No lymphokines in T-cells around loosened hip prostheses

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ABSTRACT – Research results have been contradictory about the role of lymphocytes and immune response in aseptic loosening of total hip replacement (THR). Conclusive evidence is still lacking in spite of extensive in vivo and in vitro studies. Our study was designed to check whether T-cells were activated and if they produced lymphokines in synovial membrane-like interface tissue around loosened THRs. Tissue sections were stabilized and permeabilized to allow the cytokine-specific antibodies to penetrate through the cell membrane and the membranes of intracellular organelles. This technique, combined with computer-assisted image analysis, permits the detection and quantitation of lymphokine-producing cells. We found that the number of T-cells was low, and none of the T-cells was activated, as shown by the absence of interleukin-2 receptor (IL-2R) immunoreactivity. There was no cell producing lymphokines, such as interleukin-2 (IL-2), interferon-gamma (IFN-γ), and tumor necrosis factor-beta (TNF-β). Our results suggest that T-cell-mediated immune response is not actively involved in aseptic loosening of THR.

Formation of a synovial membrane-like interface tissue is common in aseptic loosening of total hip replacement (THR). The main cell-types in the interface tissue include macrophages, giant cells, and fibroblasts. Lymphocytes usually account for less than 10% of total cells (Goldring et al. 1983, Maloney and Smith 1995). Cytokines and proteinases produced by activated macrophages have been implicated in the loosening process (Lassus et al. 1998, Takagi et al. 1998). In contrast, the effects of immune response are poorly understood. The findings from various groups have been contradictory and conclusive evidence is still lacking about the exact role of T-cells and immune response in this process (Santavirta et al. 1991a, 1993, Gil-Albarova et al. 1992, Sandhu et al. 1998).

Using a routine immunostaining technique, we first examined the expression of CD2 (cluster designation-2), a marker of resting and activated lymphocytes, and IL-2R, a marker of activated lymphocytes. Then we stabilized and permeabilized serial tissue sections to allow cytokine-specific antibodies to penetrate through cell-surface membranes, the membranes of the endoplasmic reticulum, and Golgi organelle. This technique facilitates detection of cytokine-producing cells (Sander et al. 1991). We studied the expression of IL-2, TNF-β and IFN-γ because they are the main cytokines produced by activated lymphocytes, the so-called lymphokines.

Patients and methods

Patients and samples

10 synovial membrane-like interface tissue samples were collected between the prostheses/cement-bone from the patients undergoing revision operations due to aseptic loosening of THRs. Their mean age was 70 (37–85) years, 7 were women. The indication for primary THR was arthrosis. The mean interval from primary THR to revision was 8

(4–20) years. All the revised cases were cemented THR. The femoral stems of these prostheses were made of cobalt-based alloy (Co-Cr-Mo, 7 cases) and titanium-based alloy (Ti-6Al-4V). Cultures for aerobic and anaerobic organisms were routinely taken at revision surgery on interface tissue and pseudosynovial fluid, and all the results were negative. For comparison, 10 synovial membrane samples were collected from patients with rheumatoid arthritis (RA) undergoing primary THR. Their mean age was 44 (38–76) years and 8 were women. These samples were embedded in Tissue-Tek OCT compound and snap-frozen in isopentane precooled in dry ice and stored at –70 °C pending use. 8 serial cryostat sections (6 μm thick) were cut from each sample. The first and last sections were stained with hematoxylin. Section numbers 2–6 were used for immunostaining, and section number 7 as a negative staining control.

Detection of CD2 and IL-2R positive cells

The sections were fixed in cold acetone at –20 °C for 15 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in absolute methanol for 30 min. The sections were incubated with the following reagents at room temperature: 1) normal horse serum (diluted 1:50 in Tris buffered saline (TBS) containing 0.1% bovine serum albumin (BSA); Vector Laboratories, Burlingame, CA) for 20 min; 2) mouse monoclonal antibody (MAb) against human CD2 (IgG_1, diluted 1.4 μg/mL in TBS containing 0.1% BSA; Dako, Glostrup, Denmark), and IL-2R (IgG_1, 2.0 μg/mL; Dako), respectively, for 60 min; 3) biotinylated horse anti-mouse IgG (1:100 in TBS containing 0.1% BSA; Vector) for 30 min; 4) avidin-biotin-peroxidase complex (1:100 in TBS; Vector) for 30 min; 5) a combination of 0.023% diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, MO) and 0.006% H_2O_2 for 5 min. Between the steps, the sections were washed in TBS for 3 × 5 min. Finally, the slides were counterstained with hematoxylin, dehydrated in a graded ethanol series, cleared in xylene and mounted using Diatex. Monoclonal mouse IgG_1 with the irrelevant specificity (Aspergillus niger glucose oxidase) was used in the same concentration, instead of the primary antibodies, as the negative staining control.

