Quantification of the cell infiltrate in synovial tissue by digital image analysis

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Abstract

Objective. The experience with digital image analysis (DIA) in the assessment of synovial inflammation is limited. In this study we compared DIA with two currently applied methods for the evaluation of the synovium.

Methods. Synovial tissue (ST) specimens were obtained by arthroscopy from knee joints of rheumatoid arthritis (RA) patients with variable disease expression and in control subjects. CD68+ macrophages and CD3+ T-cells were detected by immunohistochemical staining using two different labelling techniques. The labelled ST sections were quantified using three different analysis techniques: manual cell counting (MC), semi-quantitative analysis (SQA) and DIA.

Results. We observed strongly positive correlations between the three methods when examining T-cell infiltration: between MC and SQA: $\sigma = 0.91$ ($P < 0.0001$), between MC and DIA: $\sigma = 0.95$ ($P < 0.0001$), and between SQA and DIA: $\sigma = 0.83$ ($P < 0.0001$). Similarly, for the analysis of synovial intimal macrophages, positive correlations were noted between MC and SQA ($\sigma = 0.62$; $P = 0.002$), MC and DIA ($\sigma = 0.56$; $P < 0.01$), and SQA and DIA ($\sigma = 0.79$; $P < 0.0001$). Finally, the analysis of synovial sublining macrophages revealed positive correlations between MC and SQA ($\sigma = 0.95$; $P < 0.0001$), MC and DIA ($\sigma = 0.64$; $P = 0.001$) and SQA and DIA ($\sigma = 0.69$; $P < 0.0001$).

Conclusion. These three different methods generated similar results. DIA offers the opportunity of a reliable and time-efficient analysis of the synovial infiltrate.

KEY WORDS: Synovial tissue, Analysis, Immunohistochemical analysis, Image analysis, Quantification methods.

Synovial tissue (ST) biopsy specimens are being studied to provide insight into pathogenetic mechanisms underlying inflammatory arthritis and to evaluate the effects of therapeutic interventions [1]. The methods available to quantify the features of ST inflammation include: digital image analysis (DIA), semi-quantitative analysis (SQA) and manual cell counting (MC). DIA uses computerized digital image-processing techniques to extract numerical information from visual images, while SQA utilizes a limited number of scores which are assigned to a microscopic examination.

A major advantage of DIA is the reduction of both inter- and intra-observer variability [2], and the ability to quantify the density of the antigen of interest [3–5]. The role of DIA in ST analysis has not as yet been determined, since there are no studies showing that this method is superior to SQA or MC. SQA requires relatively little time and, therefore, offers the opportunity to evaluate multiple biopsy specimens from relatively large patient numbers, which minimizes sampling error. This is an advantage when considering the variation of synovial inflammation that can be found within the joint and between individuals within one diagnostic group [6–9]. SQA has been applied in relatively large studies and has been shown to be a sensitive and reproducible tool for assessing differences between patient groups as well as for the evaluation of therapeutic interventions [10–17]. There is also a highly significant correlation between semi-quantitative scores for immunohistological features of ST and scores for disease activity [9, 12]. MC is sensitive and accurate, but more time consuming than SQA [18]. Comparison of SQA and MC revealed a highly significant correlation between the two methods [19], indicating that both methods can be used. However, MC is by definition more sensitive than SQA in the detection of small changes in synovial inflammation after treatment [19].

Since there are no studies comparing the performance of DIA, SQA and MC, the aim of this study was to provide more insight into the validity of the methods to quantify the cell infiltrate in ST.

