Phenotypic characteristics of T lymphocytes in the interfacial tissue of aseptically loosened prosthetic joints

BETH HERCUS*, PETER A. REVELL
Department of Histopathology, Royal Free and University College Medical School, IRC in Biomedical Materials and Osteoarticular Research Group, Rowland Hill Street, London NW3, UK

Aseptic loosening of prosthetic joints represents a major cause for revision surgery [1]. Wear particles represent the initial stimulus in the production of a multi-cellular inflammatory membrane at the bone–implant interface. The mechanisms by which this membrane is established and its influence on bone resorption are central to understanding aseptic loosening. T cells have been identified as a component of interface tissue [1, 2] a feature confirmed in this study. Of the 15 cases studied, 12 cases (12/15) stained positive for CD3. T cell infiltration was present throughout the sections with some perivascular clustering. Multiplex PCR (MPCR) testing of eight of the 15 cases for Th1/Th2 cytokines did not show a predominance of either ''type'' of T cell response. Interleukin (IL)-2 mRNA expression was the most common feature (7/8) while IL-4 (6/8), IL-13 (6/8) and IFN-γ mRNA expression (6/8) was also prevalent. IL-5 (4/8) and IL-10 (4/8) mRNA expression was less and IL-12 (3/8) mRNA expression was the least. Recent work has shown furthermore, that activated T cells can directly stimulate osteoclastogenesis through the expression of RANK ligand [3, 4]. However, although staining for RANK ligand was a consistent feature of all cases stained, such RANK ligand expression was limited to endothelial cells. Helper T cells control and develop immune responses, their role in the inflammation seen in aseptic loosening will aid further understanding of this reaction and may also identify key points for therapeutic intervention.
© *2001 Kluwer Academic Publishers*

1. Introduction

The production of wear debris from the articulating surfaces of prosthetic joints and their subsequent migration and deposition between the bone and implant is the initial step leading to aseptic loosening. Aseptic loosening is defined as the loosening of prosthetic joints that is due neither to mechanical failure nor infection [1]. Wear particles of approximately 2–3 µm in size are phagocytosed by tissue resident macrophages. In response, these cells are stimulated to produce inflammatory mediators. This response has been demonstrated both by *in vitro* work and through analysis of human revision tissue. Production of a variety of inflammatory mediators, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α has been shown in response to wear particle challenge of monocytic cells [2, 5–7]. These mediators have a variety of effects including vasodilation, up-regulation of cellular adhesion molecules (CAMs) and increased vascular permeability, all of which encourage the accumulation of immune/inflammatory cells.

Accumulation of these cells at the site of wear particle deposition, and the production of extra-cellular matrix (ECM) by resident fibroblasts leads to the formation of an inflammatory membrane. This membrane is termed the interface membrane [1], although other names include the pseudosynovial interface, and periprosthetic membrane. The interface membrane has furthermore been implicated in contributing to the localized bone resorption that causes aseptic loosening.

As with other disorders that are characterized by bone erosion, aseptic loosening is a result of an imbalance in the normal homeostatic mechanism of bone remodeling. There is an increase in osteoclastogenesis without the reciprocal increase in osteogenesis. The cells responsible for bone resorption are osteoclasts and it is through activation of mature forms of these cells and an increase in maturation of precursor osteoclasts that increased bone resorption is mediated. However, growing evidence has implicated the fusion products of macrophages the foreign body giant cell as possessing the ability themselves to resorb bone [1]. The steps leading to the formation of cells capable of bone resorption represent a key point for therapeutic intervention.

Increasingly T lymphocytes have been identified as cellular constituents of the interface [1, 2]. Previously,

* Author to whom correspondence should be addressed.

TABLE I Defining characteristics of the T_H1 and T_H2 subsets

	Th1 cell	Th2 cell
Defining cytokines	IL-12 IFN-γ	IL-4 IL-10
Immunity	Cell mediated (DTH)	Humoral (allergy)
Effector cells	Macrophage, NK cell	B Lymphocyte, plasma cell, eosinophil
Ig Class	IgG_{2a}	IgE
Cytokines produced	IL-2, IFN-γ, TNF-α/β, GM-CSF, & IL-3	IL-4, -5, -6, -9, -10, -13, TNF-α, GM-CSF, & IL-3.
Inhibitor	IL-10	IFN-γ

they have been confirmed as key cells in the osteopenic disorder, rheumatoid arthritis [8], and are also noted for their role in inflammation [9]. T cells present in the interface are activated, and capable of producing mediators that can influence the response [2]. T cells and in particular T helper (T_H), are responsible for shaping the immune response. Through their production of key mediators, they can polarize the immune response as outlined in Table I. Cell mediated immune responses are supported by T_H1 cells and humoral responses by T_H2. Identifying a predominance if any between the two responses will allow us to begin to understand their role within aseptic loosening.

