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Quantitative histological analysis of antigen-induced arthritis in the rabbit

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Antigen-induced arthritis in the rabbit (AIAR) provides the closest experimental equivalent to human rheumatoid arthritis in terms of infiltration of synovial tissue by lymphoid cells. A method is described for quantitative histological analysis of AIAR. Measurements of total cell numbers, lymphocyte and polymorphonuclear leucocyte infiltration, and thickness of infiltrated synovium were obtained for ranges of antigen dosage and duration of arthritis. The method has been devised as part of a system for the analysis of joint swelling, synovial fluid biochemistry and cytology, cartilage proteoglycan chemistry and synovial histology on the same specimen.

Keywords: experimental arthritis, synovitis

Antigen-induced arthritis in the rabbit (AIAR) (Dumonde & Glynn 1962) is one of many animal systems used for the study of chronic synovitis and its response to drug treatment. The main advantage of AIAR over mouse and rat systems is the closer resemblance to human disease in terms of the cellular infiltrate (Doble 1986).

The inflammatory stimulus in AIAR involves a secondary immune response to locally administered antigen. It does not shed light on mechanisms of systemic autoimmune synovitis. However, in the absence of a satisfactory systemic model, AIAR remains the system of choice for the study of local cellular interactions.

The heterogeneity of synovial tissue makes quantitative histological analysis difficult. Advances have been made in the preparation of rabbit joint tissue for histological assessment (Gall & Gall 1980, Ohrt *et al.* 1986). However, methods described so far are only semiquantitative and do not cater for the simultaneous analysis of both cartilage biochemistry and synovial fluid biochemistry and cytology.

A method is described which allows each of these aspects of joint disease to be analysed quantitatively.

Materials and methods

Animals. Mature male New Zealand White rabbits weighing 3-4kg were used in all experiments.

Immunization

Animals were immunized with ovalbumin in Freund's complete adjuvant on two occa-

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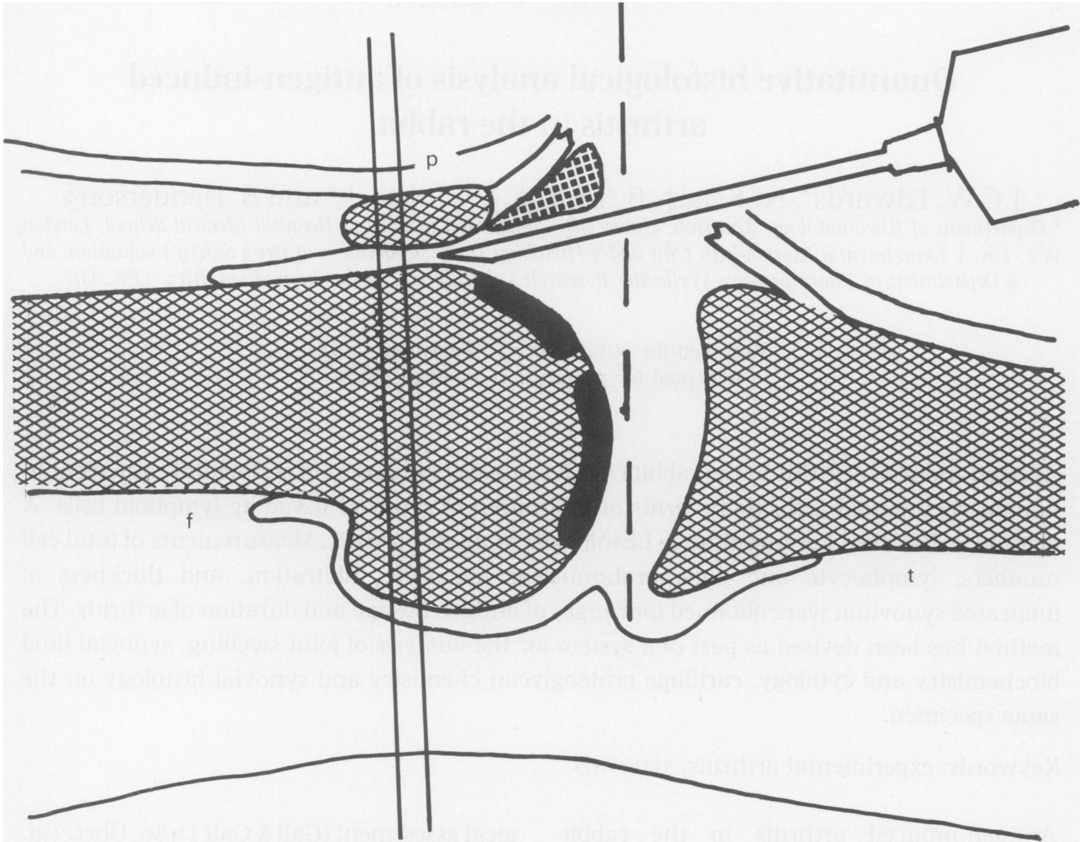