Materials and methods

Patients

Nine patients with rheumatoid arthritis (RA) (ACR 1987 criteria [20]) and both a clinically involved (CI) and a clinically uninvolved (CU) knee joint were investigated [12]. Five people without inflammatory joint disease served as the control subjects [12]. All patients gave informed consent. In all RA patients, an arthroscopic procedure [12] was performed in both knees using a small-bore 2.7-mm arthroscope (Storz, Tuttlingen, Germany) by a single skin portal in the suprapatellar pouch for both macroscopic examination of the synovium and biopsy. At each arthroscopy, synovial biopsy specimens were obtained from the suprapatellar pouch, the synovium–cartilage junction, the patellar gutters and the tibiofemoral junction using 2-mm grasping forceps (Storz). If there was macroscopic variation, samples were taken from both macroscopically inflamed and macroscopically non-inflamed regions. In all cases, an average of 20 pieces of ST were used for immunohistological analysis. ST from the five control subjects was obtained by arthroscopy with 5-mm grasping forceps. All ST samples from one knee joint were snap-frozen together *en bloc* in Tissue-Tek OCT (Miles Inc. Diagnostic Division, Elkhart, IN, USA) and immersed in methylbutane ($-70°C$). Frozen blocks were stored in liquid nitrogen until sectioned for staining. Sections ($5\,\mu m$) were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Braunschweig, Germany) and the slides were stored at $-80°C$ until immunohistochemical staining was performed.

Antibodies

Serial sections were stained with the following monoclonal antibodies (mAbs): anti-CD3 (Leu-4; Becton-Dickinson, San Jose, CA, USA) and anti-CD68 (EBM11; Dako, Glostrup, Denmark). For control sections, the primary antibodies were omitted or irrelevant isotype-matched mouse mAbs were applied.

Chemicals

Affinity-purified and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody was obtained from Dako, affinity-purified HRP-conjugated swine anti-goat Ig from Dako, and diaminobenzidine (DAB) and amino ethylcarbazole (AEC) from Sigma (St Louis, MO, USA).

Immunohistochemical staining

Two staining protocols were used. The first protocol has been described previously [9, 10, 12]. After fixation with acetone, the ST sections were incubated with the primary mAbs, then HRP-conjugated goat anti-mouse antibody was added, followed by incubation with HRP-conjugated swine anti-goat antibody. HRP activity was detected using hydrogen peroxide as substrate and AEC as dye. Subsequently, the slides were counterstained with Mayer's haematoxylin (Merck, Darmstadt, Germany) and, after washing with distilled water, mounted in Kaiser's glycerol gelatin (Merck). The second staining protocol has also been described previously [2, 5, 21, 22]. After fixation with acetone the primary mAbs were added, followed by an HRP-conjugated goat anti-mouse secondary antibody. The HRP activity was detected using hydrogen peroxide as the substrate and DAB as the dye. Slides were counterstained with haematoxylin and, after washing with acid alcohol, lithium carbonate, ethanol and xylene, mounted in DPX (BDH Chemicals, Poole, UK).

Evaluation of the slides

All sections were coded and analysed in a random order by independent observers who were blinded for the clinical data (MCK, JJH). After staining, the sections were scored according to three distinctive analysis methods, applied as described previously for MC [18, 23], SQA [23] and DIA [2]. The time that it took to complete the analysis for each method was formally measured.

MC. MC was performed on all sections by the same observer (JJH), counting eight randomly selected high power fields (HPF) per section. An HPF was defined by a 24 mm² square graticule (Olympus, Tokyo, Japan) using a $400 \times$ magnification on a binocular microscope (BX50, Olympus). For the evaluation of CD68+ macrophages, MC was performed for intimal macrophages and synovial sublining macrophages separately.

SQA. SQA was performed by two independent observers (MCK, JJH) as previously described [11, 12, 24]. The expression of CD3+ cells and CD68+ cells was scored on a 5-point scale (range 0–4). A score of 0 represented minimal infiltration, while a score of 4 represented infiltration by numerous inflammatory cells. For evaluation of the expression of CD68+ cells, SQA was performed for intimal macrophages and synovial sublining macrophages separately. Minor differences between observers were resolved by mutual agreement.

DIA. One observer (MCK) performed processing of all images, as described previously [2, 25]. Five randomly selected HPFs were chosen for the evaluation of each section. For assessment of CD68+ macrophages, each HPF contained both intimal lining layer and synovial sublining [5]. The HPF images were acquired on an Olympus microscope (Olympus), captured using a CCD (Charged Coupled Device) video camera (Sony, Tokyo, Japan), and digitized using a PV100 multimedia 16-bit colour video digitizer card, using a standardized macro program to simplify as well as to standardize the acquisition process. The resultant colour images were in a 640×480 pixel RGB format with a 24-bit resolution, enabling the use of 16 581 375 colours. For each acquisition session, the microscope, camera and computer were calibrated according to a standardized procedure. The

images obtained were stored as bitmaps without compression using a Zip disk and portable driver (Iomega, Roy, UT, USA).

