Studies of cartilage degradation in vivo

Al-Duaij, Ahmed Yousuf Mohamed

STUDIES ON CARTILAGE DEGRADATION IN VIVO

A thesis presented

by

AHMED YOUSUF MOHAMED AL-DUAIJ

for the degree of

DOCTOR OF PHILOSOPHY IN

THE UNIVERSITY OF LONDON

Department of Experimental Pathology

St. Bartholomew's Hospital Medical College

UNIVERSITY OF LONDON

1985

ABSTRACT OF THESIS

A new method has been established to study cartilage breakdown *in vivo*. Cartilage was implanted into air pouches on the backs of mice or rats and loss of proteoglycan measured biochemically.

Using the air pouch it was possible to produce various inflammatory environments either immune or non immune and examine the effects of these upon proteoglycan loss. It was found that the various type of inflammation failed to accelerate proteoglycan loss from implanted cartilage. Subsequently a variety of drugs used in the treatment of the arthropathies were examined for their effect on both inflammation and cartilage breakdown.

Using xiphisternum a difference could be shown between non-steroidal anti-inflammatory drugs (NSAID) and d-penicillamine in that, NSAID failed to protect the cartilage whereas d-penicillamine prevented proteoglycan loss. This type of cartilage was not examined further, as it was not characteristic of articular cartilage - being surrounded by perichondrium.

Later articular cartilage was implanted once again into different inflammatory situations and drug effects evaluated. It was found using this cartilage that all the drug used protected from loss of proteoglycan and whereas some inhibited inflammation (indomethacin and dexamethasone),

levamisole had no effect in contrast, d-penicillamine potentiated the inflammatory response. Finally the mode of action of the drugs in this method is discussed.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Professor D·A· Willoughby for giving me the opportunity to work in his department and for his valuable supervision and constant encouragement. His informed criticism and skill in being able to condense and clarify my often confused English have been invaluable.

I wish to thank Dr A·D·Sedgwick for his support and advice throughout this work and for reading the manuscript.

I would also like to thank Dr F·B·de Brito for providing important information relating to the work concerned with this thesis.

To Mr A·R·Moore a special debt of gratitude for his excellent technical assistance in particular the preparation of the histological material·

Above all I would like to thank my wife and daughters who have suffered so much during the preparation of this thesis and without whose support this would not have been possible.

TABLE OF CONTENTS

INTRODUCTION

HISTORICAL REVIEW OF THE LITERATURE

GENERAL INTRODUCTION

Florey (1970) pointed out the difficulty in finding an acceptable definition by most pathologists for the term "inflammation". This author considered the definition of Burden-Sanderson (1871) as the most suitable, in which inflammation is described as "the succession of changes which occur in a living tissue when it is injured, provided that the injury is not of such a degree as to at once destroy its structure and vitality". Ebert (1965) expressed inflammation as "the process that begins following a sublethal injury to tissue and ends with complete healing". More recently, Ryan and Majno (1977) defined inflammation as "the response of living tissue to local injury, that leads to the local accumulation of blood cells and fluid, and the overall process, seen against the broad perspective of evolution, is a useful one - its primary significance being (in all likelihood) that of a defence against microscopic invaders".

Inflammation was known to the ancients by the appearances it produced in the skin and other surfaces of the body. The four main cardinal signs in this situation were summarised by the Roman encyclopaedist, Cornelius Celsus, (BC 30 - AD 38), as ruber (redness), tumor (swelling), calor (heat) and dolor (pain). This definition is so precise that it has still not been improved upon, except that now a fifth sign of inflammation has been added. This is functio laesa (loss of function). This was originally attributed to Galen (AD 130 - 200), the first person who wrote extensively on inflammation, but this was since proved to be untrue and this was in fact contributed by Rudolph Virchow (1821 - 1902) in his cellular pathology, published in 1858

(Ryan and Majno , 1977).

Boerhaave (1668 - 1738) laid much emphasis on the change of state of the blood vessels in inflammation. He believed that there was an "excess of blood in the inflamed part, due to increased hydrostatic pressure and to the friction of the red arterial blood in the smallest canals".

The work of Cowper, Hallen and Spallanzani in the 17th and 18th centuries, established that the redness of the inflamed part was due to the distension of the small blood vessels, and not, as first believed, to the passage of blood into vessels not normally containing it or to the passage of blood into the vessels.

John Hunter (1794) re-introduced the important conception formerly adumbrated by Galen, that inflammation is not a disease but a useful process, and can be initiated by any injury, for instance an injury caused by pressure, friction, heat, cold or "air in wounds".

William Addison (1843) was the first to undertake microscopic studies of inflammation. The microscope allowed the observation of blood flow and its corpuscles in the web of the frog's foot. Addison showed that blood vessels responded to irritant by becoming engorged. He observed the extra-vascular appearance of white corpuscles in pus and that white cells in the inflamed foot could be immobilised along the inside of the blood vessels.

Further interest was taken in the involvement of cells and inflammation when Augustus Waller (1816 - 1870), in an additional postscript to his essay "Microscopic examination of some of the principal tissues of the animal frame, as observed

in the tongue of the living frog or toad", described pus as originating from the extravasation of the colourless or spherical corpuscles from the capillaries. Waller had described diapedesis of leucocytes, that is their emigration from the blood vessel to the tissue.

A great step forward in understanding inflammation and in diffusing knowledge of it, was taken by Cohnheim (1867) when he observed, and gave detailed and accurated descriptions of, the events to be seen during inflammation of the transparent tissue, such as the web and tongue of the frog. Much of Cohnheim's work was carried out on frog mesentery, the alveolar cornea of the frog and rabbit having been discarded as unsuitable tissue for the study of inflammation. The dilatation, especially of small arteries, and the loss of axial flow within them, was observed by Cohnheim in the inflamed mesentery. White corpuscles appeared to take up a more general disposition throughout the lumen, adhering to the vessel walls, through which their migration into extravascular tissue was closely followed. Cohnheim was aware of the growing importance of the vascular events in inflammation, which led him to propose that "without blood vessels there is no inflammation".

In 1862, Haeckel observed that the blood vessels of tethys (a gastropod mollusc) could phagocytose particles. This was followed by the classical work of Metchnikoff (1883), who was responsible for developing the concept that phagocytic cells form the defence mechanism of the metazoan body.

The concept that inflammation is a process involving both vascular and cellular components has remained with us to the present day definition. Spector (1977) defines inflammation as

"the response of the living microcirculation and its contents to injury".

VASCULAR PERMEABILITY

Numerous studies have been done to examine the increased vascular permeability seen in inflammation. The relevant investigations have been presented in tabulated form.

Investigations into the mechanisms of increased vascular permeability

Author	Year	Author's contribution to the understanding of vascular permeability.
Starling	1896	showed that protein in solution exerts osmotic pressure and the endothelial membrane is highly permeable to water and salts, but not protein.
Florey	1925	colloids pass through the cytoplasm of endothelial cells.
Chambers and Zweifach	1947	endothelial cells contract during increased vascular permeability, giving rise to interendothelial cell gaps.
Pappenheimer	1953	developed the molecular sieve theory. Permeability depends upon pores in capillary wall.
Majno, Palade and Schoeffl	1961	used electron microscopy to observe the blood tissue movement of carbon particles induced by histamine, in the cremaster muscle. Found vascular leakage occurred in venules of 20-30μ diameter and attributed leakage to the formation of gaps between endothelial cells.
Cotran and Majno	1964	delayed, prolonged vascular leakage (eg, that caused by bacteria or x-ray) was mainly confined to capillaries, for which certain unknown mediators appeared responsible.
Majno and Leventhal	1967	proposed the activation of a contractile mechanism within venular endothelial cells during increased vascular permeability induced by histamine, 5HT and bradykinin .

Renkin, Carter and Joyner	1974	histamine and bradykinin stimulate turnover rate of endothelial vesicles.

Investigations concerning humoral factors involved during increased vascular permeability in inflammation

General aspects

Author	Year	Author's contribution to the understanding of humoral factors
Eichwald	1864	described the presence of peptone, a
Hofmeister	1880	proteolytic enzyme, in pus.
Opie	1906 1922	demonstrated two proteases (leuco protease and lymphoprotease) in pleural exudates of dogs.
Krogh	1920	dilatation of capillaries in the frog tongue, induced by application of iodine, was abolished by pre-treatment with cocaine - implicated the axon reflex in the mechanism of vasodilatation.
Rous, Gilding and Smith	1930	referred all vasodilatation to the action of a single chemical substance liberated within the tissue.

HISTAMINE

Barger and Dale	1910 1911	demonstrated that histamine is produced by various organ extracts.
Dale and Laidlaw	1910 1911 1918	described some of the pharmacological properties of histamine, which included contraction of smooth muscle.
Sollman	1916	intradermal histamine produced a state similar to urticaria, characterised by formation of a localised weal.
Dale and Richards	1918	described vasodilator action of histamine. Intravenous injection of histamine produced a marked reduction in blood pressure and symptoms resembling those of anaphylactic shock.

Bloom	1921	histamine induced oedema formation.
Lewis	1927	described the "triple response" which characterised various types of injurious stimuli, and in which histamine was implicated.
Zweifach	1953	suggested that increased vascular permeability, following application of histamine to rat mesoappendix, may be a result of contraction of endothelial cells.
Spector and Willoughby	1957	demonstrated the role of histamine in the early stages of turpentine induced pleurisy and thermal injury in rats.
Anderson, Glover and Rabson	1977	showed that histamine inhibits neutrophil chemotaxis.
Lehmeyer and Johnston	1978	showed the ability of histamine to interfere with superoxide anion (O_2^-) production from human phagocytes.
Robinson	1982	showed that histamine inhibits granulocyte adherence.
Fantuzzi et al	1984	histamine inhibits lysosomal enzyme release from human neutrophils stimulated by a synthetic chemotactic peptide (N-formyl-methionyl leucyl phenylalinine).

5-HYDROXYTRYPTAMINE (5HT)

Erspamer	1954	isolated 5HT from enterochromaffin tissue of the gastro-intestinal tract.
Humphrey and Jaques	1954	showed that platelets were a rich source of 5HT.
Parratt and West	1957 a,b	inhibited 5HT-induced foot oedema in rats by pre-treatment with 2-bromo-d-lysergic acid, an antagonist of 5HT.
Spector and Willoughby	1957	demonstrated the presence of 5HT in early pleural exudates induced by turpentine in rats, but were unable to suppress the reaction with 5HT antagonists.
Collier	1958	demonstrated the wide distribution of 5HT in blood, spleen, central and peripheral nervous system.