Intracellular detection of lymphokines

After fixation with 2% formaldehyde in phosphate-buffered saline (PBS) for 20 min, the sections were washed with Earl's Balanced Salt Solution containing Ca^{++} and Mg^{++} (EBSS, Gibco, Paisley, Scotland) 3 × 5 min, followed by a wash with EBSS containing 0.1% saponin (Riedle de Haen AG, Seelze, Germany) 3 × 5 min. The endogenous peroxidase activity was blocked with 1% H_2O_2 and 2% sodium azide (NaN3) dissolved in EBSS-saponin solution containing 0.01M HEPES buffer in the dark for 60 min. After being washed in EBSS-saponin solution 3 × 5 min, the sections were incubated with the following reagents at room temperature: 1) avidin blocking solution (Vector) containing 0.1% saponin for 15 min; 2) biotin blocking solution (Vector) containing 0.1% saponin for 15 min; 3) the following mouse anti-human MAbs (diluted in EBSS-saponin solution supplemented with 0.002% NaN3) overnight: IL-2 (IgG_1, 2 μg/mL; Serotec, UK); IFN-γ (IgG_1, 4 μg/mL; PharMingen, CA); and TNF-β (IgG_{2b}, 1 μg/mL; Bender Medsystem, Austria); 4) normal horse serum (diluted 1:50 in EBSS-saponin solution; Vector) for 15 min; 5) biotinylated horse anti-mouse IgG (diluted 1:100 in EBSS-saponin solution; Vector) for 30 min; 6) premixed avidin-biotin-peroxidase complex (diluted 1:100 in EBSS-saponin; Vector) for 60 min. Between the steps, the sections were washed with EBSS-saponin solution 3 × 5 min. After incubation with DAB (0.5 mg/mL) for 5 min, the sections were washed with EBSS for 5 min. Finally, they were counterstained with hematoxylin for 30 seconds and mounted with PBS-glycerin solution. Monoclonal mouse IgG_1 and IgG_{2b} of the same subtypes but with irrelevant specificities were used in the same concentrations, instead of primary antibodies, as the negative staining control.

Quantitative microscopic assessment

Quantitative microscopic assessment was done, using a low light-charge screen mounted with a 12-bit PC digital image camera (SensiCam, Kelheim, Germany) on a Leitz Diaplan light microscope (Wetzlar, Germany). The camera was further linked to a semiautomatic Analysis Pro 3.0 image analysis and processing system (Soft Analysis System GmbH, Münster, Germany). The whole

section areas were checked. The total number of cells for each sample was recorded as the average number of cells in the first and last sections stained with hematoxylin. The positive cells labeled by different MAbs were counted in the sections without counterstaining. The image analysis was performed by 3 researchers, and every section was evaluated at least 5 times.

Results

Histological evaluation of the synovial membrane-like interface tissue

All interface tissue samples had the synovial lining-like structure, usually 1–3 cell layers thick. The stroma was characterized by histiocytosis and fibrosis. It consisted of cell-rich areas with macrophage-like cell infiltration, fibrotic areas with fibroblast-like cells and collagen fibers. Various types of wear debris, e.g., metal, polymethylmethacrylate (PMMA), and ultra-high molecular weight polyethylene (UHMWPE) particles, were seen in these samples.

Lymphocytes and lymphokines in interface tissue and RA samples

Lymphocytes were scarce in the interface tissue samples, and they never formed aggregates. The dispersed lymphocytes were usually found in the sublining and cell-rich areas with macrophage-like cell infiltration. There were no IL-2R positive cells in the interface tissue samples. All RA synovial membrane samples were characterized by lymphocyte infiltration. T-cells were scattered throughout this membrane, mainly in the sublining areas. Perivascular aggregates appeared in all RA samples. A few IL-2R positive cells were located both in the dispersed T-cells and the perivascular aggregates (Figure 1).