All sections stained for CD68+ macrophages were examined using two separate computer-assisted colour video image-analysis systems: VP32 (Video Pro 32; Leading Edge P/L Adelaide, South Australia) and Qwin (Qwin Pro V2.2; Leica, Cambridge, UK), respectively. Both systems consist of a personal computer (PC) with software (VP32 on a PC with an Intel 80486 DX processor and a Windows™ 3.1 environment, Qwin on a PC with Intel Pentium 200 MHz processor and Windows® NT 4.0 environment). DIA on the CD3 stained sections was performed only with the Qwin system.

In the macro program for the VP32 system, the synovial intimal lining layer and the synovial sublining were identified and regions that were not to be analysed were deleted. A first colour discrimination threshold was then adjusted such that a binary overlay covered both the positive stained areas and the counterstained areas. This threshold was kept constant for all measurements with the same marker. A second colour discrimination threshold was then adjusted such that a second binary overlay covered the area of positive staining. This threshold was kept constant for all measurements with the same marker. Subsequently, this field was measured. The results of measurement included the area of positive staining and the mean optical density (MOD), which is proportional to the cellular concentration of protein. The integrated optical density (IOD) is related to the total amount of antibody present and is equal to the MOD multiplied by the area of positive staining.

In the macro program for the Qwin system, the image was edited by marking the intimal layer, enabling separate analysis of the intimal layer and the synovial sublining. Two binary masks were applied using threshold values for the red, green and blue channels. These thresholds were kept constant for all measurements with the same marker. A first binary mask identified the counterstained areas as reference for the total region of tissue, and a second binary mask covered the positively stained areas. Both binary masks were individually processed to reduce the signal/noise ratio using erode, open and dilate commands. Overlapping areas between the two binary masks were identified and they were not included in the analysis. Analysis was performed on the absolute area stained, as indicated by the secondary binary mask. For the assessment of infiltration by CD68+ cells, the area was measured in pixels, the MOD was measured and the IOD was calculated by multiplication of the relatively or absolutely stained area with the MOD. For the evaluation of CD3+ T cells, an extra segmentation step was performed, which allowed the number of positive cells to be counted.

Statistical analysis

Correlations between the results obtained by the different methods to quantify the cell infiltrate were calculated using the Spearman rank test. The ability of the methods to detect differences between the three groups of ST specimens (CI joints and CU joints from RA patients and controls) was analysed using non-parametric tests. The Wilcoxon rank test for matched pairs was used to compare ST from the CI and CU joints, and the Mann–Whitney two-sample test was used to compare ST from CU joints with the controls.

Results

The groups of RA patients (mean age 66 yr, range 47–76 yr) and control subjects (mean 66 yr, range 56–76 yr) were similar with regard to age. Five RA patients were male and four RA patients were female; all control subjects were male. All RA patients were rheumatoid factor positive and had erosive disease.

It took 50 min to quantify T cell infiltration in the 21 sections by SQA and 65 min to quantify macrophage infiltration in both regions. MC took 4 h 30 min for the evaluation of T cells and 6 h 10 min for macrophages in both regions. DIA took 50 min (30 min warming-up time for the light source excluded) for the evaluation of T cells and 1 h 40 min for macrophages in both regions for the Qwin system, and 5 h 10 min for the VP32 system for the evaluation of T cells and 6 h 10 min for macrophages in both regions. The differences between the two systems were predominately caused by the differences in calculation speed of the microcomputer used by each image-analysis system.

Comparison of the two DIA systems for quantification of macrophage infiltration revealed strongly positive correlations (intimal lining layer: $\sigma = 0.90$; $P < 0.0001$ and synovial sublining: $\sigma = 0.78$; $P < 0.0001$) (Fig. 1). T-cell infiltration was not analysed using the VP32 system because of these positive correlations and because the VP32 system was faster.