POLYPEPTIDES

Menkin	1936	proposed a chemical mediator 'leucotaxine' (a polypeptide) which could alter capillary permeability. This polypeptide demonstrated in turpentine pleural exudate, was found to have no similar properties in common with histamine.
Duthie and Chain	1939	leucotaxine thought to be a mixture of polypeptides.
Rocha e Silva, Beraldo and Rosenfeld	1949	discovered 'bradykinin'. This substance was released by the action of trypsin or snake venom on plasma substrate. It was able to induce the slow contraction of guinea pig ileum.
Spector	1951	described the isolation from inflammatory exudates and hydrolysate of fibrin (by pepsin) of polypeptide fractions, some of which were able to induce vascular permeability.
Elliot, Horton and Lewis	1960 a,b	bradykinin, purified and classified as an octapeptide-induced, increased vascular permeability in guinea pigs and rabbits, and caused pain when applied to the exposed base of cantharidin blister in man.
Lewis	1961	participation of bradykinin in functional dilatation and inflammation. Believed that bradykinin was formed and destroyed in the interstitial fluid.
Zweifach	1966	observed topical application of bradykinin lead to pronounced increase in flow through blood vessels at high concentrations. Increased permeability in post capillary venules independent of histamine.
Kaplan, Mayer and Austen	1972	showed that kinins are chemotactic for neutrophils.
Nesjletti and Malik	1979	found that kinins are potent prostaglandin-releasing agents.

PROSTAGLANDINS

Bergstrom, Duner, Von Euler, Pernow and Sjovall	1959	showed infusion of prostaglandins into forearm produced erythema.

Horton	1963	prostaglandins increased vascular permeability in guinea pigs.
Willoughby	1968	prostaglandins $PGF_2\alpha$ produced veno-constriction, antagonist of vascular permeability induced by histamine, 5HT, bradykinin and lymph node permeability factor.
Willis	1969	identified PGE in rat inflammatory exudate.
Crunkhorn and Willis	1971	PGE1 and PGE2 increased vascular permeability and were as potent as histamine, bradykinin or 5HT.
Di Rosa, Giroud and Willoughby	1971	proposed sequential release of mediators, histamine, 5HT, kinins and prostaglandins during non-immune inflammation.
Velo, Dunn, Giroud, Timsit and Willoughby	1973	suggested that prostaglandins $F_2\alpha$ acts by controlling increased vascular permeability in inflammation.
Williams and Morley	1973	found that prostaglandins were less active than histamine and bradykinin at increasing vascular permeability, and that they were able to potentiate the action of these mediators. In general, potentiation was greater with bradykinin than histamine.
Moncada, Needleman, Buting and Vane	1976	demonstrated that thromboxanes were involved in vasoconstriction and were the major prostaglandin-related mediator of platelet aggregation.
Moncada, Higgs and Vane	1977	showed that prostacy lin (PGI2) caused vasodilatation and inhibited platelet aggregation.
Davidson, Ford - Hutchinson, Smith and Walker	1978	prostacylcin potentiates histamine and bradykinin in carrageenan paw oedema.
Orning, Hammerstrom and Samuelsson	1980	demonstrated that the major component of slow reacting substance of anaphylaxis are the leucotrienes C4 and D4.

Other factors found to be involved in vascular permeability

Author	Year	Author's contribution
Miles and Wilhelm. Wilhelm, Miles and Mackay	1955	showed permeability factor dilute (PF dil) which increased vascular permeability and was activated when guinea pig plasma activated.
Spector	1956	demonstrated a permeability factor associated with the more soluble globulins. Present in active exudate.
Ratnoff and Lepow	1963	showed that purified C'I esterase increases vascular permeability of guinea pig skin.
Willoughby and Spector	1964	described lymph node permeability factor. This factor extracted from lymph nodes, and was found to increase the vascular permeability.
Cochrane and Muller-Eberhard	1968	described two cleavage peptides, C_3a and C_5a, which increased vascular permeability by inducing histamine release.
Willoughby, Polak and Turk	1968	depletion of serum complement caused decreased vascular permeability responses in allergic and non-allergic inflammation.
Pick et al	1969	showed the supernatants of lymph node cells (from guinea pigs injected with tubercle baccilli) incubated with purified protein derivative, produced intense inflammatory reactions when injected into guinea pig skin (skin reactive factor).
Dumonde et al	1969	interaction of specific antigen BCG with sensitised lymphocytes produced a soluble factor, which increased vascular permeability. They proposed the name lymphokine for this factor.
Williams and Morley	1974	described the vascular permeability produced by factors released from sensitised lymphocytes. These factors have been named lymphokines.
McManus et al	1981	intravenous injection of platelet activating factor (PAF) results in acute, transient increase in systemic vascular permeability.

Author	Year	Author's contribution
Humphrey	1982	intradermal administration of PAF induces leucocyte adhesion and subsequent vessel infiltration accompanied by vessel hyperpermeability leading to regional exudation of plasma (extravasation).
Williams et al	1984	showed that interaction between neutrophils and microvascular endothelial cells leads to cell emigration and plasma protein leakage.
Brain et al	1985	described a calcitonin gene-related peptide as a potent vasodilator.

CELLULAR ASPECTS OF THE INFLAMMATORY RESPONSE

In 1871, Virchow emphasised the importance of cells in inflammation. He postulated that inflammation was a result of increased activity of cells searching for a suitable source of food in the surrounding tissue. Virchow postulated that vascular changes seen were only secondary. This concept was different to that held by Cohnheim (1867) who propsed that inflammatory cells originated from the blood stream.

In 1882, Metchnikoff stressed the role of leucocytes in inflammation and their emigration and phagocytosis as a means of removing noxious irritants. At the end of the 19th century, there was great controversy concerning the origin of inflammatory cells. Metchnikoff had used the term 'microphage' to designate the polymorphonuclear cell in the blood and tissue, in order to distinguish them from the larger mononuclear phagocytic cells - the macrophages.

A considerable amount of information has been published on physiology and origin of the polymorphonuclear leucocyte since

the days of Metchnikoff. The large numbers of granules present in the cytoplasm of these cells have been shown to contain different hydrolytic enzymes, such as acid phosphatase, acid and neutral hydrolases and peroxidases (Ackerman, 1968; Wetzel, Spicer and Horn, 1967; Bainton and Farquhar, 1968). Abundant glycogen deposits have been found in these cells with little or no smooth or rough endoplasmic reticulum present (Scott and Still, 1968; Watson and Tielemans, 1971).

Goud, Schotte and Van Furth (1975) showed that polymorphonuclear leucocytes were derived from bone marrow stem cell and that they were a separate cell line from the mononuclear phagocytic cell, the myeloblast and monoblast having very different characteristics. Several factors have also been shown to regulate polymorphonuclear cell release and production (Bierman, 1964; Kampschmidt et al, 1977; and Baum et al, 1978). As these cells are short lived, with no capacity for mitotic division (Spector and Wynne, 1975), they die in large numbers during inflammation.

A chronic inflammatory infiltrate, especially if granulomatous, consists of cells derived from three lines - the mononuclear phagocyte, the lymphoid and the fibroblast. Mononuclear phagocytes are seen as macrophages, epitheloid cells or giant cells. According to Spector and Willoughby (1974), the true architectural unit of chronic inflammation is almost always the macrophage and its derivatives, the epitheloid cell and the giant cell.

Mallory (1898), Permar (1924) and Foot (1925) proposed that the macrophage of the inflammatory reactions was derived from the endothelial cells of blood capillaries. Sabin, Doan

and Cunningham further reinforced this view in 1925. Using the hanging-drop technique described by Lewis (1925, 1926), they claimed to see the development of 'clasmatocytes' (macrophages) from the endothelium of chick embryo.

The idea that lymphocytes were capable of transforming into macrophage during an inflammatory reaction was first put forward by Metchnikoff (1888), and then by Maximow (1927). Downey (1917) had supported this view by demonstrating the ability of alleged lymphocytes to phagocytose vital dyes in the circulation, which he temporally arrested between a double ligature.

Lewis (1925, 1926), by using the hanging-drop technique, was able to observe the development of macrophages from monocytes. The hanging-drop technique consists of a drop of blood or plasma being placed onto a small glass coverslip, which is then inverted and sealed over a hollow, ground slide, and examined under the microscope..

Clark, Clark and Rex (1936) were able to show that endothelial cells were not transformed into leucocytes during microtrauma induced in a tadpole's tail by injection of croton oil. They believed that the nuclei of polymorphonuclear leucocytes 'rounded up', giving rise to the relatively high numbers of apparently mononuclear cells observed in the latter stages of the reaction.

Ebert and Florey (1939) used a modified Sandison-Clark (rabbit ear) chamber to study the extravascular development of the blood monocytes. The method consisted of fixing two glass coverslips on both sides of a hole which had been punched through the ear of a rabbit. They concluded from their experiments

that monocytes could develop and become indistinguishable from tissue histiocytes.

Volkman and Gowans (1965 a,b) studied the inflammatory reaction induced by subcutaneous implantation of glass coverslips in rats linked by parabiosis, in an attempt to find the origin of inflammatory macrophages. They were able to show that exudate cells were derived from the blood. In further studies, they demonstrated that lymphocytes did not transform to macrophages. They also showed that lymphocyte depletion, either by drainage of the thoracic duct or by x-irradiation at 400 rads, did not alter the macrophage components of the cellular exudate. However, x-irradiation at 750 rads resulted in decreased emigration of macrophages into the inflamed area, and this effect was prevented when the tibial bone marrow was shielded. However, by injecting labelled bone marrow cells, spleen cells, lymph node and thymus cells, they concluded that bone marrow, and to a lesser extent the splenic cells, were the major source of macrophages emigrating from the circulation into the inflamed site.

Spector, Walters and Willoughby (1965) used rat leucocytes labelled with either colloidal carbon or tritiated thymidine or with both markers. Since monocytes are phagocytic and have a higher rate of DNA synthesis than lymphocytes, this is reflected in a much higher uptake of tritiated thymidine and the intensity of ingested carbon particles. On the basis of these principles, these authors found the mononuclear cells of acute inflammatory exudates originated almost entirely from circulating monocytes, not lymphocytes. Spector and Willoughby (1968) demonstrated the lack of lymphocyte participation in the

reaction to incomplete Freund's adjuvant in the rat. They also investigated the reaction to complete Freund's adjuvant which was closely similar to that of incomplete Freund's adjuvant until four weeks, when foci of small round cells resembling lymphocytes appeared. Lesions of this type and age only were found to admit cells from the lymph nodes. Everett and Tylor (1968) also supported the development of macrophages from blood monocytes in inflammatory exudates.

