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Modification of the soft tissue response to implanted materials through the use of an anti-inflammatory drug

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The soft tissue response of adult rats to a drug-loaded copolymer hydrogel was studied histologically by using monoclonal antibodies specific for certain inflammatory cell types. A hydrogel was loaded with Diclofenac sodium, a non-steroidal anti-inflammatory drug, and designed to release the drug at a constant rate after implantation into muscle tissue. The sites of antibody binding were analysed automatically, by using an image analysis system, providing information on the number of inflammatory cells and their distribution relative to the hydrogel implant. The ability to measure these and other parameters is considered to be of major importance in the assessment of biocompatibility. The experiments demonstrate that Diclofenac sodium (25–30 $\mu g\,ml^{-1}$) reduced the number of macrophages and neutrophils found at the implant site compared with a PBS control. Diclofenac sodium did not have any effect on the T cell response.

1. Introduction

It is now well established that the tissue response to implanted materials represents a balance between inflammatory and repair processes, and that a large number of factors, relating to the surface chemistry of materials and to the release of substances from them, are able to influence this balance. The use of "inert" biomaterials is based on the premise that the stimulus to acute and chronic inflammation will be maximized with those materials that corrode or degrade most readily. While proving measurably successful with several materials in selected applications, this approach has its limitations and it may prove beneficial in some circumstances to control the inflammatory response in a more active way. One possible way of achieving this involves the use of non-steroidal anti-inflammatory drugs, preferably delivered to the site of inflammation.

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is widespread in many different areas of medicine. In orthopaedics, for example, they have been used in the treatment of rheumatoid arthritis for many years, and may be effective in the treatment of ankylosing spondylitis and osteoarthrosis, as well as controlling periarticular heterotopic ossification following total hip arthroplasty [1], and inhibit the remodelling of traumatized bone [2]. It is self-evident that the continued interest in the use of these drugs in this way is dependent upon a detailed knowledge of the effect of their topical or local delivery to tissues. NSAIDs have been shown to inhibit leukotaxis in response to *N*-formyl-methionine-leucine-phenylalanine (FMLP) in the rabbit anterior eye chamber model [3]. In particular the NSAID used in the present experiments Voltarol (Diclofenac sodium) is very effective in reducing the arthritic swelling of secondary lesions in the rat at very low doses ($0.4\,mg\,kg^{-1}$) [4].

The main mode of action of these drugs is well known, i.e. the inhibition of arachidonic acid metabolism and therefore prostaglandin synthesis [5]. More specifically Diclofenac sodium acts by potent cyclo-oxygenase inhibition, reduction of arachidonic acid release and enhancement of arachidonic acid uptake, thus affecting both the cyclo-oxygenase and lipo-oxygenase pathways [6] and preventing the formation of thromboxane, prostaglandins and prostocyclin. The mechanism of action of these drugs may not be solely due to prostaglandin inhibition but by directly affecting the activation of inflammatory cells [7]. Ibuprofen and Piroxicam inhibit various discrete neutrophil responses, such as aggregation, superoxide generation and lysosomal enzyme release induced by the chemotactic peptides FMLP, Concanavalin A, Phorbol myristate acetate. These actions will affect the influx of inflammatory cells into an area and there is little evidence *in vivo* to suggest that an NSAID effects the number of cells found in an area of inflammation.

A series of preliminary experiments has therefore been initiated with the object of characterizing the host response to locally delivered NSAIDs and determining their potential for controlling the biocompatibility phenomena. For this purpose the tissue response to an NSAID released from an implanted hydrogel has been examined by using a combination of immunocytochemistry and image analysis. It is now

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widely recognized that more sensitive techniques are required to assess the subtle differences between host responses to new biomaterials. Such techniques should overcome the inherent errors involved in bulk subjective assessment of histological sections that, at present, rely on observer-based differentiation of cell morphology. This paper reports on these new techniques and their use in the study of a NSAID biomaterial combination.

2. Materials and methods

The NSAID used in these experiments was Voltarol, the active part of which is Diclofenac sodium, obtained from Ciba Geigy (Horsham, UK). 2-Hydroxyethylmethacrylate (HEMA), 1-vinyl pyrrolidone (NVP), styrene, acrylamide, benzoyl peroxide and ethylene dimethacrylate, all used in hydrogel preparation, were obtained from Aldrich Chemicals (Gillingham, UK). Immobilon anaesthetic, potent neuroleptoanalgesic (C vet, Bury St Edmunds, UK)

2.1. Hydrogel synthesis

For these experiments a constant localized drug release system was required. To achieve this, the drug was encapsulated inside a device enclosed by a polymeric membrane and the rate of drug release was controlled by its permeation through the membrane wall. The device needed to be easy to handle and could be frozen and sectioned *in situ*, avoiding tissue–implant interface destruction. A hydrogel capsule drug delivery system was designed and produced to the following specifications by using the monomers: HEMA, NVP, styrene and acrylamide, at 40, 40, 10, 10 mol % respectively. 1 mol % ethylene dimethacrylate was used as the cross-linking agent and 1 mol % benzoyl peroxide was used as the initiator. An appropriate weight of each substance was placed in a test tube and deoxygenated for 10 min with nitrogen. The mixture was then placed into PTFE moulds. Two moulds were designed, one to form a tube-shaped hydrogel of external diameter 6.0 mm, the second to form plugs for the tubular hydrogel. These moulds were sealed and placed into an incubator to polymerize at 60 °C for three days. The copolymer hydrogel was removed from the moulds and hydrated in distilled H_2O. The hydrogels were rinsed in sterile phosphate buffered saline (PES) for at least two weeks before use. When hydrated the hydrogel was approximately 47% liquid.