The results comparing the three methods are summarized in Table 1 (protocol using AEC method) and Table 2 (protocol using DAB method). Generally, the two staining protocols using either AEC or DAB revealed similar results. For T-cell quantification, we observed strong correlations between MC and SQA ($\sigma = 0.91$; $P < 0.0001$), between MC and DIA ($\sigma = 0.95$; $P < 0.0001$), and between SQA and DIA ($\sigma = 0.83$; $P < 0.0001$) (Fig. 2). All three methods were able to detect differences between the three biopsy groups. However, the difference in scores for T-cell infiltration between CI compared with CU joints did not reach statistical significance using the AEC method, although the semiquantitative scores were statistically significantly different using the DAB method.

For quantification of macrophages, we also found strong correlations between the three methods. For intimal macrophages, the Spearman rank correlations were as follows: between MC and SQA: $\sigma = 0.62$ ($P = 0.002$); between MC and DIA $\sigma = 0.56$ ($P < 0.01$); and between SQA and DIA $\sigma = 0.79$ ($P < 0.0001$) (Fig. 3). For synovial sublining macrophages, the values were: between MC and SQA $\sigma = 0.95$ ($P < 0.0001$); between MC and DIA $\sigma = 0.64$ ($P = 0.001$); and between SQA

Intimal lining layer

$\rho\,0.90$

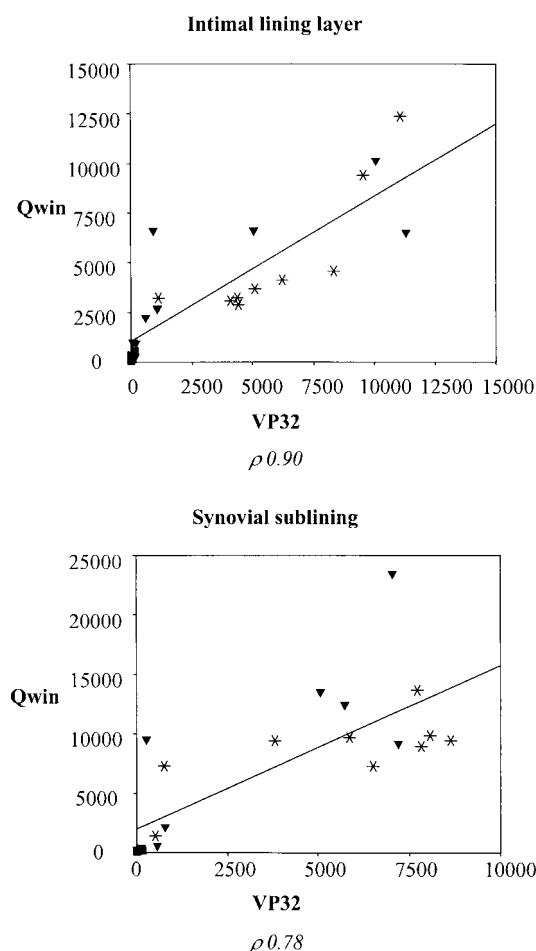**Synovial sublining**

$\rho\,0.78$

FIG. 1. Scatter diagram of the mean integrated optical density per high power field for the two digital image analysis systems (Qwin and VP32), for CD68+ macrophages in the intimal lining layer and synovial sublining for the three groups under investigation [(■) control knee joints, (▼) clinically uninvolved knee joints and (*) clinically involved knee joints] and Spearman rank correlation coefficient.

and DIA $\sigma = 0.69$ ($P < 0.0001$) (Fig. 4). The differences in scores for intimal macrophages between the three groups could be detected using all three methods (Table 1 and Table 2). However, the differences did not reach statistical significance in all cases, presumably due to the relatively small number of patients. None of the methods was clearly superior to the others in detecting differences in intimal macrophage infiltration. Moreover, all three methods were able to detect differences in infiltration by synovial sublining macrophages comparing the three biopsy groups.