Sutton and Weiss (1966) demonstrated the formation of epitheloid and giant cells from avian monocytes. Mariano and Spector (1974) studied the same subject, in vivo, following the subcutaneous implantation of glass coverslips into mice. They concluded that lymphocytes do not participate in giant cell formation. With the aid of the electron microscope, they presented evidence supporting the concept of macrophage fusion as a mechanism of giant cell formation. It was also established that emigration of fresh mononuclear cells from the circulation was required to maintain the process.

Leucocytes' movement from blood vessels into the inflammatory site and the sequence of cell influx

Author	Year	Author's contribution
Addison	1843	described the process of cell migration through blood vessels, ie, diapedesis
Arnold	1873	showed leucocytes migrating through areas of the inflamed capillary wall.
Opie	1910	showed that polymorphs migrated during the early stages of inflammation followed by mononuclear cells.
Clark, Clark and Rex	1936	studied the emigration of leucocytes through inflamed vessels using the transparent tail of the tadpole and the rabbit ear chamber technique.

Page and Good	1958	suggested that the successful migration of mononuclear cells was dependent on the presence of polymorphonuclear cells at the inflamed site.
Paz and Spector	1962	suggested that polymorphonuclear cells and mononuclear cells migrate concurrently, the polymorphs dying or moving away leaving the mononuclear cells.
Hurley, Ryan and Friedman	1966	postulated separate mechanisms exist to regulate the migration of different cell types.
Sultzer	1968	proposed the hypothesis that 'one mechanism' induced both cell types to emigrate.

Chemotaxis

Author	Year	Author's contribution
Pfeffer	1884	introduced the term 'chymitaxis' (chemotaxis) to describe the phenomena in which chemicals or biochemical substances attract and determine the direction of migrating cells.
Mussart and Bordet	1890	showed that cellular breakdown products released substances which could attract granulocytes.
Commandon	1919	showed the change from random undirectional movement upon introduction of clumps of bacteria.
Menkin	1936	proposed that inflammatory cell free exudates from turpentine pleurisy contained a substance which induced vascular permeability and leucocyte infiltration.
Menkin	1938	named this substance 'leucotaxine' because of its chemotactic properties.
Ketchel and Favour	1955	showed that some serum proteins increased leucocyte migration.
Hurley and Spector	1961	suggested that chemotaxis and leucocyte emigration *in vivo* were separate phenomena.

Boyden	1962	developed a novel technique for estimating the chemotactic effect of soluble substance.
Ward, Cochrane and Muller-Eberhard	1965	antibody antigen complexes and zymosan in the presence of fresh serum induced migration of polymorphs through a millipore filter.
Cornely	1966	chemotactic factor(s) associated with lysosomal fraction of leucocytes, which did not require the presence of serum.
Snyderman, Shin, Phillips, Gerwurtz and Mergenhagen	1969	majority of chemotactic activity in whole guinea pig serum, after treatment with endotoxin, was due to a product cleaved from C'5.
Kaley and Weiner	1971	prostaglandin E_1 chemotactic for polymorphs.
Kaplan, Mayer and Austen	1972	human plasma kallikrein directly and selectively attracted human neutrophils from mixed leucocyte populations.
Turner, Campbell and Lynn	1975	esterified arachidonic acid present in plasma membrane may be a precursor of chemotactic factors.
Goetzel, Wood and Gorman	1977	human platelet lipoxigenase generated product of arachidonic acid 12-L-OH-5, 8, 10, 14, eicosa-tetraenoic acid (12 HETE) selectively chemotactic for polymorphs and mononuclear cells.
Ford-Hutchinson, Bray, Doig, Shipley and Smith	1980	leucotriene B, chemotactic both <u>in vitro</u> and <u>in vivo</u> towards both polymorphs and mononuclear cells. Equal in potency to C5a.
Goetzel et al	1980	showed that platelet activating factor induced stimulation and chemotaxis of neutrophils.
Luger et al	1983	interleukin-1 is chemotactic for polymorphonuclear leucocytes.

Macrophage proliferation

Many studies have been done to investigate factors involved in macrophage proliferation. The table below summarises the work of various authors on macrophage proliferation in vitro.

Author	Year	Author's contribution
Virolainen and Defendi	1967	factors derived from cultures of L-cells, mitogenic for peritoneal macrophage.
Adolph et al	1975	inflammatory exudate harvested from the rat pleural cavity four hours after injection of dextran.
Hadden, Sadlik and Hadden	1975	lymphokine factor released by contact of sensitised lymphocyte with antigen.
Giroud, Pelletier and Girre	1977	factors in acute non immunological exudate from four hours pleurisy were able to stimulate macrophage DNA synthesis.
Giroud et al	1977	peritoneal macrophages stimulated to divide after treatment with serum from animals presenting acute inflammation elicited with dextran or calcium pyrophosphate crystals.
Conner, Ford-Hutchinson and Smith	1980	Macrophage mitogenic factor present in normal rat serum.

ANIMAL MODELS OF INFLAMMATION AND ARTHRITIS

Many animal models of acute (polymorphonuclear leucocyte dominated) and chronic (mononuclear leucocyte dominated) inflammation have been developed. Few of these models allow the collection of inflammatory exudate formed as the result of increased vascular permeability. Induction of experimental pleurisy in the rat was found to be the most suitable animal model for providing an acute inflammatory exudate. However, experimental pleurisy was not found to be a suitable model for the study of chronic inflammation, where the inflammation resolved after a shorter period of time.

Many animal models of chronic inflammation, such as cotton pellet granuloma (Meier et al, 1950), adjuvant arthritis (Pearson, 1956) and the subcutaneous implanted coverslip (Ryan and Spector, 1970), although being useful for the screening of therapeutic agents, do not readily provide a source of exudate representative of chronic inflammation.

On the other hand, the subcutaneous air pouch model of Selye (1953) would seem to provide a more suitable cavity for providing a chronic inflammation. Selye (1953) proposed that the formation of a cavity by the subcutaneous injection of air acted as a mould for the subsequent formation of a granulomatous membrane, into which this internal cavity could easily be transformed by the injection of irritants, such as croton oil and formalin. Subsequently this model was modified to allow its use as a routine screen for anti-inflammatory agents (Robert and Nezamis, 1957), and this was followed by the introduction of carrageenan as the injected irritant (Benitz and Hall, 1963).

Since its introduction, the carrageenan air pouch method has been widely used as an acute model of inflammation for the screening of anti-inflammatory agents, such as indomethacin (McCall and Youlton, 1974). However, only Tsurufuji et al (1978) have examined the effects of anti-inflammatory drugs on the chronic stages of this model.

Edwards, Sedgwick and Willoughby in 1981, showed that the air pouch of mouse and rat at six days after initial air injection developed a lining structure with many of the features of synovial membrane, as judged by electron microscopy and light microscopy, using haematoxylin and eosin and Van Gieson stains, esterase activity and immunofluorescent staining for Ia antigen. Sedgwick et al, 1983, showed that the six day pouch of the rat responded in a special way to different inflammatory stimuli, however, these authors only examined the acute phase.

The table below summarises some of the main animal models used for the study of inflammation and arthritis.

Model	Authors	Notes
Frog tongue	Cohnheim 1867	Observations provided classical description of vascular changes of acute inflammation.
Frog mesentery	Arnold 1873	showed leucocytes migrating through areas of the inflamed capillary wall (leakage between endothelial cells).
Pleurisy (various irritants)	Spector and Willoughby 1957	These authors showed the precise role of mediators of vascular permeability. This model was used to study acute inflammation and anti-inflammatory agents. Despite the extensive use of this model, the evaluation of the inflammatory reaction is usually

		confined to the measurement of exudate volume. However, some investigators have made quantitative measurements of cell migration (Hurley et al, 1966; Ammendola et al, 1975).
Thermal injury	Wilhelm and Mason, 1958	Studied acute vascular response, mediators and types of blood vessels.
Paw oedema (carrageenan most favoured irritant)	Winter, Risley and Nuss 1962	Used as assay to study acute inflammation and in the search for anti-inflammatory activities of different agents. This model is simple and precise, but is relatively slow, and only a single datum is obtainable from each rat.
Peritonitis (various irritants)	Van Furth et al 1973	Used for quantitative studies on the kinetics of mononuclear phagocytes during acute inflammation. Despite the simplicity of the technique, the peritoneal cavity has been poorly employed to assess the effect of anti-inflammatory drugs on cellular exudation.
Cotton pellet granuloma	Meier, Schuler and Desaulles 1950	Simple and widely employed method to study chronic inflammation and granuloma formation.
Air pouch	Selye 1953	Model of both acute and chronic inflammation, possible to study cell kinetics and vascular response.
Adjuvant induced arthritis	Pearson 1956	Immunological model, considered for a long time as the closest to human disease, used as a screen for anti-inflammatory and anti-arthritic drugs.
Moncarticular arthritis	Dumonde and Glynn 1962	A model of chronic arthritis produced by injection of fibrin into the joints of rabbit previously immunised by intradermal injection of fibrin in Freund's complete adjuvant. Other antigen may also be used.
Subcutaneous glass coverslip	Ryan and Spector 1970	Used to study adhesion and turnover of cells and response to therapeutic agents.
Collagen type-II induced arthritis	Trentham, Townes and Kang 1977	Immunological model used in rats, similar to adjuvant arthritis, but the immunogen (collagen type-II) would seem more appropriate. The arthritis was induced by injection of type-II collagen in either complete or incomplete Freund's adjuvant.

ANTI-INFLAMMATORY AND ANTI-RHEUMATIC DRUGS

Non-steroidal anti-inflammatory drugs (NSAID)

In 1763 an Oxforshire clergyman, Edmund Stone, presented a paper entitled ..... "An account of the success of the Bark of the Willow in the cure of Agues". Stone recommended a decoction of the bark of the white willow for treating 'anguish and intermitting disorder', a description of what we understand to be malaria. In 1874, T J Maclagen wrote "nature seeming to produce the remedy under climatic conditions similar to those which give rise to the disease, among the salicaceae I determined to search for a remedy for acute rheumatism. The bark of many species of willow contains a bitter principle called salicin; this principle was exactly what I wanted." Maclagen was the first physician to treat rheumatic fever with salicylates, their successful use in the symptomatic relief of chronic rheumatoid arthritis and gout being proclaimed the following year by Parisian physician, German See, who was the first to describe the anti-rheumatic properties of these compounds. A further century was to pass, however, before 'new' non-steroidal compounds became widely used in clinical medicine.