Fig. 1. General method for joint analysis. p, patella; f, femur; t, tibia. Fluid was aspirated from an anterior approach. Cartilage was removed from the solid black areas. The joint was bisected through the broken line and sections cut through the continuous tramlines.

sions, 14 days apart, by multiple intradermal injection. Arthritis was induced by the intra-articular injection of 0.05, 0.5 or 5mg ovalbumin into the left knee 5 days after the second immunization.

Analysis of joint disease

Animals were killed using intravenous thiopentone. Figure 1 summarizes the method used for obtaining various types of material from joints.

The challenged and contralateral (control) knees were exposed by an anterior skin incision. The patellar tendon was severed and the joint opened anteriorly for the aspiration of synovial fluid. The collateral and cruciate ligaments were then severed, and the joint bisected in the femoro-tibial joint line. Slices of articular cartilage were removed from the distal femoral articular surface for biochemical analysis as described elsewhere (Pettipher *et al.* 1986). The femur was excised at mid shaft and trimmed of excess muscle. The soft tissues surrounding the femur were then drawn gently down into their original position.

The distal femur, with surrounding synovial tissue, was immersed in isopentane containing solid carbon dioxide. A 2-mm slice was cut from the frozen specimen at right angles to the femoral shaft, and passing

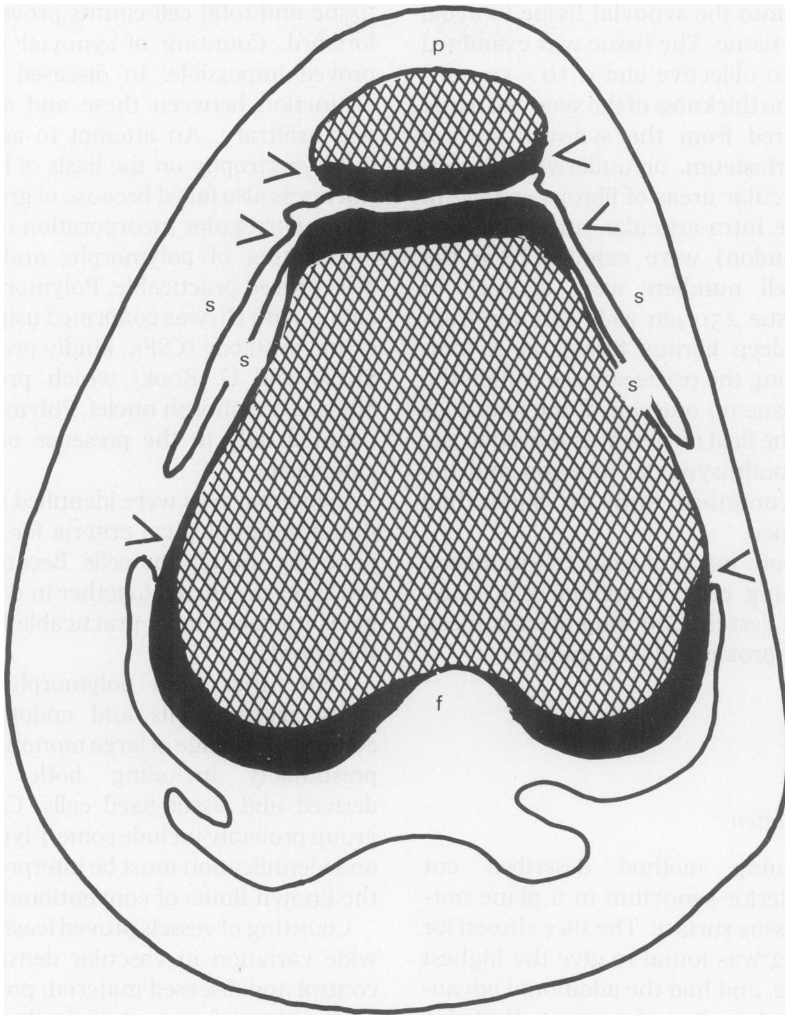

Fig. 2. Diagram of tissue section. p, patella; s, synovial tissue suitable for quantitative analysis; f, femur. Arrowheads show sites of erosion.

through the upper third of the patella and the posterior articular surface of the condyles (Fig. 2) using a fine-bladed precooled hacksaw. The slice was thawed and decalcified in saturated EDTA and embedded in paraffin for conventional sectioning and H&E staining.