Discussion

In this study we compared three distinct analysis methods and two staining protocols for the evaluation of ST infiltration by CD3+ T cells and CD68+ macrophages. In general, the results obtained by one method showed strong correlations with results obtained by the other methods. DIA using the Qwin system and SQA were clearly more time efficient than MC.

Manual counting (MC) of cells is a sensitive and accurate method, but the laborious character limits wide application of this technique. Recognition of this limitation of MC resulted in a study performed on limiting the number of HPFs used in MC analysis [18]. This study showed that reliable measurement of T-cell infiltration may be obtained from analysis of 17 randomly selected HPFs from three different biopsy specimens, and even the analysis of eight HPFs appeared to be reliable [18]. The data presented here support this conclusion by showing good correlations between limited MC on the one hand and either SQA or DIA on the other. However, in our hands, the MC analysis of eight HPFs of a single tissue section still took, on average, considerably more time than the other two methods.

SQA is a reliable and cheap method that allows the analysis of the entire ST sections in a time-efficient fashion. It has been shown for a number of markers that reliable results can be obtained by examination of only six sections from different biopsy specimens [26]. It is possible to evaluate these sections within a few

TABLE 1. Grouped results of the three analysis methods: manual cell counting [MC (mean number of cells)], semi-quantitative analysis [SQA (mean score on a 0–4 scale)], and digital image analysis {DIA [mean number of CD3+cells and mean integrated optical density for CD68+ cells]}. Data are shown for CD3+T cells and CD68+ macrophages stained with AEC in the synovial intimal ining layer and synovial sublining in the three groups

		Controls (C)			Clinically uninvolved (CU)			Clinically involved (CI)			C−CU	CU−CI
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max		
T cells	MC	0	0	1	7	0	23	55	9	123	<0.02	<0.01
	SQA	0	0	0	0.6	0	3	1.1	0	4	<0.05	ns
	DIA	3.2	1.6	6	23.5	3.8	65.2	60.5	29.6	77.6	<0.01	<0.005
Macrophages	MC	4	3	5	16	3	33	19	11	39	<0.05	ns
Intimal	SQA	1	1	1	1.3	0	2	2.2	1	4	ns	<0.05
Lining	DIA	384	64	815	4111	243	10 120	5165	2862	12 352	<0.01	ns
Macrophages	MC	16	4	44	106	10	276	193	36	316	0.02	ns
Synovial	SQA	0.5	0	1	1.9	1	4	2.8	1	4	0.05	ns
Sublining	DIA	128	57	300	7915	223	23 395	8554	1401	13 700	<0.005	ns

TABLE 2. Grouped results of the three analysis methods: manual cell counting [MC (mean number of cells)], semi-quantitative analysis [SQA (mean score on a 0–4 scale)], and digital image analysis {DIA [mean number of CD3+cells and mean integrated optical density for CD68+ cells]}. Data are shown for CD3+ T cells and CD68+ macrophages stained with DAB in the synovial intimal lining layer and synovial sublining in the three groups