In 1963, Winter, Risely and Nuss described the anti-inflammatory action of a new compound called indomethacin. Indomethacin was be
found to/effective in suppressing carrageenan paw oedema and cotton pellet granuloma in rats. There were many attempts to link the anti-inflammatory action of aspirin and aspirin-like drugs with the ability to inhibit endogenous mediators of inflammation. Aspirin was labelled an anti-defensive drug by Collier in 1969, owing to its ability to inhibit the defensive

reactions of the body, such as fever, pain and inflammation. This author proposed that its anti-defensive action might arise from antagonism of kinins, slow reacting substance A and C, prostaglandin $F_2 \alpha$, arachidonic acid, adenosine triphosphate or acetylcholine.

In three pioneering papers, NSAID were shown to inhibit prostaglandin biosynthesis in guinea pig lung homogenates (Vane, 1971), perfused dog spleen (Ferreira et al, 1971) and in human platelets (Smith and Willis, 1971). This inhibition was confirmed in other systems, both in vivo and in vitro (Vane, 1974; Flower and Vane, 1974 a,b). Vane (1971) has postulated that NSAID exert their pharmacological actions through inhibition of prostaglandin biosynthesis in tissues. This proposal was to serve as a mainspring in the synthesis of many NSAID with the ability to inhibit prostaglandin synthesis. It has since been recognised that PGE_2 is cytoprotective for the gastro-intestinal tract, suggesting that many of the cyclo-oxygenase inhibitors lead to adverse side-effects (Rainsford et al, 1980).

New developments in the field of NSAID were initiated by Hamberg and Samuelsson (1974). These workers described mammalian lipoxygenase enzyme as providing a competitive route for the oxygenation of free arachidonic acid, this enzyme being unaffected by either aspirin or indomethacin. The products of lipoxygenase pathway were subsequently implicated as mediators of inflammation. 12-hydroxy-5, 8, 10, 14, eicosatetraenoic acid (12 HETE) was found to be chemotactic for polymorphs (Geotzel et al, 1977), in company with the hydroperoxy acids (HPETE) and the corresponding HETE (Goetzel and Sunn, 1979). More recently, leukotriene B, another lipoxygenase product

(5,12 hihydroxy 6, 8, 10,14 eicosatetraenoic acid) has been shown to be more chemotactic then either the HETE's or HPETE's (Ford-Hutchinson et al, 1980).

Recognition of lipoxygenase products as important mediators in the inflammatory process lead to the development of agents which inhibit their synthesis. Higgs et al, 1979, described a compound, BW755C, which inhibits both cyclo-oxygenase and lipoxygenase, and another potent anti-inflammatory agent, benoxaprofen,was developed. This was able to inhibit both enzymes but with preferential activity against lipoxygenase (Walker and Dawson, 1979).

Steroidal anti-inflammatory drugs

Hench et al, in 1949, proposed that an anti-rheumatic substance - X - was released during pregnancy and jaundice, which was one of the adrenal hormones. They showed that certain clinical and biochemical features of rheumatoid arthritis could be markedly improved by daily adrenal hormone. Steroids were found to inhibit both polymorphonuclear and mononuclear leucocytes' migration (Thompson and Van Furth, 1970; Watnick et al, 1974). Anti-inflammatory steroids were also found to inhibit polymorphonuclear leucocytes' adherence (MacGregor, 1976) and chemotaxis (Rivkin et al, 1976). Lysosomal enzymes and free radicals release were also inhibited by steroidal anti-inflammatory (Lehmeyer and Johnston, 1978; Smith and Iden, 1980).

Glucocorticoids are indirectly able to inhibit phospholipase A_2 and thereby prevent the biosynthesis of a number of pro-inflammatory mediators, such as the prostaglandins, thromboxanes and leukotrienes (Nijkamp et al, 1976; Tam et al, 1977).

This action depends upon steroidal interaction with specific receptors, and the modulation of ribonucleic acid (RNA) and protein synthesis with the target cell (Danon and Assoulin, 1978; Flower and Blackwell, 1979; Carnuccio et al, 1980).

The anti-phospholipase effect of corticosteroids was found to be exerted by a 'second messenger' synthesised and secreted by the cells in response to steroid stimulation. In 1980, Blackwell et al isolated this material from rat peritoneal macrophages and showed it to be a protein with a molecular weight of 15,000 daltons. They subsequently named the material Macrocortin. In 1980, Hirata et al isolated a protein with similar properties and a molecular weight of 40,000 daltons from glucocorticoid-treated rabbit neutrophils, and this protein was named Lipomodulin. In 1980, Blackwell et al showed that macrocortin exists as a pre-formed store within the rat peritoneal macrophage and its secretion is induced by glucocorticoids.

Anti-rheumatic compounds

Anti-rheumatic drugs are defined as those drugs which have shown efficacy as disease-modifying anti-rheumatic drugs (DMARD) (Stecher and Carlson, 1984). In order to be classifed as DMARD, a drug need not necessarily effect a cure, but rather must retard or stop the underlying progression of the disease (Roth, 1982). The term DMARD has replaced the term SAARD, or slow-acting anti-rheumatic drugs, which appeared in the literature for a brief period.

Antimalarials

Aminoquinolone antimalarials have been used to treat rheumatoid arthritis since 1951 (Zvaifler, 1971). Freedman (1956) showed that after sixteen weeks of treatment, patients treated with chloroquine sulphate were significantly better than a control group. Chloroquine is neither analgesic nor anti-inflammatory in animal models and its mode of action is unknown. It accumulates in lymphocytes and reduces the responsiveness of lymphocytes of patients with rheumatoid arthritis to phytohaemagglutunin (Panayi et al, 1973). There was a fall in the titre of rheumatoid factor (antibody to normal tissue gamma globulins present in 80% of rheumatoid sera) in patients on chloroquine, and it is of considerable interest that there was a significant correlation between changes in rheumatoid factor and changes in clinical state. Such a correlation has not been found with other drugs of this type.

Gold

It was not until 1961 that the question of gold's efficacy was finally answered, when the Empire Rheumatism Council (1961) conducted a well controlled double-blind study with gold sodium thiomalate in a large patient population. The results from this study showed that, by practically all criteria, improvement was observed. The mode of action of gold compounds is still uncertain. Gold compounds have been suggested to be therapeutic by virtue of inhibition of lysosomal enzyme activity (Di Martino and Walz, 1977) and release (Finkelstein et al, 1977), inhibition of prostaglandin synthesis (Stone et al, 1975) or an immunosuppressive action.

The most recently considered hypothesis is that gold compounds are immunosuppressive. This view is supported by the observation that gold sodium thiomalate inhibited the development of adjuvant-induced arthritis in rats (Walz et al,1971), depressed mitogen-induced lymphocyte blastogenesis (Harth et al, 1977; Simon et al, 1977) and clinically appeared to depress immuno-globulin synthesis after long term therapy (Lorber et al, 1974).

D-penicillamine

The use of penicillamine in the treatment of rheumatoid arthritis was pioneered two decades ago by Jaffe (1963). D-penicillamine has been demonstrated to be effective in the treatment of rheumatoid arthritis (Multicentre Study Group, 1978). A number of observations in treated patients have suggested that D-penicillamine might exert an immunosuppressive action and, thus, act by suppressing the on-going immune response that underlies the chronic inflammation. In this regard, therapy with D-penicillamine often results in decreased rheumatoid factor titres (Jaffe, 1965), immunoglobulin levels (Bluestone and Goldberg,1973) and concentrations of circulating immune complexes (Jaffe, 1965).

There has been considerable speculation that the efficacy of penicillamine in rheumatoid arthritis is due to its copper chelating and copper mobilising properties (Arrigoni-Martelli, 1982). Other studies have suggested an effect on collagen biosynthesis (Trentham et al, 1978), lymphocytes (Merryman and Jaffe, 1978), mononuclear phagocytic function (Devrines et al,1982), complement (Mellbye and Munthe, 1977), antibody responses (Hunneyball et al, 1978) and cell mediated immunity (Stecher et al, 1981). As is the case for the other DMARD, the mechanism of action of

D-penicillamine has not been defined.

Levamisole

Levamisole, an antihelminthic agent with immunostimulating activity, has been used experimentally for the treatment of rheumatoid arthritis since 1974 (Ansell, 1978; Huskisson et al, 1976). Levamisole has been shown to produce an effect in some patients with rheumatoid arthritis measurably superior to placebo or the first-line drugs, and with a delayed onset similar to gold or penicillamine (El-Ghobarey et al, 1978).

The mode of action of levamisole is still unclear, although several hypotheses have been propsed (Goldstein, 1978) and numerous effects on monocytes have been demonstrated (Wynne et al, 1981; Barada et al,1982). The occurrence of sudden unpredictable agranulocytosis limits the uncontrolled practical use of levamisole as an anti-rheumatic, despite the fact that the drug appears to prevent progression of the disease (Stecher and Carlson, 1983).

Rheumatoid arthritis

The rheumatic disease has been recognised since the 5th century before Christ, under the general title of arthritis. The term 'rheumatism' was probably introduced by Galen in medieval times. In the works of Hippocrates, rheumatic fever is briefly described under the generic name arthritis. Guillaume de Baillou (or Ballonuis) was the first to use the term rheumatism, in the sense of an acute polyarthritis which has no connection with gout, and he is, therefore, often referred to as the Father of Rheumatism. In 1676, Thomas Sydenham, the

greatest clinical physician of his century, gave the first full description of acute rheumatism. William Heberden (1802) was one of the first to point out that acute rheumatism is largely a disease of childhood. It was Bouillaud (1836), the French physician, in his work "Nouvelles researches sur le rheumatisme articulaire", who first established a fundamental basis upon which modern ideas of the disease are based. Salicylate treatment was introduced by Maclagan of Dundee in 1876, and aspirin in 1899.

Rheumatoid arthritis is a chronic symmetrical polyarthritis. Although the principal manifestations occur in the joints, it is a multi-system disease. It is associated with the presence of rheumatoid factor in the blood, although it is not clear whether this is cause or effect (Berry, 1983). Despite innumerable sophisticated microbiological investigations, no infectious agents - bacterial or viral - have been identified to the general satisfaction of workers in the field (Sokoloff, 1984).

Synovial joints

The general anatomical features of a typical synovial joint are, articular cartilage lying on sub-chondral bone plate and non-articular surfaces within the joint covered by synovial tissue. The whole joint is enclosed by the capsule, which is continuous with the periosteum covering the bone (Vernon-Roberts, 1980).