2.2. Loading the hydrogel

500 mg of Diclofenac sodium was dissolved in 4 ml of 96% ethanol (125 mg ml^{-1}). Hydrogel cylinders were cut to 1 cm lengths and sealed at one end with a hydrogel plug. 40 µl of the drug solution was placed into the lumen of the cylindrical hydrogel, which was then sealed with another hydrogel plug. The plugs were kept in place by using a small amount of silicone adhesive (Silastic medical adhesive type A, Dow Corning, Mi, USA). For control samples, PBS was loaded into hydrogels in place of the drug.

2.3. Drug release

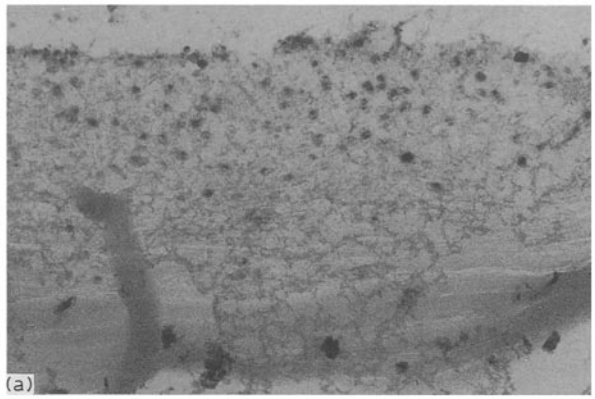
The release of Diclofenac sodium was examined *in vitro* and *in vivo*. With *in vitro* studies, the drug-loaded hydrogel was placed in a universal container with 5 ml of PBS and rotated at room temperature for 24 h. After this time the hydrogel was removed and placed into a new universal container containing 5 ml of fresh PBS, this was repeated each day for 11 days. The amount of Diclofenac sodium released into the PBS was determined by using high performance liquid chromatography (HPLC) [8]. The procedure was repeated five times. *In vivo* drug release was examined by using a rat implantation model. British Standard no. 573 (The evaluation of medical devices, part 2) was observed for the care of these animals. Samples were implanted bilaterally into the dorso-lumber musculature, in black and white hooded lister rats of the Liverpool strain and retrieved at 1, 2, 5, 6, 7, 9, 12, 19, 26, 33, 40, and 47 days after implantation. The amount of drug remaining in the hydrogel was determined by the same HPLC technique. One rat with two hydrogel implants was examined for each time period.

2.4. Implantation for host response studies

Hydrogel samples were autoclaved at 121 °C for 20 min, then loaded with drug or PBS, sealed and left in PBS for two days before implantation. The animal model used was the adult black and white hooded Lister rat of Liverpool strain. For each of the time periods, 1, 2, 5, 7, and 14 days, two drug-loaded and two PBS-loaded hydrogels were implanted bilaterally into the dorso-lumber musculature, two hydrogels per rat. The animals were anaesthetized by using small animal immobilon. An incision was made along the midline and the muscle exposed. Two pockets were created in the muscle by blunt dissection, one either side of the midline, an implant placed into each pocket which was then closed with one Dexon suture. The wound was closed with silk. At the appropriate time periods, animals were sacrificed by using a standard procedure.

2.5. Histology and image analysis

The implant and surrounding muscle tissue was explanted from the rats and frozen by using cardice and isopentane, for cryostat sectioning. As a hydrogel by design contains a high percentage of water it was possible to section the tissue with the implant *in situ*. Serial sections 7 µm thick were cut perpendicular to the cylindrical plane of the hydrogel. Two staining techniques were used to identify the major inflammatory cells on these serial sections. The first involves a rapid enzymatic technique to detect chloroacetate esterase. This enzyme is found almost exclusively in neutrophils and mast cells (Fig. 1a). The second, an avidin–biotin immunohistochemical technique, was used to stain for macrophages (Fig. 1b), T lymphocytes, IL2 receptor sites on T cells and B lymphocytes. Full details of these procedures are published elsewhere [9].



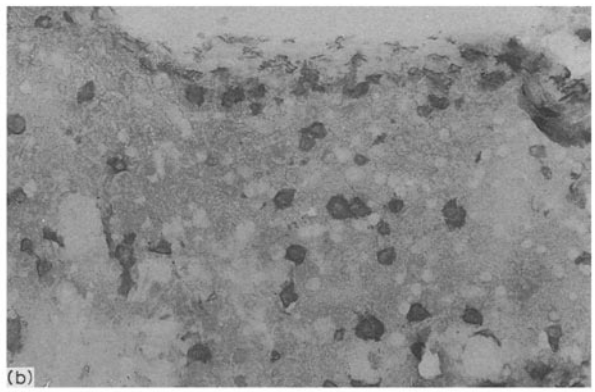


Figure 1 (a) Neutrophils stained by using the chloroacetate esterase technique (× 320). (b) Macrophages stained by using immunocytochemistry technique (× 320).