|		Controls (C)			Clinically uninvolved (CU)			Clinically involved (CI)			C−CU	CU−CI
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max		
T cells	MC	1	0	1	10	0	54	27	2	102	<0.01	<0.05
	SQA	0.4	0	1	1.2	0	3	2.3	1	3	<0.05	<0.05
	DIA	1.5	0	3.2	16	1	44.6	38.2	7.2	95.2	<0.02	0.05
Macrophages	MC	9	2	20	15	0	35	19	8	30	ns	ns
Intimal	SQA	1	1	1	1.3	0	3	2.2	1	4	ns	0.05
Lining	DIA	1471	25	4016	2786	55	12319	9375	2991	18154	ns	<0.01
Macrophages	MC	9n	3	19	35	0	118	64	23	144	ns	0.05
Synovial	SQA	1	0	2	1.4	0	3	2.4	1	4	ns	<0.05
Sublining	DIA	626	9	1574	5237	46	27015	12731	2892	13818	ns	<0.02
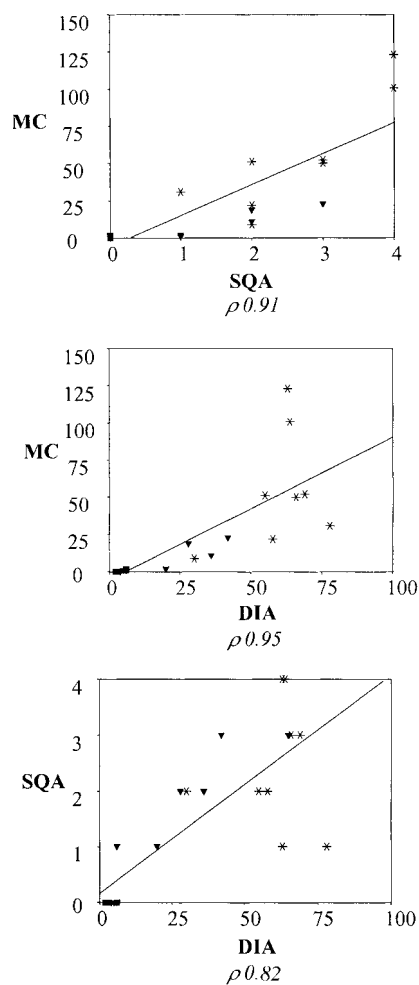
FIG. 2. Scatter diagram of the scores as found with manual cell counting [MC (mean number of cells per HPF)], semi-quantitative analysis [SQA (mean score per section)] and digital image analysis [DIA (mean number of cells per high power field] representing CD3+ T cells for the three groups under investigation [(■) control knee joints, (▼) clinically uninvolved knee joints and (*) clinically involved knee joints] and the Spearman rank correlation coefficient.
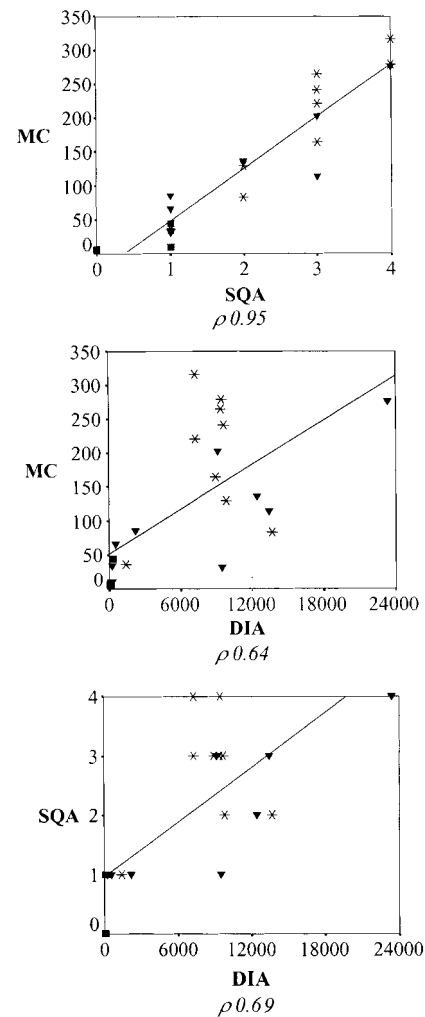