Articular cartilage

The articular surface of each bone is covered by a layer of hyaline cartilage, which acts as a resilient cushion. It

is fixed to the underlying sub-chondral bone by collagen fibres. Cartilage contains relatively few cells (chondrocytes) but a lot of extracellular material. This extracellular matrix consists largely of fibres (collagen) and ground substance (proteoglycans). Cartilage has no nerve or blood supply, and therefore receives nutrients via diffusion from the synovial fluid, sub-chondral bone or the surrounding membrane (Hascall, 1981). Articular cartilage exhibits the properties of being both firm and flexible. This flexibility or resilience to compressive forces can be directly related to proteoglycan content (Kempson et al, 1970) and the rigidity and tensile strength is related to collagen content (Kempson et al, 1973).

Cartilage proteoglycans are complex glycoproteins composed of a protein core comprising 7-12% of the total weight, to which a large number of polysaccharide chains (glycosaminoglycans) are covalently linked, giving a molecular weight of approximately 2.5×10^6. In hyaline cartilage proteoglycans, the constituent glycosaminoglycans are chondroitin sulphate and keratan sulphate (Faltynek and Silbert, 1978). Most proteoglycans are present in the extracellular matrix asmultimolecular aggregates. Aggregates consist of many proteoglycan subunits bound to a single molecule of hyaluronic acid by an ionic interaction. The chondrocytes produce the matrix during growth and, despite their small numbers, maintain the tissue during adult life. The ability of chondrocytes to regulate their surrounding matrix,and to respond to changes in that matrix by both anabolic and catabolic processes, is critical for tissue maintenance and function in both normal and pathologic tissues (Kimura et al, 1980).

Capsule and ligaments

The joint is enclosed by a fibrous tissue capsule attached to the bone near the periphery of the articular cartilage and in continuity with the periosteum. Thickening of the capsule forms named ligaments which serve to stabilise and strengthen the joint and to limit certain movements.

Synovial tissues

The capsule is lined by synovial tissue, which invests all the non-articular surfaces within the joint. The synovial tissue normally possesses variable numbers of folds and finger-like synovial villi and comprises a thin synovial layer, which forms the lining of the non-articular joint surface and an underlying subsynovial supporting layer composed of fibro-fatty connective tissue of varying thickness which merges with the capsule. The cells (synoviocytes) of the synovial layer are divisible into two principal types - type A cells have the appearance of cells engaged in phagocytic activity, whereas the type B cells have the appearance of cells engaged in synthetic activity (Barland et al, 1962).

Cartilage degradation

Irreversible cartilage destruction in rheumatoid arthritis is usually associated with a chronic hypertrophic synovitis, the rheumatoid pannus that invades over and into the cartilage, eroding the cartilage matrix mainly under its advancing edge (Harris, 1981). The superficial lining layers of that pannus consist of synovial cells of both the phagocytic or macrophage-like-(A)-type and the fibroblastic-(B)-type (Barland et al, 1962).

Its deeper portions, that extend to the cartilage, contain, together with proliferating blood vessels, abundant fibroblasts and a heavy infiltrate of mononuclear phagocytes, B and T lymphocytes and plasma cells, with only rare occasional granulocytes (Kobayashi and Ziff, 1975; Ishikawa and Ziff, 1976). Cartilage invasion occurs at the cartilage/pannus junction by an ingrowth of macrophage-like cells and fibroblasts accompanied or not by proliferating small blood vessels (Poole et al, 1976 and Woolley et al, 1977).

The above mentioned observations suggest that macrophages and fibroblasts, and possibly also the related synovial cell types, are among the main effector cells of irreversible cartilage erosion in rheumatoid arthritis. Under cell culture conditions, each of these cell types has the capacity to produce and secrete enzymes lysing the main constituents of the cartilage matrix - collagenase (Wahl et al, 1974; Rauer et al, 1975; Werb and Burleigh, 1974 and Dayer et al, 1976), and proteoglycan-degrading metal-dependent neutral proteinase (Hauser and Vaes, 1978; Huybrechts-Godin and Vaes, 1978; Peeters-Joris et al, 1981).

The enzymatic mechanism of cartilage matrix degradation

The table below summarises the main observations of enzymatic cartilage degradation.

Author	Year	Author's contribution
Barnett	1956	showed that when hyaluronidase was injected into rabbit joint, which is then vigorously exercised, there was a loss of articular cartilage ground substance.
Thomas	1956	showed that there was a droop in the

		rabbit's ears following intravenous injection of papain, and there was chondroitin sulphate in the rabbit's blood and urine.
Dingle	1962	proposed that lysosomal enzymes might degrade the matrix of articular cartilage.
Hammerman et al	1967	showed that rheumatoid synovial membrane contains much higher content of lysosomal enzymes than the normal membrane.
Janoff et al	1970	showed that neutrophil granule enzymes degraded glycosaminoglycan of cartilage matrix.
Malemud et al	1975	demonstrated that at least part of the proteoglycan degrading activity of the neutrophil granule function is due to elastase and cathepsin.
Kobayashi and Ziff	1975	proposed that the major cause of cartilage erosion in rheumatoid arthritis is a degradation of matrix by macrophages and possibly by fibroblasts.
Kempson et al	1976	showed that cathepsin B is active against collagen as well as proteoglycan.
Barrett et al	1977	reported that the neutrophil serine proteinases degrades collagen and proteoglycan of the articular cartilage.
Werb et al	1977	reported that adherent rheumatoid synovial cells produced collagenase and plasminogen activators.
Dingle	1979	showed that the synovial membrane has both acid enzymes (the cathepsins B, D and N) and the neutral metalloproteinases and collagenase, serine proteinases were also found in low activity. Significant activities of cathepsins B, D and F were found in articular cartilage, though there were only trace activities of neutral proteinases such as elastase, cathepsin G and plasmin.
Dayer et al	1979	showed that high levels of collagenase and prostaglandin E2 were released into culture medium from adherent rheumatoid synovial cells isolated from the superficial layer of synovium.
Barrett	1981	suggested that the agents of cartilage matrix degradation are proteinases.

McGuire	1982	suggested that collagenase is the only specific enzyme that acts at neutral pH to degrade the native helix of collagen.

Prostanoids

It has been suggested that prostaglandins play a role in cartilage metabolism in inflammation (Trange, 1980) as protective (Zurier and Ballas, 1973) and destructive (Teitz and Chrisman, 1975) agents. They are secreted in increased concentrations by untreated rheumatoid synovium and cultured rheumatoid synovial cells (Robinson et al, 1975). It has been shown in vitro that rheumatoid synovium secretes substances that inhibit proteoglycan synthesis by fragments of articular cartilage (Lippiello et al, 1978) and it is likely that this effect is mediated by prostaglandins or related substances, since this inhibition is suppressed by indomethacin. In 1982, Metrovic showed, using cultured bovine articular chondrocytes, that PGE_2 inhibits, whereas PGI_2 stimulates proteoglycan and protein synthesis respectively.

Free radicals

Polymorphonuclear leucocytes and macrophages have been shown to release in vitro superoxide radical (Goldstein et al, 1975 and Johnston et al, 1976), which may initiate lipid peroxidation in synovium and cartilage, causing damage to both tissues via the generation of highly toxic intermediate substances and hydroxyl radicals (Hemler and Lands, 1980 and Polgar and Taylor, 1980). Hydroxyl radicals may induce changes in cell membranes

causing cell necrosis and release of proteolytic enzymes, and may depolymerize hyaluronic acid, proteoglycans and collagen fibres, leading to fibrillation of articular cartilage and further loss of the matrix macromolecules (Greenwald et al, 1976).

Lymphokines

Various factors secreted *in vitro* by the lymphocytes, polymorphonuclear leucocytes and macrophages have been described and probably play an important role *in vivo*, particularly in synovial inflammation (Kelly et al, 1981 and Wallach, 1978). These factors may attract polymorphonuclear leucocytes and macrophages, stimulate phagocytosis, cell division, the production and release of proteolytic enzymes and prostaglandins, or stimulate proteoglycan biosynthesis (Castor and Whitney, 1978).

Catabolin

Catabolin is the name coined by Dingle and colleagues (1979) for a protein, or a family of proteins, capable of stimulating intact living cartilage to degrade its extracellular matrix. Catabolin does not impair the viability of the chondrocytes, nor does it immediately modify the mitotic activity of these cells.

Catabolin is an acidic protein with a molecular weight of 21,000, produced by pig mononuclear cells when they are cultured with concanavalin A (Saklatvala et al,1983). Recent studies have confirmed that purified porcine mononuclear cell catabolin is a form of interleukin-1 (Saklatvala et al, 1984).

Interleukin-1

Over the past twenty-five years, there has been an ever-expanding interest in the mediators that control the immune response (Lawrence and Landy, 1969). One of these is produced by macrophages and was first called lymphocyte activating factor (Gery et al, 1971), but is now designated interleukin-1 (Mizel and Farrar, 1979). Mizel and his colleagues have shown that interleukin-1 is a single polypeptide chain of molecular weight 12,000 - 16,000 daltons, and it is produced by the mouse macrophage cell line. This macrophage-derived mediator (interleukin-1) has many in vitro biologic activities, including the augmentation of lectin stimulated thymocyte proliferation (Gery et al, 1972), enhancement of production of lymphocyte-derived lymphokines, such as interleukin-2 (Smith et al, 1980), chemotactic factors (Oppenheim et al, 1979), migration inhibition factor (Yamamoto and Onoue, 1979), promotion of in vitro antibody production by B-lymphocytes, and function as a second signal that may serve to amplify immunologic reactions (Wood, 1983). Interleukin-1 is mitogenic for cultured fibroblasts (Schmidt et al, 1982) and chemotactic for polymporphonuclear leucocytes (Luger et al, 1983). It was found that interleukin-1 induces fever when injected into rabbits (Rosenwasser et al, 1979) and is inhibited by antiserum to endogenous pyrogen (Murphy et al, 1980). It has been shown that interleukin-1 stimulates hepatocytes to produce acute phase proteins (Sztein et al, 1981).

Mizel and his colleagues (1981) have shown that interleukin-1 activates the production of collagenase and prostaglandins by synovial cells. Gowen et al in 1984, have shown that human interleukin-1-like material mediates cartilage breakdown and

production of collagenase and prostaglandins by human chondrocytes. Interleukin-1 also mediates bone resorption (Gowen et al, 1983).