The system used to analyse the stained tissue sections was a Joyce Loebl Mini Magiscan image analyser, linked to a Carl Zeiss Jenaval microscope at × 200 magnification. The video input to the system was achieved by linking a Hitachi KP140 solid state black and white camera to the microscope. The slides were analysed by using GENIAS (General Purpose Image Analysis Software) to give access to many image manipulation and analysis options. Image manipulation includes functions to separate clumps of cells and count them as individuals, an essential function in any cell counting technique. Full details of this technique are in press [10].

From each section a series of 20 black and white images were captured and stored. These 20 images correspond to a measured distance 624 μm away from the implant, providing a total analysed area of 2.06×10^6 μm^2 for each section. A large number of parameters were selected and measured in this analysed area, including total cell number, area, circularity, and centre of gravity (x and y co-ordinates of an object). This provides relative position and enables overall cellular distribution around the implant to be calculated. A number of other measurements (up to 25) were made. The results from each tissue section were stored in a data file for analysis. SAS (Statistical Analysis Software, SAS Institute Inc., Cary, USA), ver. 6.03, was subsequently used to plot the data shown in the next section.

3. Results

Fig. 2a shows the amount of Diclofenac sodium released in the *in vitro* study. The results are expressed as the mean and standard deviation of five experiments. Fig. 2b shows the amount of Diclofenac sodium remaining in the hydrogel at the measured time periods *in vivo*.

The cellular response to all implants predominantly involved the neutrophil, macrophage, and T cell. No B cells were observed in any tissue sections. The "total number of cells" refers to a measure of the number of cells observed in the two-dimensional 2.06×10^6 μm^2 area. The bivariate graphs of Figs 3 and 4 show the distribution of each cell type counted around each implant.

For the hydrogel/PBS control, Fig. 3a shows the distribution of neutrophils around the implant site as a function of time. There are more neutrophils at the 1 and 2 day time periods, (maximum 286 cells at 2 days), than at the 5, 7, and 14 day. The numbers of cells are greatest near the implant interface. The macrophage response (Fig. 3b) seems fairly constant with time, with a maximum of 608 cells at 5 day time period. They appear to be evenly distributed throughout the tissue. There is a large T cell response (Fig. 3c, d), which is evenly distributed throughout the area measured. The maximum number of T cells counted with CD8 type antibody was 735 at 2 days. The maximum number of T cells counted using the IL2 receptor antibody was 1659 at 1 day. The numbers of cells appears to tail off by 7 and 14 days.

For the hydrogel/Diclofenac sodium, there were very few neutrophils; Fig. 4a shows cell numbers increasing to a maximum of 182 cells at 2 days, then tailing off to zero by 7 and 14 days. Those cells that were counted at the 1, 2 and 5 day periods were concentrated at the implant–tissue interface. The neutrophil response has completely disappeared by 7 and 14 days. The number of macrophages (Fig. 4b) is also low, but constant for all time periods up to 7 days. The maximum number of macrophages, 334, was found at 14 days, by which time their distribution has changed from fairly even throughout the area measured to an accumulation at the implant surface. The T cell (Fig. 4c, d) response was very large at 1 day; 949 cells with the CD8 type antibody and 1661 cells with the IL2 Receptor antibody. The number of T cells counted with both antibodies decreased with time.

4. Discussion

After the first day, a constant drug delivery rate of 25–30 μg ml^{-1} per day was achieved *in vitro* by using this hydrogel design (Fig. 2a). This result led to the loaded hydrogels being left to stand in PBS for two days before implantation, therefore missing this "burst" effect *in vivo*. The *in vivo* drug release experiments confirm that the drug was being released into the animal (Fig. 2b). Comparing Fig. 3a with Fig. 4a, the 1 and 2 day time periods show a change in the pattern of neutrophil distribution around the implant due to the drug. Neutrophils concentrate at the site of