Despite the theoretical problem of ice artefact, cell morphology was excellent and the freezing step minimized tissue trauma and increased speed and simplicity of handling.

Synovial tissue from the infrapatellar region was maintained in the frozen state and used for qualitative immunohistochemical study (not described here in further detail).

Quantitative analysis

The chondrosynovial junction of the femoral surface of the patello-femoral joint was identified. The first field to be examined was taken

far enough into the synovial tissue to avoid transitional tissue. The tissue was examined using a ×40 objective and a 10×10-mm² graticule. The thickness of the synovial tissue was measured from the synovial surface down to periosteum, or underlying fibrous tissue. Avascular areas of fibrous synovium (forming the intra-articular surface of ligament or tendon) were exluded from the analysis. Cell numbers were counted in bands of tissue 250 μm wide running from surface to deep fibrous tissue, at 2.5mm intervals along the tissue surface. When the length of tissue on one side of the joint was exhausted the field of view was moved to the opposite chondrosynovial junction and the procedure continued until 10 bands had been examined.

Counts were made of total cell numbers, synovial lining cells, lymphoid cells, polymorphs and vessels. Counting of each specimen took approximately 15 minutes.

Results

Tissue orientation

The sectioning method described cut through anterior synovium in a plane normal to the tissue surface. The slice chosen for cell counting was found to give the highest cell numbers, and had the additional advantage of showing sites of pannus. Posterior synovium (and synovium cut in previous studies using sagittal sectioning) showed variable obliquity of sectioning and was not used for analysis.

The pattern of cellular infiltration in anterior synovium on either side of the patella was stereotyped and hence suitable for analysis. Examples of minimal and severe synovitis are given in Figs 3 and 4. Infrapatellar adipose synovium was more variable and was used only for qualitative immunocytochemistry.

Cell identification

Measurement of the thickness of synovial tissue and total cell counts proved straightforward. Counting of synovial lining cells proved impossible. In diseased tissue any distinction between these and deeper cells was arbitrary. An attempt to assess lining cell hypertrophy on the basis of lining layer thickness also failed because of gross variability and irregular incorporation of fibrin.

Counting of polymorphs and lymphoid cells proved practicable. Polymorph identification with HE was confirmed using a monoclonal antibody (CSF5, kindly provided by J. Steele and G. Rook) which preferentially binds to polymorph nuclei. Polymorphs were counted only in the presence of an intact cytoplasm.

Lymphoid cells were identified by conventional morphological criteria for small lymphocytes and plasma cells. Because the two cell types occcurred together in dense aggregates it proved most practicable to combine the counts.

Cells other than polymorphs, lymphocytes, plasma cells and endothelial cells comprised a range of large mononuclear cells presumably including both monocyte-derived and tissue-fixed cells. Cells in this group probably include some T lymphoblasts and identification must be interpreted within the known limits of conventional histology.

Counting of vessels proved feasible, but the wide variation in vascular density in both control and diseased material, precluded the recognition of any statistically significant changes.

The study was not designed to assess pannus or erosion on a quantitative basis, but the sectioning technique allowed qualitative assessment of changes at the margins of both patello-femoral and tibio-femoral joints.