Mononuclear cell factor

In 1977, Dayer, Russell and Krane, demonstrated that human mononuclear cells in culture produce a factor which can stimulate the production of collagenase by the adherent rheumatoid cells. Dayer, Robinson and Krane (1977) found another factor produced by mononuclear cells, and its production was enhanced by phytohaemagglutonin. This factor stimulates the production of prostaglandin E2 and has a molecular weight similar to that of the first factor. In 1980, Meats and his colleagues found that the culture mediums from explants of normal and rheumatoid human synovium contained a factor which is capable of stimulating the production of prostaglandin E2 and plasminogen activator by chondrocytes of human articular cartilage.

Deshmukh-Phadke et al (1980) reported that rabbit peritoneal macrophages, when activated with lipopolysaccharides, release a factor which stimulates the chondrocytes to produce collagenase and the neutral proteases. This factor has a molecular weight ranging from 13,000 - 15,000 daltons. Another factor produced from stimulated rabbit peritoneal macrophages, and with a molecular weight of 30,000 daltons, was found by Ridge, Ornosky and Kerwar in 1980. Addition of this factor to cartilage in culture, results in the synthesis of collagenase and neutral proteases, both in latent form which can be activated by either trypsin or plasmin.

In 1980, Meats et al discovered another factor, named the synovial factor. Synovial factor is identified as an activity

present in the medium of cultured explants of human normal or rheumatoid synovial tissue, that stimulated the production of prostaglandin E2 and plasminogen activator by isolated human chondrocytes. Elford and his colleagues in 1985, showed that mononuclear cell factor and synovial factor are closely related to, or are identical with, interleukin-1.

MATERIALS AND METHODS

A. ANIMALS AND MAINTENANCE

Mice

Adult male outbred SAS/4 mice weighing between 30-40 grams were used. The mice were obtained from the colony at St Bartholomew's Hospital, and were housed in plastic cages. The mice were kept in a temperature-controlled environment and fed special dietary pellets, FFG(M) diet for scientific research animals, (E Dixon & Sons, Ware Ltd) and fresh water ad libitum.

Rats

Adult male outbred Wistar rats weighing between 200-250 grams were used. These animals were obtained from the colony at St Bartholomew's Hospital. Adult male inbred Fisher rats of the same weight were also used and were obtained from Charles River UK Ltd, Kent, UK. Their housing and dietary regime was similar to that of the mice.

Rabbits

Adult male rabbits of the New Zealand White strain weighing between 3-4 kg were used. They were obtained from Animal Suppliers (London) Ltd, Pottersheat Road, Welwyn, Hertfordshire, and they were housed in galvanised steel cages (24' x 18' x 18'). They were fed on special 'diet 18' (Dixon & Sons, Ltd) and allowed fresh water ad libitum.

B. EXPERIMENTAL MODELS OF INFLAMMATION

B1 The formation of different aged pouches in the rat

The air pouch as described by Edwards et al (1981) was used.

Animals were anaesthetised with anaesthetic ether (May and Baker, Ltd, London) and their dorsal surface cleaned with 70% alcohol and shaved. Using a 20 ml disposable syringe (Gillette) and 25G x 15/16 gauge needle, 20 ml of air was injected subcutaneously into the tissue of the back in the midline to produce an air pouch. Pouches older than 1 days had 10 ml of air injected into the pouch every 3 days to keep the cavity open.

B2 The formation of 6 day old pouches in mice

The dorsal surface of lightly anaesthetised mice were cleaned with 70% alcohol and shaved. Using a 5 ml disposable syringe (Gillette) and a 25G x 15/16 gauge needle, 5 ml of air was injected subcutaneously into the dorsal surface of each animal. Later, 2.5 ml of air was injected into the pouch after 3 days to keep the cavity open.

B3 Carrageenan

Carrageenan was used to produce inflammation in the pre-formed air pouches of mice and rats. Carrageenan, viscarin 402 (lot 272500) was kindly provided by Marine Colloids Inc, Springfield, USA. This was dissolved in a sterile solution of 0.9% sodium chloride containing Benzylpenicillin 100 units/ml (sodium, BP Glaxo, Greenford, England) and Streptomycin 100 ug/ml (sulphate, BP Glaxo, Greenford, England). The solution was stirred continually at 37^{o} C so that it was homogenous. A concentration of either 1% or 2% W/V was used for injection. Mice received 1 ml of 1% carrageenan into pouches, whilst rats received either 2 ml of 2% (high dose) or 2 ml of 1% (low dose) of carrageenan.

B3a Sera collection

Blood was collected from the terminal bifurcation of the aorta of rats. Animals were anaesthetised with ether and the abdomen was opened with sterile scissors. The viscera were removed to reveal the aorta. A 21G x 1 'Gillette' needle was fixed to a 10 ml disposable syringe and carefully inserted through the aortic bifurcation with the common iliac arteries and the blood was collected in the syringe. To avoid haemolysis, blood was poured carefully along the side of a 10 ml Sterilin container (Sterilin, Teddington, Middlesex, England). After one hour at room temperature, the sample was centrifuged at 300g for ten minutes and the serum was removed with a sterile plastic pipette (Alpha Laboratories Ltd, Eastleigh, Hants, England). Sera was subsequently stored at -20° C.

B3b Exudate collection

Exudate was collected from all animals receiving an inflammatory insult. Anaesthetised animals were killed by cervical dislocation and an area of the pouch was cleaned with 70% alcohol before being shaved. A small incision was made using sterile scissors. The exudate was collected using a sterile plastic pipette and poured into 30 ml sterile Sterilin containers. Heparin solution (Sigma Chemical Company) was added to the collecting tubes (50 units/ml final concentration). The exudate volume, together with the total leucocyte count, was assessed for each animal.

B3c Cell viability test (Trypan blue technique)

The viability of cells were tested using trypan blue. One volume of cell suspension was mixed with one volume of trypan

blue solution (0.1%) for five minutes. A smear was made and the percentage of cells with blue colouration was noted. This gave an index of cell death.

B3d Storage of exudate

Exudate was centrifuged at 800g for ten minutes at 4^{o} C and the free exudate stored at -20^{o} C. Smears were prepared from the sedimented cellular pellets.

B4 Arthus reaction

B4a Preparation of anti-bovine serum albumin (anti-BSA)

(i) Immunisation of rabbit to obtain the anti-BSA

The anti-BSA was obtained from rabbits. Rabbits were first immunised by injecting 10 mg of BSA (Sigma Chemical Company, USA) in 0.5 ml of sterile saline, mixed well in the syringe with 0.5 ml of complete Freund's adjuvant (Difco Laboratories, USA). The hind foot pads received 0.15 ml each and the remaining 0.7 ml was divided between three skin sites - each flank and over the neck. The injections were repeated every week for eight weeks. After the final week, blood was removed from the rabbit via the ear vein. The blood was centrifuged at 1000 x G for twenty minutes to obtain the serum.

(ii) Isolation of anti-BSA from blood serum

Twenty-five millilitres of serum were mixed gently with anhydrous sodium sulphate (Na_2SO_4 - 18g/100 ml serum) using a Pasteur pipette. This precipitated the γ - globulin. After mixing, the solution was incubated for 45 minutes at 37^{o} C. The precipitate was then centrifuged at 6,000 - 10,000 rpm for twenty minutes. The supernatant was discarded and

precipitate dissolved completely in distilled water. The clear solution obtained was then dialysed at 4^{o} C in 20 mm Visking tube (Gallenkamp) in phosphate buffered saline, pH 7.4, in a five litre flask. Dialysis went on for 30 hours with six changes of buffer in between. After dialysis, the solution was passed through the sepharose-BSA conjugated column.

(iii) Preparation of the sepharose-BSA conjugated column

a) 4 g of sepharose 4B activated with cyanobromide (Pharmacia, Fine Chemical, Uppsala, Sweden) was washed through with 800 ml of 1.0 mM hydrochloric acid (HCl) in a sintered glass funnel.

b) The sepharose was washed through with 800 ml of 0.1 M sodium bicarbonate to remove acid.

c) BSA (90 mg) was prepared in 15 ml of 0.1 M sodium carbonate and optical density was measured at 280 nm to obtain a reading for 90 mg BSA.

d) Sepharose was mixed with BSA solution in a small beaker and stirred gently for 16 hours at 4^{o} C.

e) The supernatant was removed and optical density was measured again to make sure that sepharose binding sites were saturated. More BSA was added if necessary.

f) Sepharose-BSA was placed into a sintered glass filter funnel and washed through in sequence with the following:-

200 ml 0.1 M sodium bicarbonate ($NaHCO_3$)

800 ml phosphate buffered saline (pH 7.4)

800 ml glycine - HCl buffer (pH 2.3)

g) A suspension of the sepharose-BSA conjugate in PBS was prepared in a small beaker and then poured into the column whose mouth had been plugged with clean glass wool. The sepharose-BSA was rammed down gently with a glass rod, so as to pack firmly.

h) Finally, the column was washed with 1.0 litre of PBS pH 7.4

Composition of buffers:

phosphate buffered saline (pH 7.4)

sodium chloride (Na Cl) 42.5 g

disodium hydrogen phosphate $\left(Na_2\ HPO_4\ 12H_2O\ (M/15)\right)$ 96.5g

potassium dihydrogen phosphate $\left(KH_2\ PO_4\ (M/15)\right)$ 8.7g

make up to 10 litres with distilled water

glycine - HCl buffer (pH 2.3)

glycine M/10 2.25 g

sodium chloride M/10 1.76 g

hydrochloric acid M/10 200 ml

make up to 10 litres with distilled water

(iv) Isolation of anti-BSA in sepharose-BSA conjugated column

The column was surrounded by a jacket of circulating cold anti-freeze glycol at 4^{o} C. Attached to the top of the column was a long rubber tube leading to a volumetric flask containing the buffer. The solution which had been dialysed over night was applied with a Pasteur pipette gently to the sepharose-BSA gel. When the sample had been absorbed onto the column, the column was washed through with PBS (pH 7.4). The rate of elution was 10 seconds per drop. After the first 40 ml of eluent had been collected, the rate of elution was increased

to 8 seconds per drop. A further 40 ml of eluent was collected and a small quantity of the solution (1 in 10 dilution) was tested for its optical density (280 nm). When there was no protein in the eluent the anti-BSA could be released from the column.

(v) Release of anti-BSA from column

When all the unwanted protein had been eluted, glycine-HCl buffer (pH 2.3) was applied to the column slowly. The rate of elution of the buffer was 10 seconds per drop. With the acidic buffer passing through, the anti-BSA was released from the BSA and collected. When 25-30 ml of eluent had been collected, a small quantity was taken out and diluted 50 times. The optical density (280 nm) of the diluted solution was measured so as to check the protein concentration and, thus, obtain the total amount of antibody collected. Using 1.0 cm square cuvettes, 13 absorbance units = 10 mg/ml (rabbit antibody).