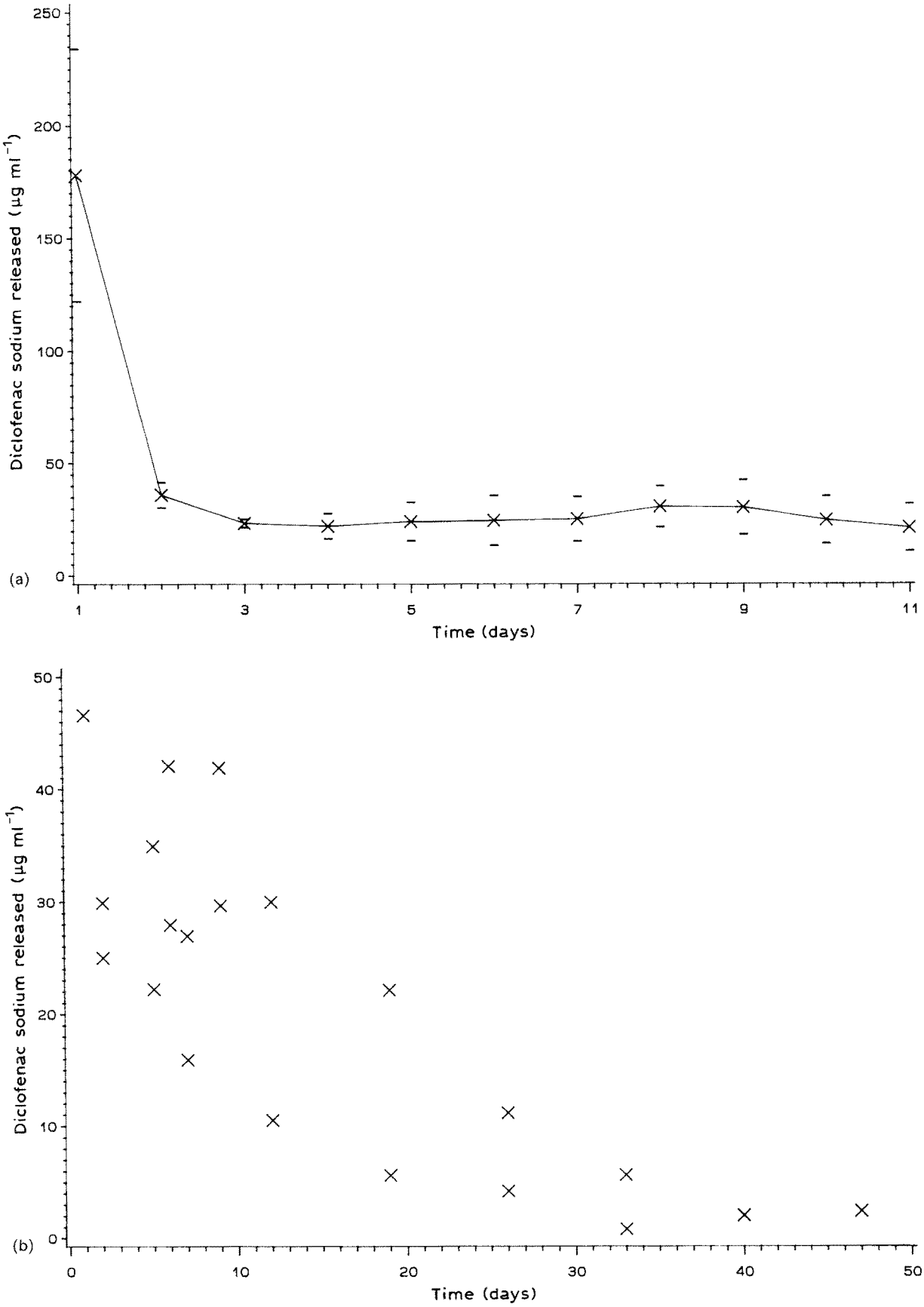


Figure 2 (a) Diclofenac sodium released from copolymer hydrogel *in vitro*. (b) Diclofenac sodium remaining in the hydrogel following implantation.

the drug-loaded hydrogel, compared to a more gradual tailing off with distance from the implant site in the PBS/hydrogel. The number of cells is less at all time periods in the drug-loaded hydrogel than the PBS/hydrogel, despite the differing distribution pattern. The combination plot of neutrophil results (Fig. 5a) demonstrates this effect on total neutrophil cell numbers.

Analysis of the macrophage distribution, Fig. 3b and 4b, around an implant showed that the pattern of

distribution of these cells was unaffected by the NSAID up to seven days. The drug-loaded hydrogel distribution pattern appears to show a peak at the implant at 14 days. The precise reason for this peak is unclear but it could be due to local changes in drug concentration. The total numbers of these cells did differ in each type of hydrogel. A combination plot (Fig. 5b) of the macrophage results shows that there was a reduction in the total number of macrophages at all time periods in the presence of the NSAID. From