No cells in the interface tissue samples showed typical perinuclear immunoreactivity for the lymphokines investigated (Table). Very weak membrane or extracellular staining was occasionally detected, but such staining was never concomitant with clear intracellular immunoreactivity. In RA samples, a small subset of T-cells producing lymphokines, such as IL-2, IFN-γ, and TNF-β, were found scattered in the sublining areas or in the perivascular aggregates. IL-2-producing and TNF-β–producing T-cells showed more generalized cytoplasmic signals, while the staining for intracellular IFN-γ was always more restricted and often formed small perinuclear dots (Figure 2).

Total number of hematoxylin-stained cells, and the number of CD2, IL-2R, IL-2, IFN-γ, and TNF-β positive cells

Sample	Total number of cells	CD2	IL-2R	IL-2	IFN-γ	TNF-β
RA-1	61880	6745	186	62	130	99
RA-2	48365	9915	290	58	44	48
RA-3	70380	13091	352	141	99	56
RA-4	40528	9240	203	41	49	49
RA-5	62594	11642	250	56	150	75
RA-6	76908	14228	385	123	277	77
RA-7	58684	10798	293	65	147	94
RA-8	87720	11403	351	123	158	53
RA-9	75361	10551	301	241	181	75
RA-10	57477	9714	287	86	172	63
IT-1	70176	1404	0	0	0	0
IT-2	67320	2491	0	0	0	0
IT-3	57174	2058	0	0	0	0
IT-4	67691	1015	0	0	0	0
IT-5	58885	1531	0	0	0	0
IT-6	76628	3448	0	0	0	0
IT-7	68896	2825	0	0	0	0
IT-8	64872	2530	0	0	0	0
IT-9	58767	1058	0	0	0	0
IT-10	63126	1263	0	0	0	0

RA: the synovial membrane samples from patients with rheumatoid arthritis undergoing primary THR.
IT: the synovial membrane-like interface tissue from patients undergoing revision THR due to aseptic loosening.

Discussion

Studies on the presence of lymphocytes and the expression of lymphokines in the interface tissue around loosened THR are a good way to show host-responses to the materials used in THR. Since cells in the interface tissue can take up cytokines from other sources, it is important to confirm that the lymphokines are produced in situ before any conclusion is drawn about the role of an immune response in the loosening process.

In this study, we stabilized and permeabilized tissue sections to permit specific MAbs to penetrate through the cell surface membrane, the cytosol, and the membrane of the endoplasmic reticu-