Antibody is normally expressed as $\mu g\ N_2$

$$= \frac{\text{mass of antibody}}{6.25}\ \mu g\ N_2$$

Eg, if O.D reading is X, then actual O.D of solution is 50X (because of dilution of 50X).

13 absorbance units = 10 mg/ml

= 10 x 1000 µg/ml

$$= \frac{10 \times 1000}{6.25}\ \mu g\ N_2/Ml$$

$$50X \text{ absorbance units} = \frac{10 \times 1000 \times 50X}{6.25 \times 13} \mu g\ N_2/ml$$

After collecting the anti-BSA in glycine - HCl buffer (pH 2.3), the pH was adjusted to 7.0 with glycine - NaOH buffer (pH 11.3). At pH 7.0, the solution contained some precipitate. The solution was then dialysed over night in PBS (pH 7.4) with four changes in between.

Glycine - NaOH buffer (pH 11.5)

Glycine - M/10	1.84 g
NaCl M/10	1.43 g
NaOH M/10	1.02 g

Make up to 500 ml.

(vi) Concentration of antibodies

After dialysis, the solution was filtered (millipore filter - 0.45 μm, Bedford, Massachusetts, USA), to remove the precipitate. The supernatant was then concentrated. The concentrating apparatus consists of a separating funnel, a bent graduate pipette and a 5 litre flask, connected tightly. The protein solution was put into 7 mm visking tubing which was secured by strong rubber bands at the junctions. After making sure that there was no leak, the flasK was evacuated by an electric pump (care was taken not to burst the visking tube). The flask was then left to concentrate at $4^{o}C$ for 12 hours. After concentration, the antibodies were diluted out 1:1 and then dialysed over night in PBS with three changes of PBS in between.

After dialysis over night, the solution was filtered (millipore 0.45 μm). A small quantity of the filtrate was

diluted with PBS (1:50) and optical density was measured. The total amount of antibody was calculated. Aliquots of the antibody were placed into small test tubes and stored at -20° C. Each aliquot was calculated in such a way, so that one test tube would give enough antibodies for one group of six animals. This was necessary to avoid unnecessary thawing and freezing, which may lead to denaturation of protein.

B4b Induction of active Arthus reaction in the air pouch

Rats were sentitised by weekly injection of 0.4 ml emulsion containing 0.2 ml saline and 5 mg bovine serum albumin (BSA: essentially globulin-free, from Sigma Chemicals Co, Ltd, London) mixed with an equal volume of complete Freund's adjuvant; 0.2 ml emulsion was injected into each of the rat hind paws, using 19G 1½" gauge needle. After three weekly injections, the animals were left for four days, anaesthetised with ether and then injected with 20 ml air into the subcutaneous tissue of the back. Pouches older than one

day had 10 ml air injected into the same cavity to keep it open every three days. At different time intervals (1, and 6 days) after the initial air injection, animals were re-anaesthetised with ether and 1 ml sterile saline containing 1 mg BSA was injected into the pouch. Groups of animals were killed at different times after antigen challenge, and the total leucocyte number and volume of exudate in the pouch was measured. Exudate cell smears were stained with May-Grünwald-Giemsa and differentially counted.

B4c Induction of reverse passive Arthus reaction in the pouch

Groups of rats were injected with 20 ml air in their backs.

Six day old pouches received 10 ml air after three days from inducing the pouch to keep the cavity open. At different time intervals (1 , and 6 days) after initial air injection 5 mg bovine serum albumin (BSA) in a volume of 0.2 ml phosphate buffered saline (PBS) was injected intravenously via the tail vein into each rat. Twenty minutes later, rats were challenged with purified anti-BSA (1.0 ml of a solution containing 5 mg protein N_2 /ml PBS) by injection into the preformed different aged air pouches. After six hours of sensitisation, animals were killed and the total leucocyte number and volume of exudate in the pouches were measured.

B5 Prolonged immune inflammation in the pouch

B5a Preparation of the pertussis-adjuvant emulsion

Equal volumes of bordetella pertussis vaccine (Wellcome) and Freund's incomplete adjuvant (Difco, USA), were mixed in a sterile beaker using a homogeniser until a homogenous white emulsion was formed. The emulsion was tested for homogeneity by placing one drop of emulsion in a beaker containing water. If the drop did not disperse in water, this indicated a homogenous, well formed emulsion.

B5b Animal sensitisation

Outbred rats were anaesthetised with ether and 0.5 ml emulsion containing (10×10^{10}) organisms was injected into each of the rat hind paws, using a 19G $1\frac{1}{2}$" gauge needle (Gillette) fixed on a 2 ml disposable syringe.

B5c Preparation of pure bordetella pertussis vaccine

Visking tubing(Gallenkamp) was washed with distilled water and boiled in a beaker containing distilled water for ten

minutes. One end of the visking tubing was knotted and the vaccine was introduced from the other end. The tubing was placed in a 5 litre flask containing PBS. The vaccine was dialysed for 24 hours at 4° C. This procedure was to remove the preservative from the vaccine.

B5d Formation of 1 and 6 day pertussis-inflamed pouch

To form the six day pouch in the sensitised animals, 20 ml air was injected into the dorsal subcutaneous tissue of the anaesthetised rat (according to the method described by Edwards et al, 1981) six days after sensitisation. Three days later, a further 10 ml air was injected into the air pouch to keep it open. Twelve days after sensitisation, the animals were challenged with 1 ml dialysed vaccine, injected into the pre-formed pouch using a disposable syringe and a 25G x 15/16 gauge needle. To form a one day pouch in the sensitised animals, 20 ml air was injected eleven days after sensitisation.

B5e Sera and exudate collection

The same procedures used to collect sera and exudate from carrageenan-inflamed animals were applied to animals of pertussis-immune inflammation.

B5f Non-sensitised animals

Non-sensitised animals were challenged as for sensitised animals and used as controls.

B6 Quantitative assessment of air pouch inflammation

B6a Exudate volume

Volume of the collected exudate was measured using a

graduated test tube.

B6b Total leucocyte count

Total leucocytes of the collected exudate were counted using a Coulter counter (Coulter Electronics Ltd, England). 40 µl exudate was put in a Coulter pot and three drops of Zap-oglobin (Coulter Electronics Ltd, England) were added to haemolyse any red blood cells that might be present in the sample. 20 ml Isoton-II (Coulter Electronics Ltd, England) were added and the contents were mixed and read using a Coulter counter. Random samples were checked for accuracy using an improved neubeur haemocytometer. Exudate leucocytes were diluted with a 1% acetic acid solution containing crystal violet crystals. The diluted sample was counted using an improved neubeur haemocytometer and light microscope. When an exudate contained a high number of cells, the sample was diluted in phosphate buffered saline.

B6c Exudate smears

Smears were made in duplicate from the pellets remaining after the storage of the centrifuged exudate. The smears were left to dry in air, stained with May-Grünwald-Giemsa.

May-Grünwald-Giemsa

After fixation, cell smears were washed in water, then stained with May-Grünwald (BDH Chemical Ltd, Poole, England) diluted 1:1 in water for five minutes, and then in Giemsa (BDH Ltd) 1:10 for fifteen minutes. Samples were again washed in distilled water, then air dried.

C. CARTILAGE

C1 Mouse xiphisternal cartilage

Autologous xiphisternum was used as a source of cartilage. To obtain this, a mouse was anaesthetised with ether and the area around the xiphisternum was cleaned with 70% alcohol and shaved. A small incision was made in the upper abdominal area using sterile scissors to reveal the muscular layer. A small region of the muscular layer (0.7 cm) overlying the xiphisternum was cut and a light pressure applied until the xiphisternum was revealed. It was removed with great care by cutting at the point where the sternal bone is connected to the cartilage. The wound was then sutured with chromic catgut and the overlying skin edges closed with 7.5 mm Michel Clip (Thackray Ltd, London). The cartilage was prepared for implantation by removing any adipose tissue and attached sternal bone. The cleaned cartilage (0.5 cm approximately) was washed four times in medium 199 containing benzylpenicillin (100 units /ml) and streptomycin (100 µg/ml) before being implanted into the pouch. When minced cartilage was used, the intact xiphisternum was cut into six pieces (0.15 cm diameter approximately) in a petri dish containing medium 199 with antibiotics, as above.

C2 Rat articular cartilage

Cartilage was obtained from the femoral heads of donor rats. Donor animals were anaesthetised with ether and killed by cervical dislocation, and their hind dorsal surface was cleaned with 70% alcohol and shaved. The skin layer was removed with sterile scissors. An incision was made using a sterile scalpel

blade (no. 22) diagonally across the gluteus maximus muscle to reveal the sciatic nerve. Pressure was applied to the knee and the femoral head was dislocated from its position. The cartilage cap was carefully removed from the femoral head using sterile bone forceps. The cartilage was washed in sterile medium 199 (Flow Laboratories, England) containing 100 units/ml benzylpenicillin and 100 µg/ml streptomycin, before being used for implantation. Cartilage from both sides of donor animals was used.

C3 Mouse articular cartilage

In experiments where mouse articular cartilage was used, the same procedure was used as in rats, except that the area of implantation was closed with a 7.5 mm Michel Clip. Because of the small size of mouse femoral cartilage, only whole intact cartilage was used for implantation.

C4 Preparation of dead cartilage

Cartilage samples were placed in a small sterile polythene bag and wrapped carefully. This was placed in a 250 ml beaker containing the freezing mixture (solid carbon dioxide and isopentane) for fifteen seconds. The freezed bag was placed directly in a beaker containing warm water ($35^{o}5$) to thaw cartilage. This process was repeated for five cycles.

C5 Cartilage immunisation

Some groups of rats were immunised against femoral head cartilage. Cartilage caps were obtained,as described previously, and finely minced using a sterile scalpel blade. The minced cartilage was suspended in sterile saline in a clean beaker. The cartilage suspension was mixed with an

equal volume of incomplete Fruend's adjuvant (Difco, USA) then homogenised using a clean homogeniser until a good thick white emulsion was formed. 0.25 ml emulsion was injected into each foot pad of outbred Wistar rats using a 19G 1½" gauge needle (Gillett) and a 1 ml disposable syringe.

C6 Cartilage implantation

C6a Mouse intact xiphisternum

The air pouch was cleaned with 70% alcohol and shaved. A small incision was made in the cavity using sterile scissors and the incision opened by blunt dissection. The autologous intact xiphisternum was inserted into the pouch using sterile forceps. Immediately the opening was closed with a 7.5 mm Michel Clip (Thackray Ltd, London). The wounded area was cleared daily with 70% alcohol until the wound healed.