Furthermore, it has been shown that activated T cells possess the ability to directly stimulate osteoclastogenesis through the expression of RANK ligand (RANKL) [3, 4]. Ligation of RANK expressed on the surface of mature and precursor osteoclasts represents the most potent stimulator of osteoclastogenesis. Knockout mice lacking the gene for either RANK or RANKL display severe osteopetrosis and have stunted growth [3]. T cells and in particular T helper cells (Th) represent key cells in the development and control of any immune reaction. This study aims to begin to identify the phenotype of T cells found within the interface membrane.

2. Experimental procedures

2.1. Sample group

Fifteen cases were randomly selected from cases received between 1996 and 2000 inclusive for immunostaining, eight of these cases were examined using multiplex PCR (MPCR). At the time of retrieval samples were frozen while suspended in isopentane (BDH) to prevent crystallization within the tissue. Samples were first frozen in liquid nitrogen and then transferred and maintained at $-70\,^{\circ}$C.

2.2. Immunostaining

Sections for immunostaining were mounted on polylysine slides (BDH). Sections were dried for 20 min prior to fixation in a mixture of acetone/methanol (50/50 v/v) at $-20\,^{\circ}$C for 10 min. Sections were washed with 1% phosphate buffered saline (PBS) three times for 5 min each time. Sections were first blocked for 30 min with 0.05% bovine serum albumin (Sigma) diluted in PBS (PBS/BSA) and then incubated with the primary antibody diluted in PBS/BSA for 1 h at room temperature. The anti-human mouse monoclonal antibody raised against CD3 (DAKO) at a dilution of 1 in 50 was used to demonstrate T cell distribution within the interface. While the anti-human goat polyclonal antibody raised against RANKL (Autogen Bioclear) at a dilution of 1 in 25 was used to identify its cellular expression. After incubation, slides were washed in PBS as before, and blocked for 20 min using PBS/BSA. Secondary antibodies used were biotinylated anti-mouse IgG (Vector), and biotinylated, and anti-goat IgG (Vector), respectively for the two primary antibodies. Antibodies were diluted in PBS/BSA at a 1 in 100 dilution, the incubation period was 1 h at room temperature. Sections were washed as before in 1% tris buffered saline, pH 7.6 (TBS, Sigma). Sections were then incubated with the conjugate streptavidin alkaline phosphatase diluted in TBS at 1 in a 100 dilution for 1 h. Sections were washed using TBS. The sections were incubated with the substrate for 20–30 min. The substrate was made by diluting one tablet each of Fast Red and Napthol phosphate (Sigma) in 1 ml of distilled water. Sections were washed, counter-stained for 3 min with heamatoxylin, and mounted using aquamount (BDH).

TABLE II RT-PCR reaction mixture quantities

Reagent	Volume per reaction (μl)
RNase inhibitor	0.5
5 × RT buffer	10
DNTPs	20
Reverse transcriptase	1

2.3. Reverse transcription

RNA for use in reverse transcription was extracted using the TRIZOL reagent method (GIBCO BRL). Three water baths were equilibrated to 42, 70 and 95 °C. On ice, 1–2 μg of mRNA was pipetted into an RNase free reaction vial. To this was added sterile water for a final volume of 14.5 μl. To each vial was added 4 μl of random hexamer (50 μM, Bioline). The tubes were incubated at 70 °C for 5 min and then quickly chilled on ice. Reaction mixture made up as stated in Table II, was added to each vial.

The total reverse transcription mixture was incubated at 42 °C for 1 h. At the end of this time, the RT mixture was heated up to 95 °C and quickly chilled on ice.

2.4. Multiplex PCR

The MPCR reactions were set up with test samples and controls using reagents provided in kit (MPCR kit for Human TH1/TH2 Cytokine Set 2, Bio-Stat Research), and in the quantities shown in Table III. All equipment and reagents used were RNase free.

The thermocycle profile is shown in Table IV.