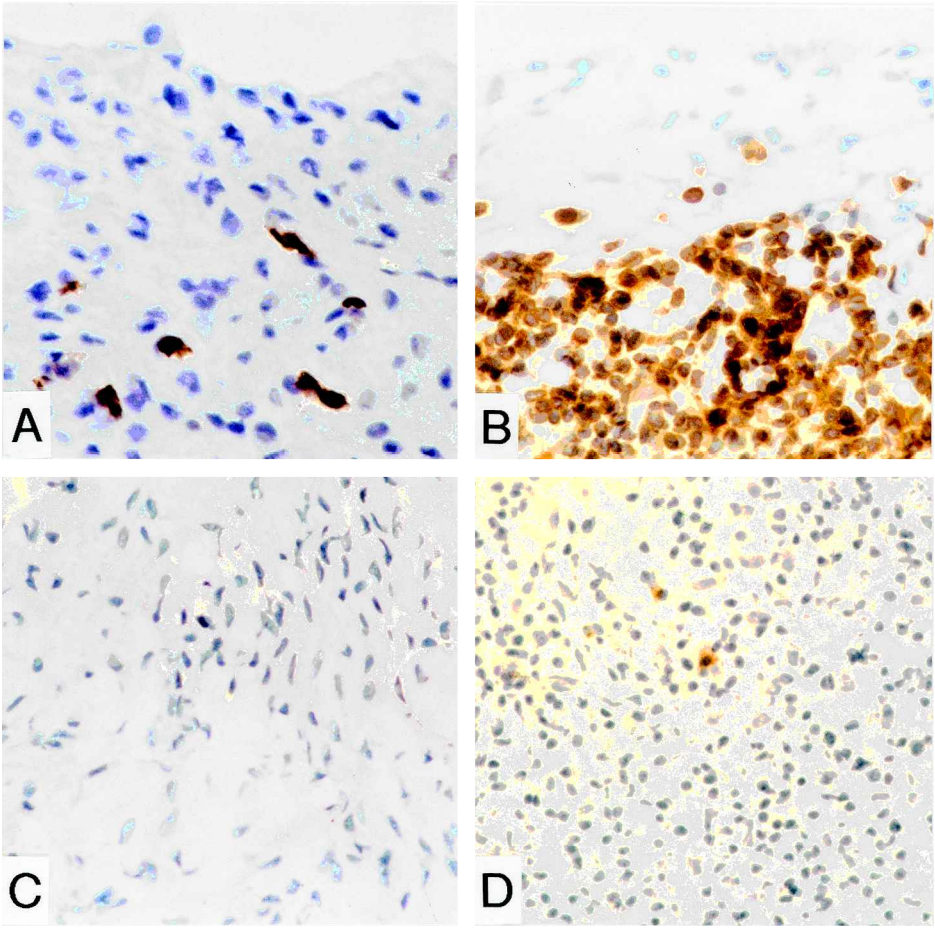

Figure 1. Detection of CD2-positive T-cells and IL-2R-positive activated T-cells with avidin-biotin-peroxidase complex method (counterstained with hematoxylin).
A. CD2 positive T-cells were rare in the interface tissue sample (original magnification, ×400).
B. Large number of CD2 positive T-cells in the RA sample (×400).
C. No IL-2R-positive T-cells in the interface tissue sample (×250).
D. A small subset of IL-2R-positive T-cells in the RA sample (×250).

lum and the Golgi organelle (Dolhain et al. 1993, Andersson et al. 1994, Ulfgren et al. 1995). The staining pattern in producer cells is distinctly different from the membrane signals generated by the mere binding of cytokines to their receptors on target cells, which helps to distinguish cytokine-producing cells from cytokine-binding ones. Perinuclear staining reflects the accumulation of cytokines in the Golgi apparatus of producer cells (Andersson et al. 1994, Lundberg et al. 1997). In cultured cells, intracellular staining has been shown to correlate with the production of cytokine mRNA and cytokine secretion in the supernatant (Dolhain et al. 1993, Raqib et al. 1995). In our study, all MAbs were carefully selected to avoid staining of cytokine-binding target cells (Ulfgren et al. 1995). Negative staining controls have confirmed the specificity of the protocol. On the basis of these facts, we conclude that the methodology is a well-validated immunologic technique.

The results concerning the role of immune responses to the biomaterials used in THR have been contradictory (Chadha et al. 1995, Haddad et al. 1996, Goodman et al. 1998, Weyand et al. 1998). The reported percentages of lymphocytes have varied in interface tissue from aseptic loosening of THR (Lalor et al. 1991, Jiranek et al. 1993, Boynton et al. 1995). Gil-Albarova et al. (1992)

