the activity of $IL\text{-}2R^+$ mononuclear cells. The observed results could result from fluctuations in the activity of two different populations of mononuclear cells. One that is largely $CD8^+$ mediating remission, while a population expressing high levels of IL-2R alpha chain mediates an inflammatory response resulting in an increase in disease activity. As the IL-2R is expressed on the majority of activated mononuclear cells, exacerbation of disease activity may result from a generalized inflammatory reaction that can be suppressed by a $CD8^+$ T cell. Conversely, the inverse relationship between the levels of sCD8 and sIL-2R may reflect the maturation of a single subset of mononuclear cells that mediates the disease within the joint. It is also possible that subpopulations of $CD8^+$ T cells may differ in their regulation of the splicing of the CD8 alpha transcript expressing either more or less CD8 in soluble form. It is known that within the CD8 T cell population there is marked heterogeneity with respect to cytokine requirements. A subset of $CD8^+$ T cells, termed helper-independent, are capable of producing IL-2 and proliferating in response to antigen without the co-operation of the $CD4^+$ subset. In contrast to other cytotoxic $CD8^+$ cells these cells do not require exogenous IL-2 and express IL-1 receptors on their cell surface (Klarnet *et al.*, 1989).

Immunophenotyping of T cell subsets has been performed in the peripheral blood, synovial fluid and synovial membrane of patients with RA. It is generally agreed that synovial membrane contains an excess of $CD4^+$ lymphocytes relative to $CD8^+$ cells (Duke *et al.*, 1983; Thoen *et al.*, 1989). However, the synovial fluid of patients with RA has been shown to contain high numbers of $CD8^+$ T cells. It has been suggested that this represents selective passage of $CD8^+$ cells from the synovium because these cells do not interact with dendritic cells to the same extent as $CD4^+$ T cells (Duke *et al.*, 1982). Controversy exists on the CD4/CD8 ratio in the PB of patients with RA. Some studies have found a high CD4/CD8 ratio associated with increased disease activity that returns to normal levels with treatment (Veys *et al.*, 1981). However, others have found the CD4/CD8 ratio to be within the normal range during active RA (Thoen *et al.*, 1989).

TNF-α has been shown to potentiate $CD8^+$ T cell function *in vivo* and *in vitro* (Ranges *et al.*, 1987; Asher, Mule & Rosenburg, 1989) and high levels have been found in synovial joints both by bioassay and immunoassay (di Giovine, Nuki & Duff, 1988; di Giovine *et al.*, 1988). We investigated the relation between synovial fluid sCD8 and levels of TNF-α. The results showed that levels of sCD8 were weakly correlated only with TNF-α levels in RA synovial fluid. We extended this observation by culturing synovial fluid MNC with human recombinant TNF-α and found that TNF potentiated the release of sCD8 from these cells. TNF-α has been demonstrated in RA synovial fluid by bioassay and immunoassay but was unrelated to markers of disease activity (di Giovine *et al.*, 1988).

Few reports are available on the levels of sCD8 in other diseases. An increased serum level of sCD8 in childhood Hodgkin's disease seems to dictate a poor prognosis (Pui *et al.*, 1989). Acute infectious mononucleosis induced by Epstein–Barr virus is associated with high levels of serum CD8 that progressively diminish with the resolution of the disease. Levels of sCD8 closely followed the changes in the proportion of T cells that were CD8/DR positive (Tomkinson *et al.*, 1989).

Interestingly, CD8 beta chain transcripts have also been found lacking the sequences coding for a transmembrane domain raising the possibility that CD8 beta protein may also be released and form soluble CD8 alpha/beta heterodimers (Norment & Littman, 1988).

Receptor shedding has been demonstrated for several cell surface receptors including insulin, epidermal growth factor, colony-stimulating factor-1 receptor, MHC class I antigen and the IL-2R alpha chain. If, as in the cases of the IL-2R, the cell-free receptor retains ligand affinity it may mediate the down-regulation of the cellular response to the ligand (Kondo *et al.*, 1988; Symons *et al.*, 1988). Whether sCD8 retains the ability to bind class I MHC has not been determined. Clearly, if sCD8 binds to cell surface MHC class I molecules, it may inhibit the interaction of $CD8^+$ T cells with antigen-presenting cells leading to a down-regulation of $CD8^+$ T cell activation or function. This may play a role in the immunopathogenesis of RA where regulatory subsets of $CD8^+$ T cells may be functionally suppressed by high levels of soluble CD8 antigen present in the inflammatory synovium.

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