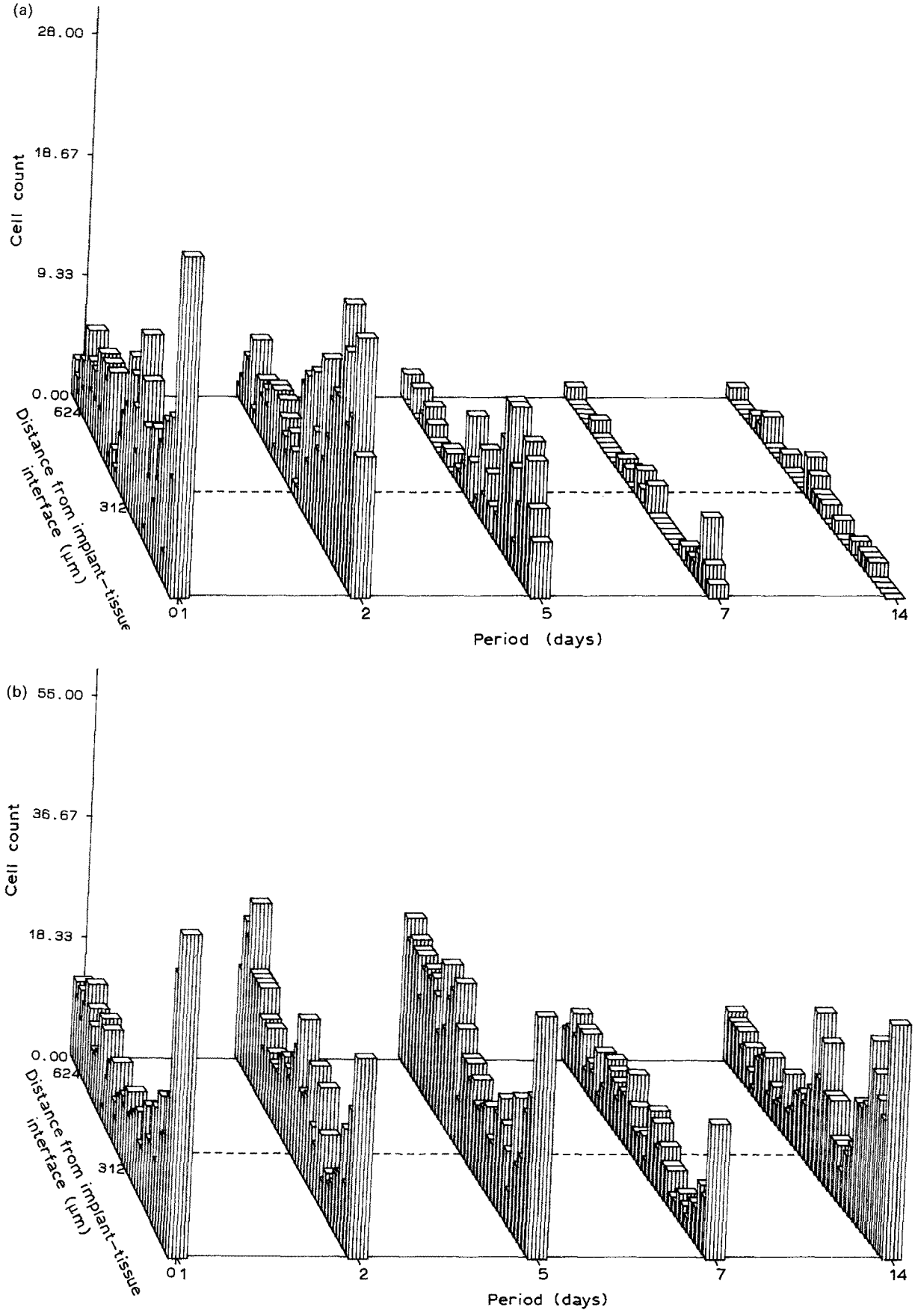


Figure 3 Cell distribution around implants of hydrogel equilibrated in PBS: (a) neutrophils; (b) macrophages; (c) T-lymphocyte (CD8 receptor); (d) T-lymphocyte (IL2 receptor).