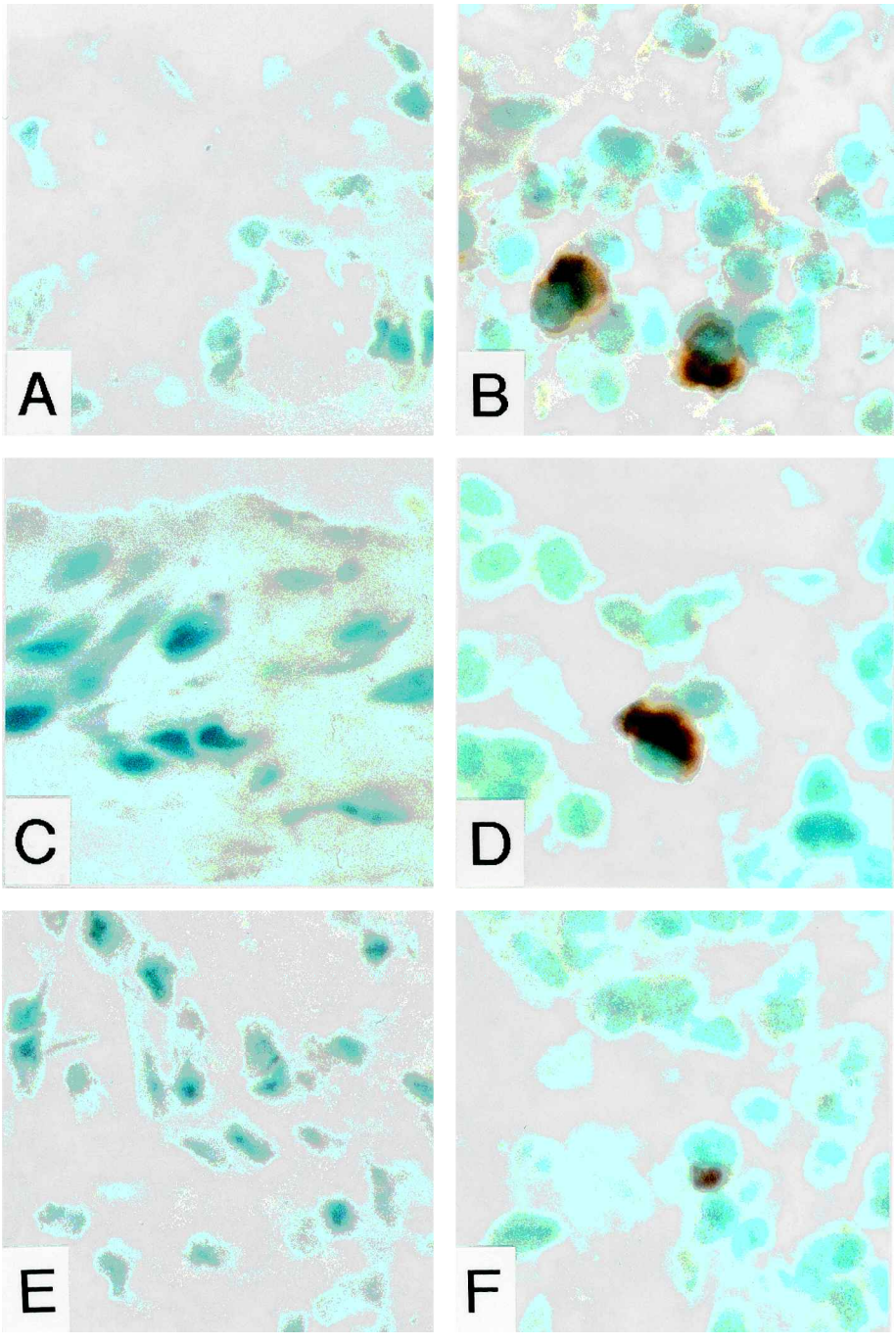

Figure 2. Intracellular lymphokine detection in formaldehyde-fixed, saponin-permeabilized tissue sections (×600, counterstained with hematoxylin).
A. No cell producing IL-2 in the interface tissue sample.
B. IL-2-producing cells in the RA sample.
C. No cell producing TNF-β in the interface tissue sample.
D. TNF-β-producing cell in the RA sample.
E. No cell producing IFN-γ in the interface tissue sample
F. IFN-γ-producing cell in the RA sample.

found a higher number of total T-cells and IL-2R positive T-cells in the patients with loosened THR than in those without loosening. They speculated that the delayed hypersensitivity might be involved in the loosening process. Sandhu et al. (1998) injected UHMWPE particles into the knee joints of immunocompetent and immunodeficient animals, and found that T-cells were not directly involved in the UHMWPE-particle-induced inflammation. In a mouse model with varying immunological capacities, subcutaneous injection of PMMA particles led to formation of a granuloma consisting of macrophages and giant cells, regardless of the degree of immunodeficiencies. The authors concluded that the immune system did not play an important role in the cellular reaction to wear-debris (Jiranek et al. 1995).

Our findings show that in the interface tissue samples from aseptic loosening of cemented THR, T-lymphocytes are scarce, only accounting for about 3% of total cells. There is no sign of lymphocyte activation, as shown by the absence of IL-2R immunoreactivity. Intracellular cytokine staining showed that there is no cell in the interface tissue that produces lymphokines, such as IL-2, IFN-γ, and TNF-β. Previous in vitro studies have demonstrated that polyethylene and methylmethacrylate particles only induced minimal T-cell responses (Santavirta et al. 1991a,b, Goodman et al. 1994). These results confirm the immunologic inertness of implant-associated biomaterials, and suggest that the T-cell-mediated immune response is not actively involved in aseptic loosening of THR.

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