Reproducibility

To assess reproducibility a study was made in which five blocks were cut sequentially in a plane perpendicular to the femoral shaft from each of five joints, starting at suprapatellar pouch level and progressing to mid

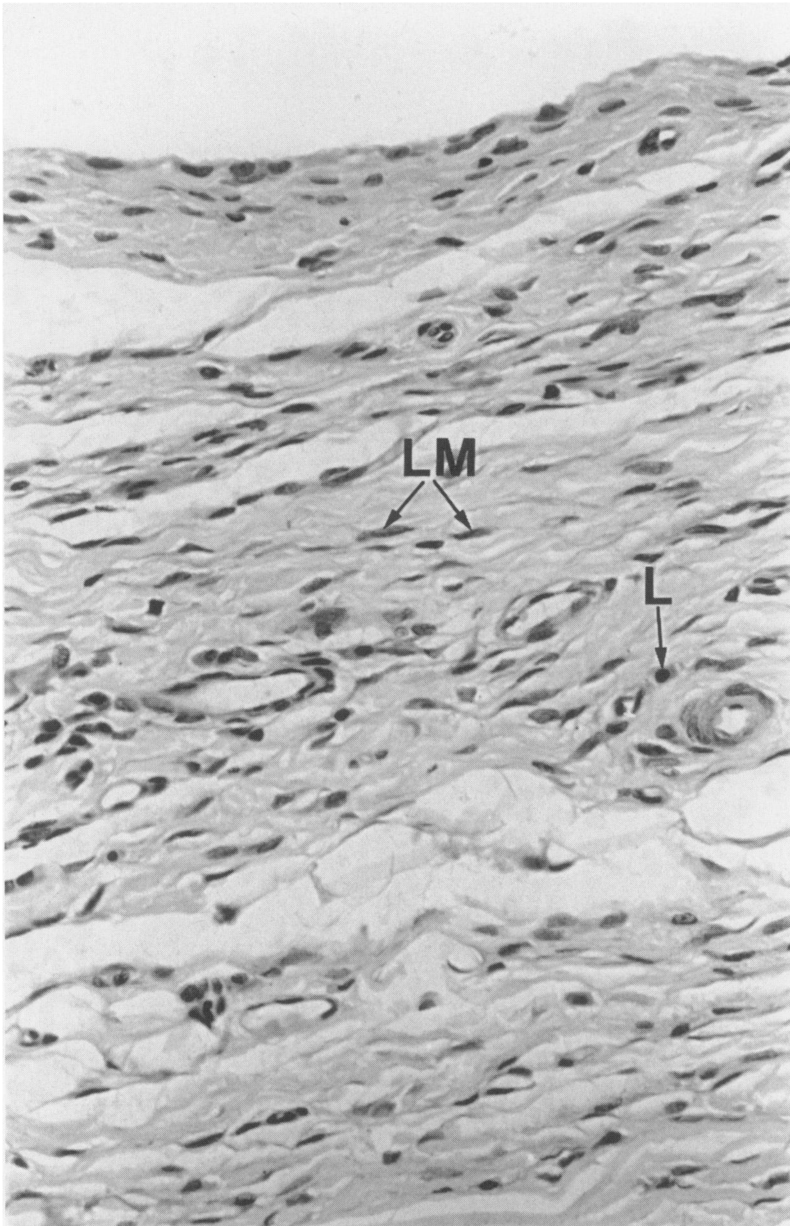

Fig. 3. Photomicrograph of a rabbit knee section showing minimal synovitis. LM, large mononuclear connective tissue cell; L, lymphocyte. H&E × 300.