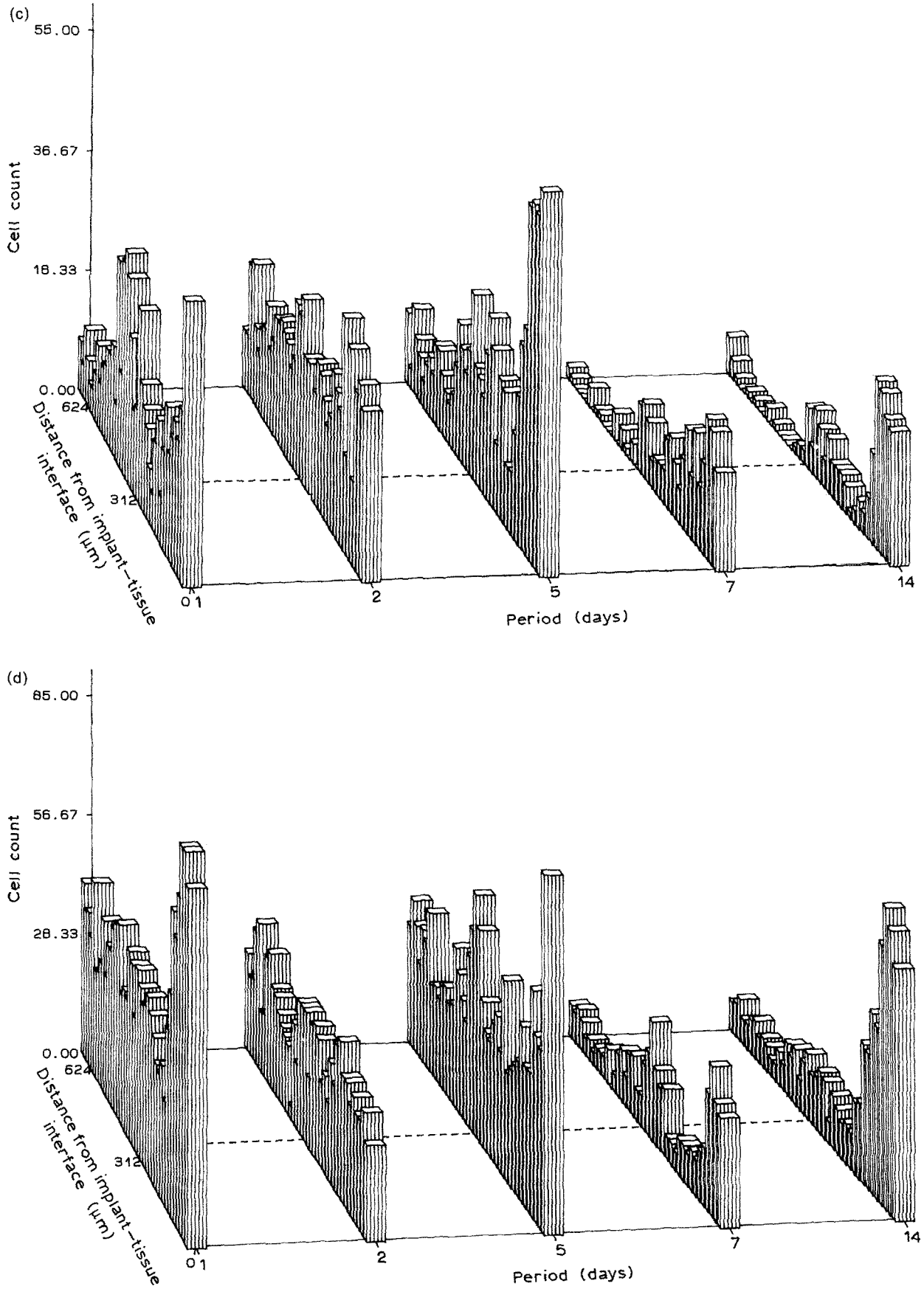


Figure 3 Continued

these results it appears that Diclofenac sodium does have an effect on the tissue response to the hydrogel, reducing the total number of cells found at the implant site, and also altering their distribution with respect to the implant. This ability to modulate the number of inflammatory cells at an implant site makes NSAIDs very interesting for their potential to improve the biocompatibility of materials.

For T cells the modulation of the response was not quite as clear cut. Examination of combination plots,

Fig. 5c, d, shows that the drug did not appear to have much effect on T cell numbers. The discrepancies between the two antibody results for T cells demonstrate that, as with any system of analysis there are many parameters to consider. The two monoclonal antibodies have quite different antibody binding sites. These two monoclonals were used to demonstrate the different activation states that exist in cell populations around implants. From these results it appears that on activation of the T cells there is a down regulation in