2.5. Visualization of MPCR

Ten microliters of MPCR product or 10 μl of the DNA marker was mixed with 2 μl of loading buffer (BDH). Each 12 μl sample was loaded onto a 2% agarose gel

TABLE III MPCR reaction mixture quantities

Reagent	Volume per assay (μl)
Distilled water	30.5
Buffer	5.0
Primers	5.0
DNA polymerase	0.5
Specimen cDNA	5.0
DNTP	4.0
Mineral oil	50.0

TABLE IV Thermocycler profile

Temp (°C)	Time	Cycles
96	1	
56–58	4	2
94	1	
56–58	2.5	26–30
70	10	1
20	Soak	

(Sigma) containing 0.5 μg/ml ethidium bromide (Sigma). Samples were electrophoresed for 25 min at 150 V. Gels were visualized and photographed under UV light.

3. Results

3.1. T cell

Immunostaining for CD3 a pan T cell marker demonstrated a T cell presence in 12 out of the 15 cases studied. The percentage of the T cell infiltrate varied between cases from 3% to 36% of total cells in the section. The upper limit here is higher than in previous studies [2], but as shown in Table V a high percentage T cell infiltrate was only seen in two cases. In positive cases, T cells were present diffusely throughout the section, showing some clustering around vessels (Fig. 1). In one case (442/99), large non-perivascular clusters of T cells were seen (Fig. 2). T cells were present both in areas where there were deposits of wear debris, and where there were not.

3.2. RANK ligand

RANK ligand is a potent stimulator of osteoclast maturation and activation and has previously been shown to be expressed by activated T cells [3, 4]. All 15 cases stained for RANKL showed positive endothelial staining (Fig. 3), but no positive mononuclear cell staining. Positive endothelial staining was a feature of both small and large vessels.

TABLE V Positive CD3 staining as a percentage of the total cells per section

Case number	Percentage of T cells per section
76/96	12
79/96	11
10/97	10
15/97	16
284/97	33
343/97	10
6/98	0
12/98	0
19/98	14
442/99	36
467/99	5
468/99	3
28/00	0
29/00	12
160/00	6

3.3. Multiplex PCR

MPCR was used to identify if T cells present at the interface were polarized to either a Th1 or Th2 response. Results were inconclusive with respect to this polarization, as neither group of cytokines predominated, a summary of the results is shown in Table VI. IL-2 mRNA was the most commonly expressed (7/8), but IL-4, IL-13 and IFN-γ were also strongly expressed (6/8). IL-5 and IL-10 were only expressed in four cases each, while IL-12 was the least expressed (3/8). Fig. 4 shows the mRNA expression of each cytokine as a percentage of the total number of cases tested.

4. Discussion

T cells are undoubtedly a common constituent of interface tissue, as has been demonstrated in this study and by the work of others [1, 2]. In this study, immunostaining showed that T cell distribution within the interface was diffuse, and did not appear to be restricted to any particular area. A common feature was the clustering of T cells around vessels. This likely represents T cell extravasation into the inflammatory site, before migration within the interface. To date, little work

TABLE VI Results of MPCR for Th1/Th2 cytokines, results are either positive or negative, no further quantitation was performed[a]

Case no	IL-4	IFN-γ	IL-2	IL-5	IL-13	IL-12	IL-10
96/79	+	+	+	−	+	−	+
97/284	−	+	−	−	−	−	−
97/343	+	+	+	−	+	−	+
98/19	−	−	+	+	+	−	+
99/442	+	−	+	+	+	+	−
99/467	+	+	+	−	+	−	+
99/468	+	+	+	+	+	+	−
00/160	+	+	+	+	−	−	−
Total	6	6	7	4	6	3	4
Percentage	75	75	87	50	75	37	50

[a]Percentage of cytokine expression was calculated by dividing the number of cases expressing that cytokine with the total number of cases tested for it.

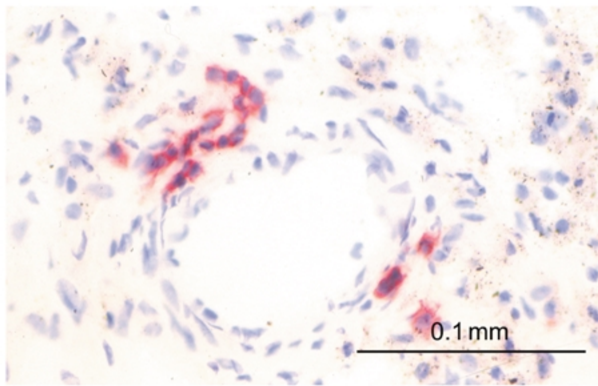

Figure 1 Positive CD3 immunostaining showing clusters of T cells around a vessel in the interfacial tissue.