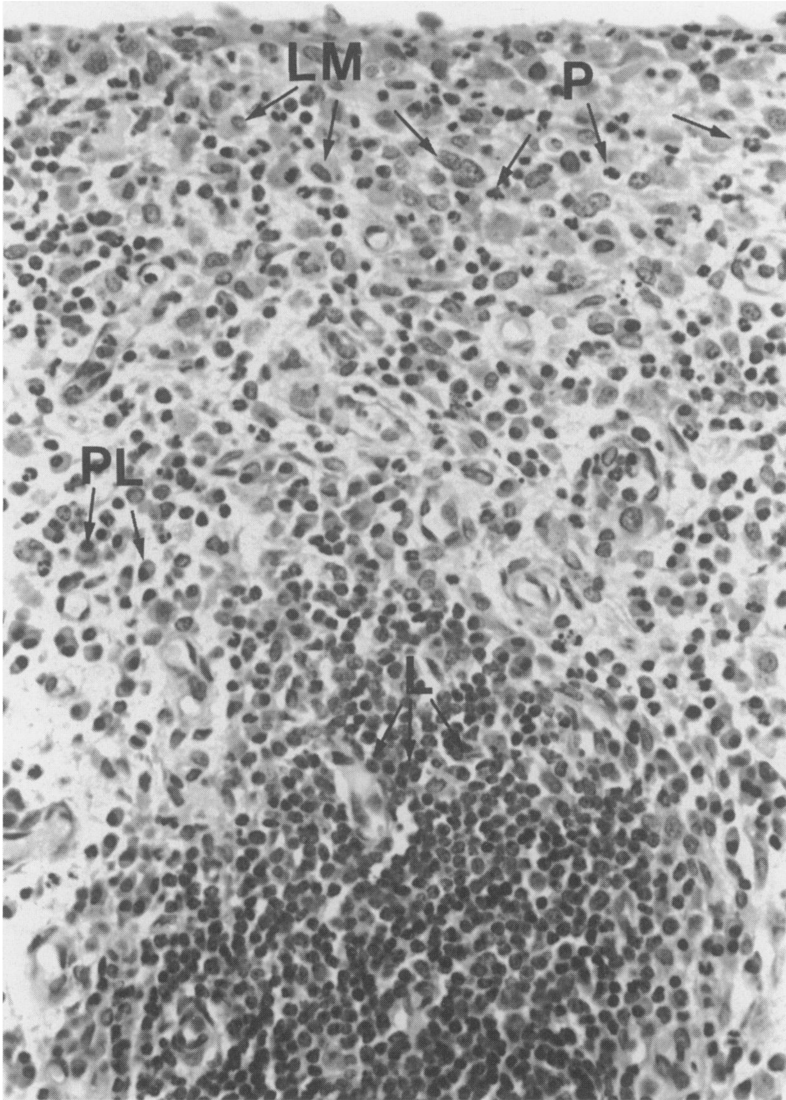

Fig. 4. Photomicrograph of a rabbit knee section showing severe synovitis. LM, large mononuclear connective tissue cell; L, lymphocyte; P, polymorphonuclear leucocyte; PL, plasma cell. H&E × 300.

condyle. Cell counts per 250 μm band peaked at the patellar level.

The five joints were ranked on the basis of cell counts at the patellar level. Of 60 counts for total cells, lymphoid cells, or polymorphs made on blocks from other levels, only nine were discordant for the original ranking, and none of these by more than one ranking position. Thus, although cell counts vary within a joint, it appears that the counts at the patellar level are a good guide to the joint as a whole.

Repeated polymorph counts on the same sections gave figures reproducible to within 20%. This is far smaller than the biological variation within a group of animals, in which counts varied by as much as 0–34%.

Variation in lymphoid cell counts within groups of animals was smaller and sampling error in counting could have contributed more significantly to variance. In a study of 29 arthritic joints 21 days after intra-articular antigen, lymphoid cell counts were repeated on coded sections in a random order. Cell counts were ranked into ten grades. On recounting, 17 counts fell into the same grade, eight fell one grade either side and four fell two grades either side. These results suggest that sampling error in counting makes a contribution to variance, but it is still small in comparison with the biological variation within groups.

All experiments were counted by one observer, using coded sections. All counts were compared with controls within an experiment. Although repeated counting of standard sections suggested that counts could be treated as absolute values, it is not recommended that values obtained by different observers, or from different experiments, should be compared.

During the development of the technique studies have been performed with groups of approximately five animals. With groups of this size, statistically significant differences in lymphoid cell counts required differences in mean counts of 70 per 250 μm band. Smaller differences could be important in therapeutic studies. On the basis of standard deviations an approximate estimate of group numbers required to demonstrate smaller differences at the 0.05% level with 90% power can be made. A difference in mean counts of 50 lymphocytes per 250 μm band would require 10 animals per group to detect, and a difference of 30 would require groups of 28 animals.

Normal tissue

In a group of six normal joints synovial tissue thickness was 105 ± 33 (mean ± s.d.) μm. The mean total cell count was 53 ± 6 (mean ± s.d.) μm. Most joints showed occasional small lymphocytes but mean counts for 10 fields were less than one in all joints. Polymorphs were seen estremely rarely and mean counts were less than one in all joints.

Experimental groups

Two studies using the morphometric technique will be described to give an indication of the range of results obtained.

In the first study the appearance of the synovium was analysed at serial time-points after the intra-articular injection of antigen (Figs 5 and 6). The technique gives a measure of both the increasing tissue thickness and the increasing cellularity with time. Polymorphs appear in the tissue at 4 h, then subside, to reappear as the lymphoid cell infiltrate becomes prominent. Lymphoid cell numbers increase steadily over 20 days.