FIG. 3. Scatter diagram of the scores as found with manual cell counting [MC (mean number of cells per HPF)], semi-quantitative analysis [SQA (mean score per section)] and digital image analysis [DIA (mean number of cells per high power field)] representing CD68+ macrophages in the intimal lining layer for the three groups under investigation [(■) control knee joints, (▼) clinically uninvolved knee joints and (*) clinically involved knee joints] and the Spearman rank correlation coefficient.
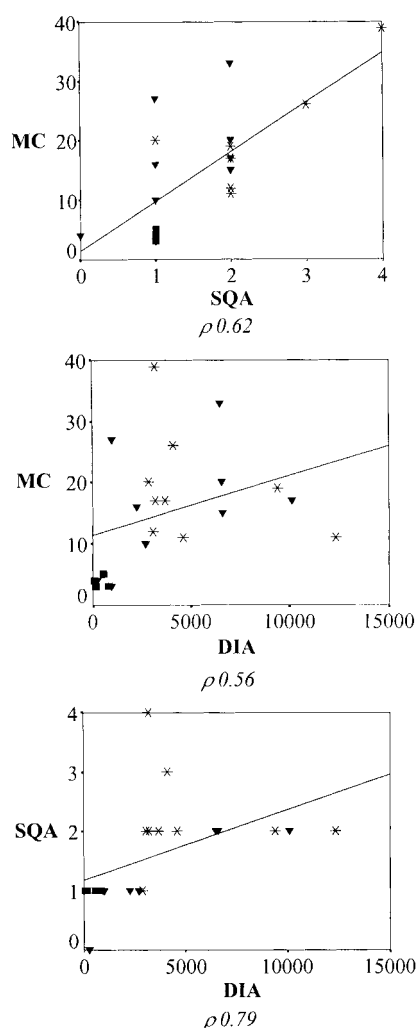
FIG. 4. Scatter diagram of the scores as found with manual cell counting [MC (mean number of cells per HPF)], semi-quantitative analysis [SQA (mean score per section)] and digital image analysis [DIA (mean number of cells per high power field)] representing CD68 + macrophages in the sublining for the three groups under investigation [(■) control knee joints, (▼) clinically uninvolved knee joints and (*) clinically involved knee joints] and the Spearman rank correlation coefficient.

minutes when different biopsy specimens are snap-frozen together *en bloc*, as in the present study. The results presented here are similar to those from a previous study on different patient material, which showed a highly significant correlation between results obtained by MC and SQA for the evaluation of macrophages and T cells [19]. In some patients demonstrating a decrease in serum levels of C-reactive protein and global subjective scores for disease activity, reduced numbers of cells were found in serial biopsies by MC analysis, whereas the SQA scores remained the same [19], indicating that MC is more sensitive for detecting small changes in synovial inflammation. Similarly, the present study shows that MC and DIA appear to be more sensitive

than SQA for detecting differences between CI and CU joints. Thus, SQA is a relatively conservative system.

DIA is a new technique under development for the analysis of ST [2–5]. The major advantage of this analysis method is the standardization of image acquisition and processing, minimizing variance, and the ability to quantify the actual stained area together with staining intensity. Quantification by DIA enables a detailed analysis in a time-efficient fashion. When applied to the quantification of CD3 + T cells, it takes on average 3–4 min per section. It is, however, possible that quantifying T-cell infiltration could be difficult when intense T-cell infiltration is present, since CD3 + T cells tend to aggregate in dense clusters. First, it is probably important to use thin sections to include only a few overlapping positively stained cells, facilitating automated quantification. Second, we used a segmentation step after the process of selection and the optimization of the signal-to-noise ratio. The advantage of this segmentation step is that the program divides dense areas of cells that are all positive for a specific marker via an algorithm into separate 'cells' that are compatible with the average size of a cell. Using this system, we found excellent correlations between DIA and the other techniques, although we included patients with maximal T-cell infiltration in this study. In addition, the use of DIA did not result in loss of the ability to detect differences between the three groups under investigation. It can be anticipated that it is difficult to distinguish between intracellular and extracellular staining. This is presumably not relevant in studies on the relationship between features of ST on the one hand and disease activity on the other, but could be important in pathogenetic studies. In such studies, conventional microscopic examination remains essential. It should also be noted that, although immuno-histological staining for intimal macrophages and fibroblast-like synoviocytes can be quantified, lining layer thickness cannot be directly measured by DIA. Major disadvantages of DIA are the cost and the expertise required to operate a DIA system.

In conclusion, all three analysis methods generated comparable results. MC is a sensitive method for detecting small differences between groups. When compared to MC, a major advantage of SQA is the time-efficient review of an entire tissue section. DIA, as a new technique, can be an attractive, sensitive, and time-efficient method for the quantification of both the number of cells stained and the staining intensity. However, a major disadvantage of DIA is the cost and expertise required for the introduction and maintenance of both software and hardware. Whichever system is used, it is essential that the investigators are appropriately trained and use reliable controls. Further research in this area should evaluate the comparability, advantages and disadvantages of methods for detecting biologically relevant molecules, such as cell adhesion molecules and cytokines, and for the evaluation of morphological issues such as vascularity.

Acknowledgements

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