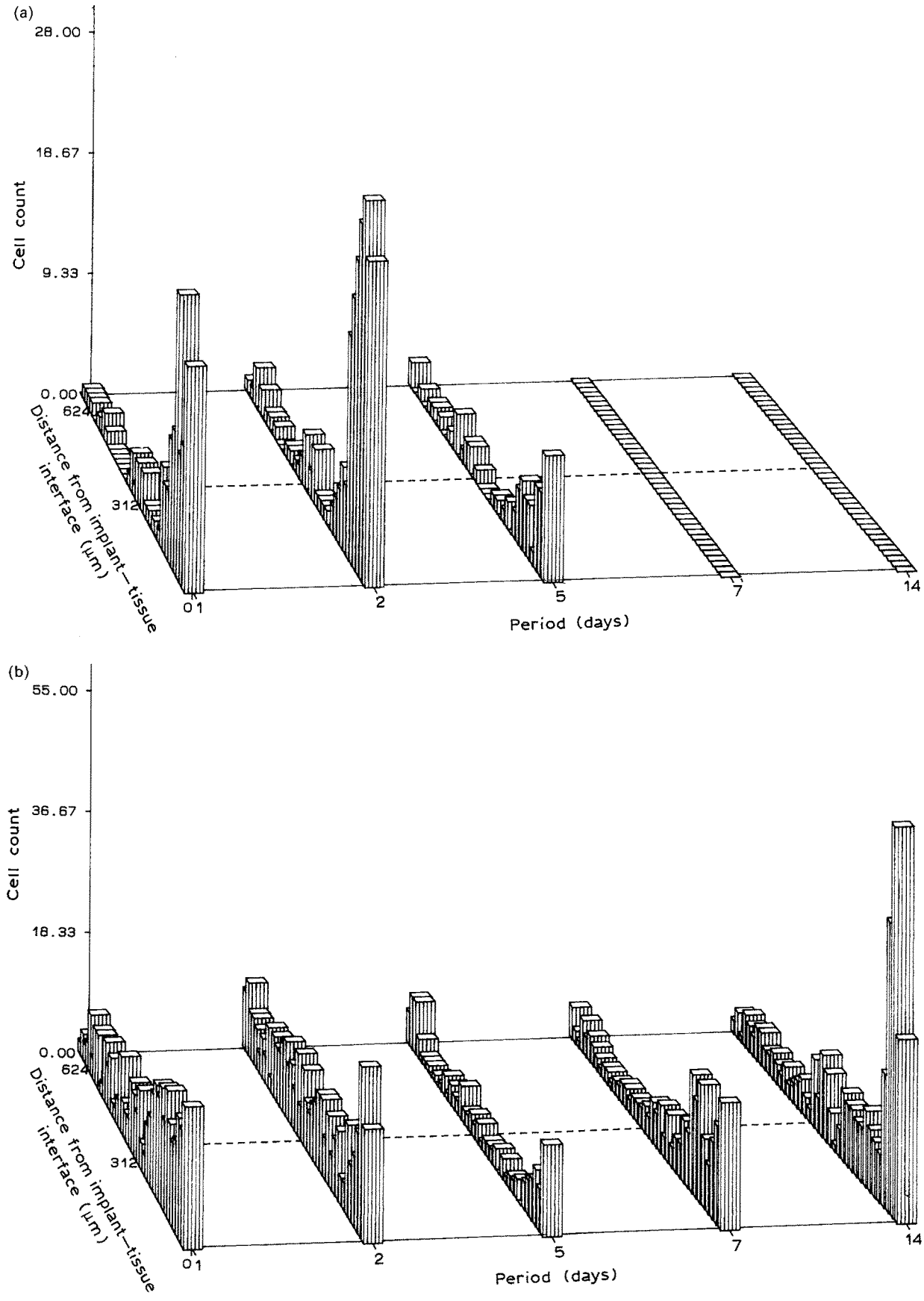


Figure 4 Cell distribution around implants of hydrogel equilibrated in Diclofenac sodium: (a) neutrophils; (b) macrophages; (c) T-lymphocytes (CD8 receptor); (d) T-lymphocytes (IL2 receptor);

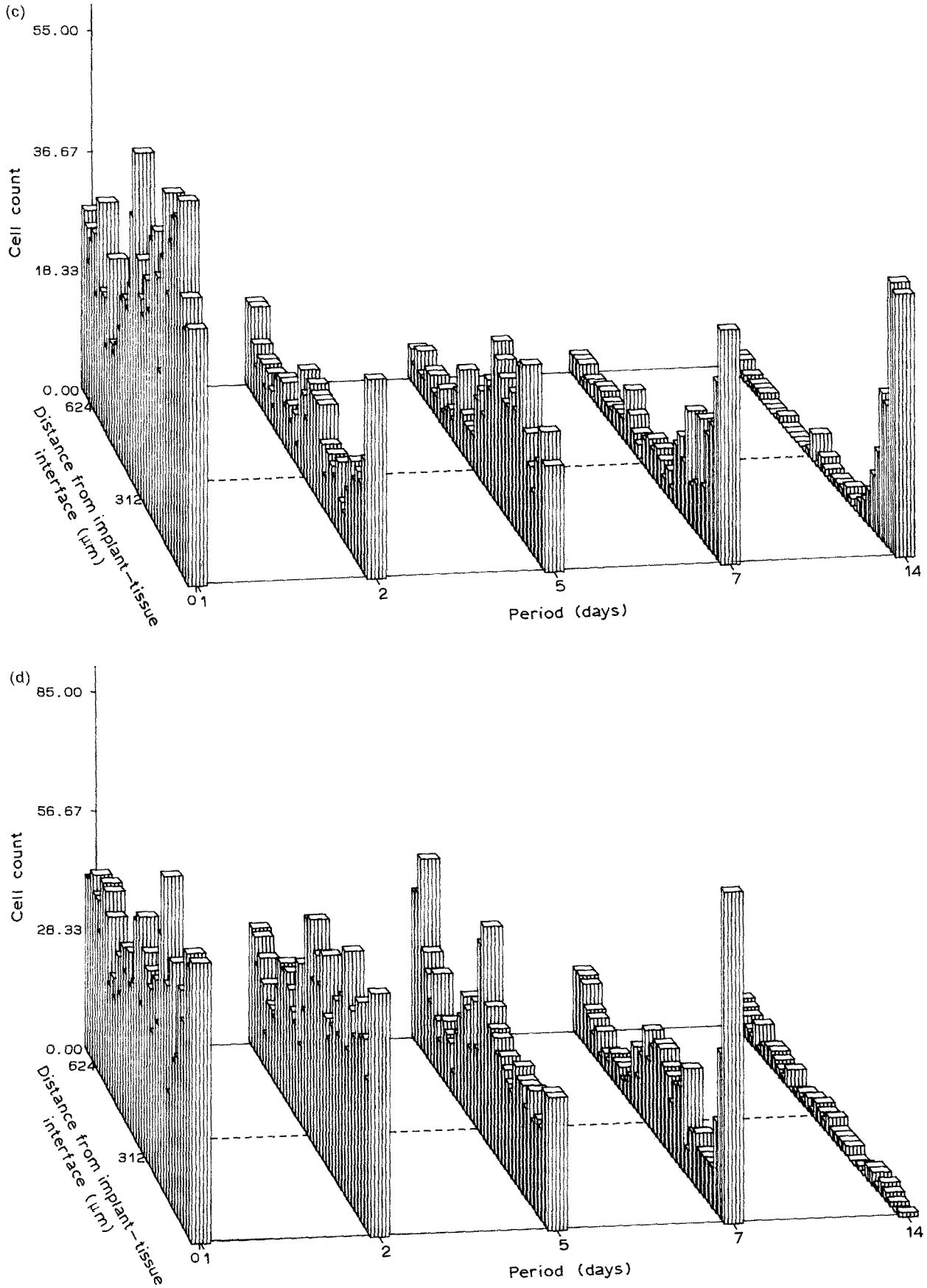


Figure 4 Continued

the number of T cell receptor sites highlighted by CD8 antibody and a concomitant increase in IL2 receptor sites. Examination of Fig. 5c,d shows that the two lines, T cells and IL2 receptors, although at different magnitudes, follow the same pattern. This T cell response is possibly due to the hydrogel acting as a thymus-independent antigen, e.g. as a mitogen like concanavalin A, non-specifically stimulating proliferation of T cells. Work is currently being carried out *in vitro* to confirm this theory. This effect would also