In the second study animals received either 0.05, 0.5 or 5mg of antigen intra-articularly. The appearance of the synovium at 20 days post injection was compared for the different doses (Figs 7 and 8). This study indicates that the inflammatory reaction is related to dose, but also emphasizes the variability of the response, particularly at low dose levels. It confirms the previous suggestion that a consistently aggressive synovitis is only achieved with a dose of 5mg of antigen (Howson *et al.* 1986).

Differences between tissue width at 4h and after 12 days, between total cell counts at 4h and after 12 days, and between lymphoid

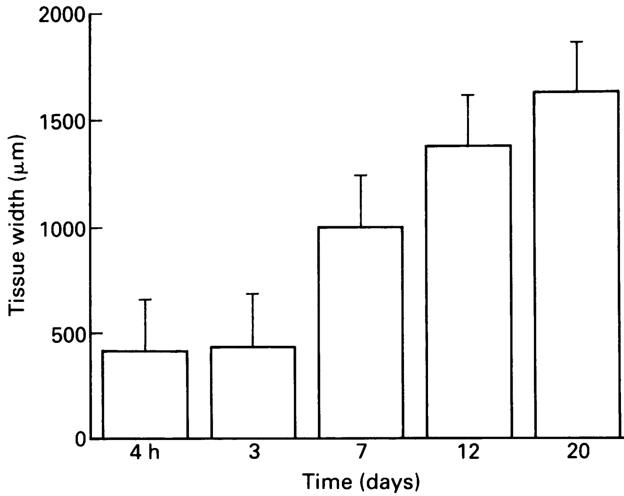

Fig. 5. The relationship between the width of synovial lining and the duration of arthritis. Mean and standard error for tissue width is shown at 4h, 3, 7, 12 and 20 days (three animals per group).

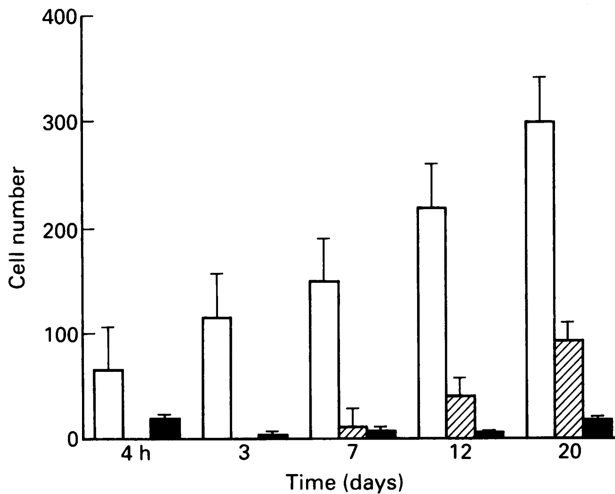

Fig. 6. The relationship between cell numbers in synovial lining and the duration of arthritis. Means and standard errors for total cells, lymphoid cells and polymorphs are shown at 4h, 3, 7, 12 and 20 days (three animals per group). □, Total cells; ■, lymphoid cells; ■, polymorphs.

cells at 4h and at 20 days, were significant at the 5% level. Differences between tissue width at the three dose levels, between total cells at 0.05 and 5mg doses, and between lymphoid cells at 0.05 and 5mg doses were significant at the 5% level. Other differences follow the same trends but were not statistically significant.

Discussion

This study demonstrates that it is possible to obtain quantitative histological information on experimental arthritic joints used for biochemical and cytological analysis of cartilage and synovial fluid (Pettipher *et al.* 1986). This is particularly useful in relation

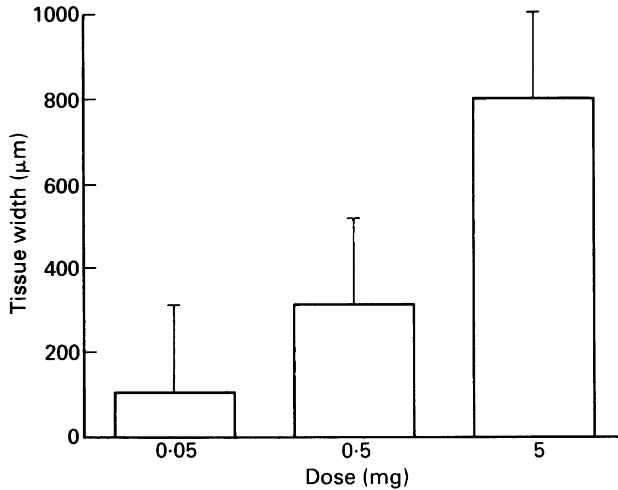

Fig. 7. The relationship between the width of synovial lining and the dose of intra-articular antigen. Mean and standard error for tissue width is shown for 0.05, 0.5 and 5mg doses of antigen (seven animals per group).