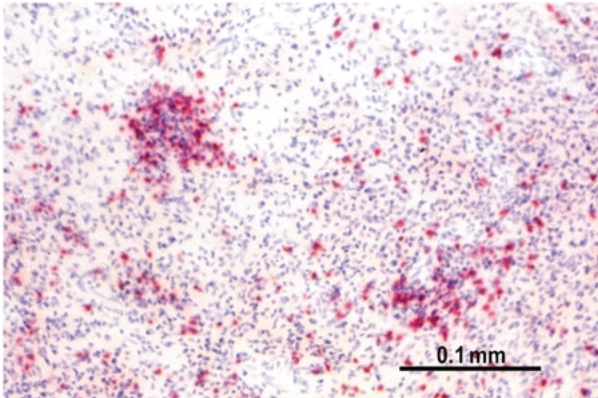

Figure 2 Positive CD3 immunostaining showing large clusters of T cells with no evidence of vacularization.

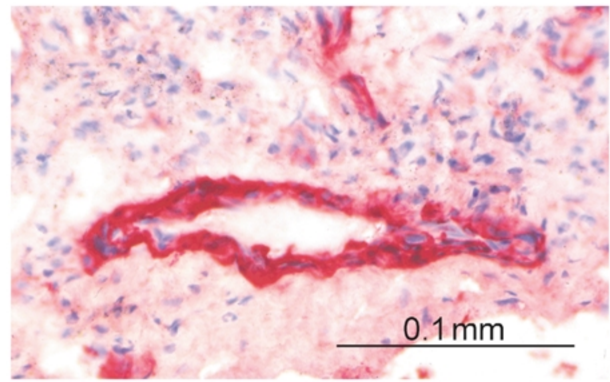

Figure 3 Positive endothelial staining for RANK ligand.

has been carried out to identify the role of the T cell in aseptic loosening. Another study has looked at T cell proliferation within the interface and whether this is polyclonal or oligoclonal, and also assessed IFN-γ production. It was shown that T cell proliferation was mainly oligoclonal and that these cells were functionally activated and transcribing genes for the cytokines IL-2, IFN-γ and in a few cases IL-4 [2].

This study begins to describe the phenotypic characteristics of T cells present within the interface and in particular the helper T cell cytokine profile. In rheumatoid arthritis the predominant T helper response is ''type'' 1 (Th1) [8], and in inflammation both Th1 and Th2 responses contribute [9], although in certain circumstances one or other ''type'' can predominate. Allergic inflammation is associated with a Th2 response while Th1 responses are involved with delayed type hypersensitivity (DTH) reactions. The highly cellular nature of the interface membrane suggests a type 1 response, as T cell production of IFN-γ is the most potent stimulator of macrophage activity. However, this was not confirmed in this study which showed that neither ''type'' of response predominated over the other. MPCR highlighted the expression of IL-2 mRNA in seven of the eight cases examined. This is of interest since IL-2 has been previously undetectable using immunohistochemistry. Failure to demonstrate the expression of the IL-2 protein is likely due to its short half-life.

RANKL expression was not a feature of mononuclear cells, but was highly expressed by endothelial cells. The expression of this ligand has previously been demonstrated, and the lack of expression by T cells in this study, may be due to the high expression of RANKL on the endothelial cells. RANKL expression by endothelial cells has not been described before, and further work will