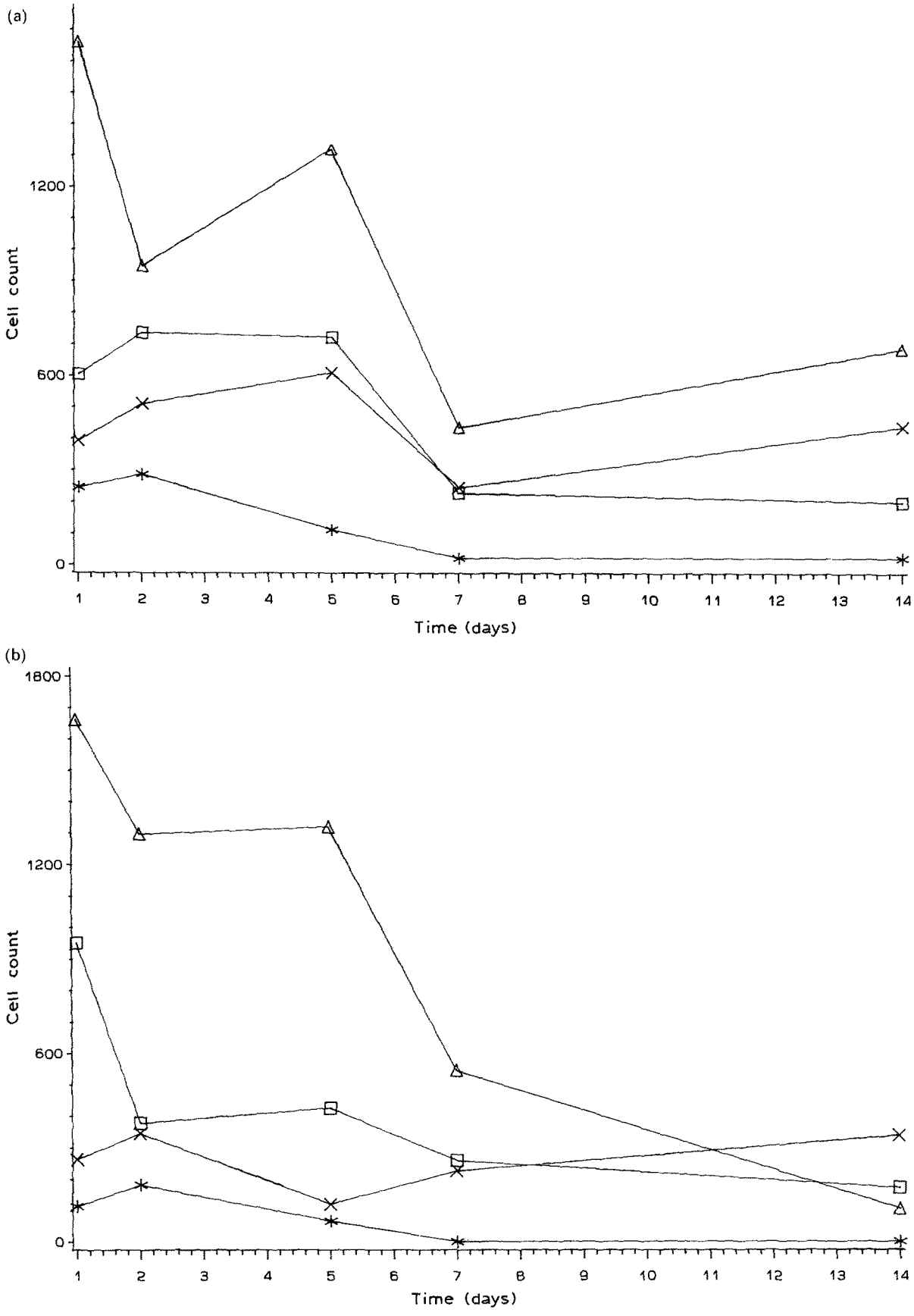


Figure 5 Total cell numbers around implants as a function of time: (a) hydrogel equilibrated in PBS; (b) hydrogel equilibrated in Diclofenac sodium. Cell type: (×) macrophages, (*) neutrophils, (□) T cells, (△) IL2 receptors.

explain why this T cell response was not accompanied by a larger macrophage response.

These results also demonstrate, that automatic quantification of histological sections by using a combination of immunocytochemistry and image analysis can be an effective way to assess tissue responses, while at the same time eliminating the variation inherent in human assessment. The image analysis system detailed here provides more data than could ever be produced manually. It not only provides cell counts,

but cell distribution, total cell area and other parameters in a fashion that can be easily and consistently repeated.

5. Conclusions

Diclofenac sodium did affect the soft tissue response to the copolymer hydrogel by modulating the numbers of macrophages and neutrophils found at the implant site. It did not affect the T cell response, which is possibly independent of the macrophage and neutrophil response.

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