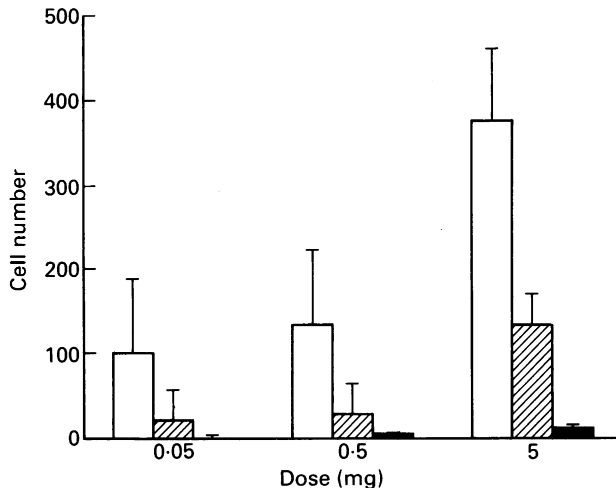

Fig. 8. The relationship between the cell numbers in synovial lining and the dose of intra-articular antigen. Means and standard errors for total cells, lymphoid cells and polymorphs are shown for 0.05, 0.5 and 5mg doses of antigen (seven animals per group). □, Total cells; ■, lymphoid cells; ■, polymorphs.

to the study of cytokines, prostanoids and other mediators, their role in the cellular events in joint disease, and the modifying effects of drugs (Pettipher *et al.* 1986, Henderson & Higgs 1987).

Histological changes can be graded using scoring systems based on reference slides (Gall & Gall 1980). Such scoring systems indicate gross differences, but are insensitive: the counting system described above is more precise. Moreover, it gives measures which can be related to individual cellular events. As an example, counts for polymorphs probably bear a linear relationship to the total

number of polymorphs in the synovium. Taken in concert with measurements of synovial fluid polymorph numbers and mediator levels, these measurements can be used to test hypothetical mechanisms of polymorph influx.

Unless cells are counted, misleading conclusions may be drawn. This is demonstrated by the attempt to assess synovial lining cell numbers and thickness. What appears to be a distinct cell layer is so ill defined and variable, and so interrupted by fibrin clumps, that no measurements could be obtained which are representative of the tissue as a whole.

The main limitation of the technique is in the identification of lymphocyte subpopulations and monocyte derived cells. T and B lymphocytes are not distinguished and larger T lymphoblasts may be missed. Monocyte derived cells cannot be reliably distinguished from tissue fixed cells. Such analysis will require histochemical or immunohistochemical staining. Currently, class II antigen positive cells are identifiable, using monoclonal antibodies, but reliable identification of T lymphocyte populations and monocyte derived cells will require further work. Stains such as non-specific esterase (α-naphthyl acetate) for monocyte derived cells have proved unreliable for quantitative assessment in rabbit tissue. When good reagents are available the method can be adapted for immunohistochemistry using surface decalcification (EDTA) of unfixed tissue.

Howson *et al.* (1986) raised doubts about the validity of AIAR as a model for human rheumatoid arthritis. They conclude that the lesion is too acute and involves cartilage necrosis at high antigen dosage. We agree that chondrocyte death is seen at high dosage, but we have not found the severe lesions which they report, including death from serum sickness. Relatively little is known about acute events in severe cases of rheumatoid arthritis and the changes seen in AIAR over a period of weeks remain closer to those in the human than other available animal models.

To date, animal models of arthritis have tended to be used as methods for assessing 'inflammation' with no attempt to isolate individual events within this process. Experience has shown that suppression of inflammation in human disease may be both ineffective in preventing long-term damage and inseparable from toxic unwanted effects. New therapies must be directed at specific events such as T cell influx. In order to hope to see effects on such events in animal models, more specialized methods are needed. The method described above provides a practical step in this direction.

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