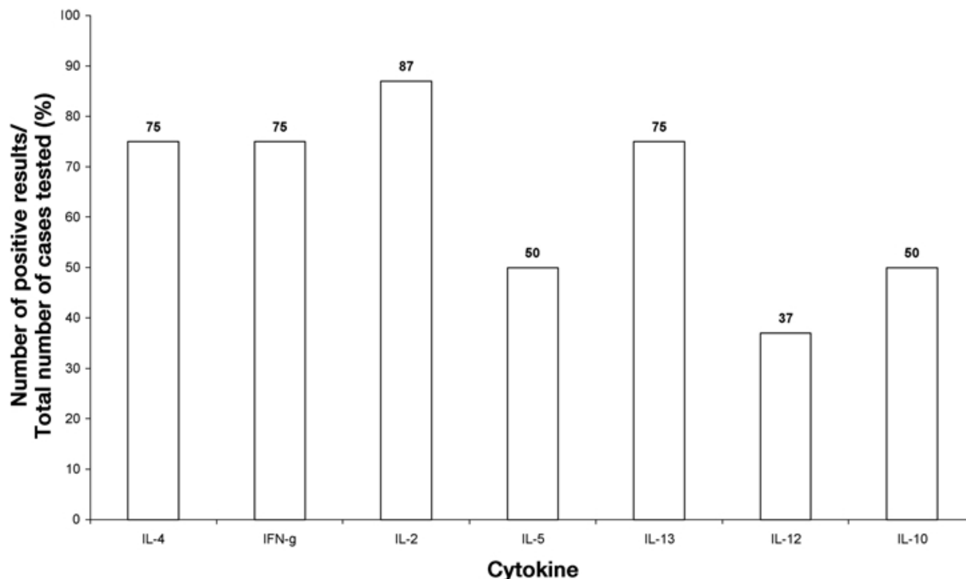

Figure 4 MPCR results showing the expression of each cytokine as a percentage of the total number of cases tested.

be required to investigate its role here in aseptic loosening.

5. Conclusions

The role of T cells within aseptic loosening is as yet undefined, evidence provided by this study and others [1, 2] have shown these cells to be a common component of the interface membrane. Although they are not as prevalent as the major two cell types macrophages and fibroblasts. T helper cells in an immune reaction are responsible for developing that response into either a cell mediated/Th1 or humoral/Th2 response. Cytokines produced by these cells mediate this development through activation or inhibition of different cell types. However, due to the pleiotropic nature of these mediators, their effects on local cell types may differ depending on the environment in which they are acting. T cells are functionally activated in the interface shown here in this study, they are present throughout the interface tissue and therefore capable of influencing most cells present. A strong relationship between Th1 cells and macrophages exist through the production of IFN-gamma; and IL-2, respectively promoting the proliferation and activation of each other. Both cytokines have been demonstrated here as a common feature of cases tested, this relationship may represent a major component of macrophage activation within the interface, and may provide a key point for the attenuation of inflammation in aseptic loosening. More extensive studies are required to expand this field of knowledge.

Acknowledgments

The authors thank the EPSRC for financial support in the form of core support to the IRC in Biomedical Materials and a studentship for B.H.

References

1. N. AL-SAFFAR and P. A. REVELL, *J. Long-Term. Effects. Med. Implants* **9** (1999) 319.
2. C. M. WEYAND, A. GEISLER, A. BRACK, M. E. BOLANDER and J. J. GORONZY, *Lab. Investig.* **78** (1998) 677.
3. Y.-Y. KONG, U. FELGE, I. SAROSI, B. BOLON, A. TAFURI, S. MORONY, C. CAPPARELLI, J. LI, R. ELLIOTT, S. MCCABE, T. WONG, G. CAMPAGNUOLO, E. MORAN, E. R. BOGOCH, G. VAN, L. T. NGUYEN, P. S. OHASHI, D. L. LACEY, E. FISH, W. J. BOYLE and J. M. PENNINGER, *Nature* **402** (1999) 304.
4. D. L. LACEY, E. TIMMS, H-L. TAN, M. J. KELLEY, C. R. DUNSTAN, T. BURGESS, R. ELLIOT, A. COLOMBERO, G. ELLIOT, S. SCULLY, H. HSU, J. SULLIVAN, N. HAWKINS, E. DAVY, C. CAPPARELLI, A. ELI, Y.-X. QIAN, S. KAUFMAN, I. SAROSI, V. SHALHOUB, G. SENALDI, J. GUO, J. DELANY and W. J. BOYLE, *Cell* **93** (1998) 165.
5. J. Y. WANG, B. H. WICKLUND, R. B. GUSTILO and D. T. TSUKAYAMA, *Biomaterials.* **17** (1996) 2233.
6. S. M. HOROWITZ, W. T. LUCHETTI, J. B. GONZALES and C. K. RITCHIE, *J. Biomed. Mater. Res.* **41** (1998) 468.
7. S. STEA, M. VISENTIN, D. GRANCHI, G. CIAPETTI, M. E. DONATI, A. SUDANESE, C. ZANOTTI and A. TONI, *Cytokine* (2000) 1575.
8. M. SALMON and J. S. GASTON, *Br. Med. Bull* **51** (1995) 332.
9. L. S. DE CLERCK, *Acta Clinica Belgica* **52–6** (1997) 388.

Received 14 May
and accepted 21 May 2001