C6b Millipore half chamber

This technique was developed for experiments where minced xiphisternal fragments were used. Without using this technique, the complete retrieval of the implanted pieces was difficult. A sterilised millipore filter (pore size 5 μm) was attached to a millipore ring (13 mm diameter) with millipore MF cement (Millipore Corporation, Bedford, Massachusetts, USA). The fragments of cartilage were then placed in millipore half chambers, and implanted into the preformed air pouch as usual.

C6c Rat articular cartilage

In experiments where the articular cartilage were used, the same procedures were used as in mice, and the incision was closed with a 12.5 x 2.5 mm Michel Clip. There was

no need to use the millipore half chamber technique in experiments where minced articular cartilage was used, because its convenient size allowed retrieval.

C6d Subcutaneous implantation

Cartilage was implanted into the subcutaneous tissue of some rats, without prior air pouch formation, through a small incision on the hind flank. The tissues were parted by blunt dissection and closed with a Michel Clip.

D. HISTOLOGY

Samples of air pouch, superficial inguinal lymph node, spleen and implanted cartilage were removed from one animal in each experimental group. Tissues were fixed in 10% buffered formal saline (pH 7.0).

This contained:

Na_2HPO4	162.0 g
NaH_2PO4	87.5 g
NaCl	212.5 g
HCHO	2.5 ml

This was made up to 25 litres with distilled water.

Tissues were processed for paraffin wax embedding according to the following schedule:

70% alcohol	1½ hour	
90% alcohol	1½ hour	
100% alcohol	1 hour	(four changes)
Toluene	1½ hour	(two changes)
Molten 56°C paraffin wax	1½ hour each	(two changes)

The alcohol used was 74 OP industrial alcohol. Blocks were vacuum-impregnated with molten wax for half an hour before being embedded in fresh wax. Sections were cut at 5µm on Leitz base-sledge microtome, floated onto a 48°C water bath and picked directly onto glass microscope slides. Sections were stained with Harris haematoxylin and eosin, and by the alcian blue methyl green pyronin technique. The toluidine blue technique was used to stain cartilage samples and the MSB technique was used to stain fibrin.

D1 Demonstration of the anatomical site of increased vascular permeability and the number of vessels involved

A colloidal suspension of carbon (Gunther Wagner, Pelikan Werke, Hanover, Germany - Batch C11/1431a) was administered intravenously at a dose of 1.0 ml/100 g body weight, fifteen minutes before killing the animals. A piece of the pouch lining tissue attached to the skin was then excised and fixed in formal saline. The fixed skin was subsequently dehydrated in ethanol and cleaned in cedar wood oil for 48 hours (Spector, Walters and Willoughby, 1965). Using this method, the vascularity of different aged linings could be assessed. To study the resorptive capacity of linings for particular matter, 0.2 ml colloidal carbon suspended in 5 ml saline was introduced into air pouch for 30 minutes before killing. The tissue was then fixed in formal saline and embedded in paraffin for histology.

D2 Haematoxylin and eosin

Sections were de-waxed with two changes of xylene for two minutes each, then hydrated with descending concentrations of

alcohol (absolute, 90% and 70%) to tap water. Sections were stained with Harris haematoxylin for five minutes, then washed in tap water and differentiated in 1% acid alcohol for approximately 30 seconds. At this stage, a microscopical check was made for direct nuclear staining. Sections were washed with tap water for five minutes and stained with 1% aqueous oesin for three minutes, then dehydrated with ascending alcohol concentrations (70%, 90% and absolute). Sections were cleared in two changes of xylene and mounted in chlophonium resin.

Results

Nuclear stained with blue colouration and cytoplasm with shades of pink.

D3 MSB (Martius, scarlet, blue) technique for fibrin

D3a Reagents

Martius yellow (acid yellow 24) C.I.10315

Brilliant crystal scarlet (acid red 44) C.I. 16250

Soluble blue (methyl blue) (acid blue 93) C.I. 42780

D3b Preparation of solutions

i)	martius yellow (acid yellow 24)	0.5 g
	phosphotungstic acid	2.0 g
	95% alcohol	100 ml
ii)	brilliant crystal scarlet (acid red 44)	1.0 g
	glacial acetic acid	2.0 ml
	distilled water	100 ml
iii)	phosphotungstic acid	1.0 g
	distilled water	100 ml

iv)	methyl blue (acid blue 93)	0.5 g
	glacial acetic acid	1.0 ml
	distilled water	100 ml
v)	glacial acetic acid	1.0 ml
	distilled water	100 ml

D3c Technique

Sections were taken to water, mercury pigment was removed with iodine thiosulphate treatment, and nuclei were stained by the celestin blue-haematoxylin sequence. Sections were differentiated in 1% acid alcohol, then washed well in tap water and were then rinsed in 95% alcohol. Sections were stained in Martius yellow solution for two minutes, then rinsed in distilled water and were then stained in brilliant crystal scarlet solution for ten minutes and rinsed in distilled water. Sections were treated with phosphotungstic acid solution until no red remained in the collagen and then rinsed in distilled water. Methyl blue solution was used to stain sections until collagen was sufficiently coloured, then they were rinsed in 1% acetic acid and deydrated through alcohols. Finally, sections were cleared in xylene and mounted with chlophonium resin.

D4 Alcian blue, methyl green-pyronin technique

D4a Reagents

i) 1% alcian blue in 0.1 M hydrochloric acid (pH 1.0)

ii) methyl green-pyronin

iii) - solution A: A 2% aqueous solution of methyl green was made and all the methyl violet was extracted with chloroform. The solution was washed with several

changes of chloroform in a separating funnel until the chloroform remained colourless. From the chloroform washed solution, the following was freshly prepared:-

2% aqueous methyl green (chloroform washed)	10.0 ml
5% aqueous pyronin Y	17.5 ml
distilled water	250.0 ml

- solution B: Acetate buffer solution, pH 4.8, was prepared by mixing 119 ml 0.1 M sodium acetate with 0.1 M acetic acid. The working solution was prepared by mixing equal volumes of solution A and B in a Coplin jar.

D4b Technique

Sections were taken to water and then stained with alcian blue for two minutes and then washed with distilled water. Sections were stained for 30 minutes with the working solution of methyl green and pyronin, then rinsed with distilled water for a few seconds and blotted lightly until dry. Sections were dehydrated with acetone for not more than one minute, then rinsed with equal parts of acetone and xylene and cleared in pure xylene. Finally, sections were mounted with chlophonium resin.

D5 Toluidine blue technique

D5a Reagent: veronal acetate buffer (pH 4.5) containing 0.25% W/V toluidine blue.

Veronal acetate buffer

Stock (A); veronal acetate stock solution.

1.94 g sodium trihydrate (MW136) and 2.94 g sodium barbitone

(MW206) were dissolved in 100 ml distilled water.

Stock (B); 0.1 M HCl (MW36.46).

0.85 ml hydrochloric acid was dissolved in 100 ml distilled water. Veronal acetate buffer solution was prepared by mixing 5 ml stock A, 10.5 ml stock B and 7.5 ml distilled water, and the pH of the solution was adjusted to 4.5. 57.5 mg toluidine blue was added to the veronal acetate buffer solution.

D5b Technique

Sections were taken to water, then stained with the buffered toluidine blue solution for 30 seconds and washed with distilled water. Sections were dehydrated rapidly, then cleared in pure xylene and mounted with chlophonium resin.

D5c Results

Metachromatic areas of red purple.

Orthochromatic areas of blue.

D6 ^{35}S-sodium sulphate uptake by cartilage cells in vitro

The ^{35}S-sodium sulphate supplied in sterile aqueous solution (The Radio-Chemical Centre, Amersham, UK) was used to detect the synthesis of DNA by cultured cartilage. The isotope solution was diluted in medium 199 to give a final concentration (in culture vessel) of 15 µci/ml. Cartilage was removed from the air pouch at the end of the implantation period and then incubated in the culture vessel (Sterilin Ltd, Middlesex, England) for twelve hours. The cultures were washed thoroughly with medium 199 to remove residual radio-activity.

The tissue was fixed for one hour in 3:1 absolute alcohol to acetic acid, and then buffered 10% formal saline for

24 hours before normal tissue processing. Sections were cut and the same procedures used to prepare the histological samples were applied.

D6a Autoradiography

Glass slides were thoroughly cleaned by soaking in a solution comprising potassium dichromate (100 g) and concentrated sulphuric acid (100 ml) made up to a final volume of one litre with distilled water. When the slides were perfectly wettable by water, they were washed and dipped into a solution containing gelatin (5 g) and chrome alum (0.7 g) made up to one litre with distilled water. The slides were drained and allowed to dry. The tissue sections were then mounted directly on these slides, which, when prepared in this way, had the property of forming a good wet adhesion with the applied radiosensitive emulsion. Paraffin sections were de-waxed in xylene and taken to water through absolute, 90% and 70% alcohol. Autoradiographic stripping film (AR 10, Kodak) was cut into strips (2.5 cm x 3.5 cm) and floated onto a solution sucrose (200 g) and potassium bromide (0.1 g), which was made up to one litre with distilled water. One volume of this solution was diluted with nine volumes of distilled water and mixed thoroughly before use at room temperature (16 - 21^{o} C). The emulsion was left on the solution for at least three minutes and then floated onto the sections, with the radiosensitive slides facing the sections. The sections were then dried thoroughly and stored in the dark for seven days at -20^{o} C, allowing exposure of the radioactive material to the radiosensitive film. After exposure, the film was developed in a solution composed of Metol (BDH), 40 g for five

minutes and washed for one minute in water. This was followed by fixation in MB Amfix (May and Baker), of which one volume was diluted in four volumes of distilled water. Sections were then washed in a large bowl of water, then air dried over night. Then the autoradiographs were re-hydrated by washing in water, stained with Harris' haematoxylin for fifteen minutes, differentiated in acid alcohol for ten seconds and 'blue' in tap water for twenty minutes. Then they were air dried and mounted in polymount.

E. RADIAL IMMUNODIFUSION FOR DETERMINATION OF α 1 - GLYCOPROTEIN

This method is based on the original technique of Mancini et al (1965) with a few minor modifications.

E1 Preparation of buffer and agar

Barbiturate buffer was prepared by dissolving 9 g sodium diethyl barbiturate in 200 ml distilled water; 65 ml 1/10M HCl were added together with 0.5 g sodium azide as preservative. The total volume was then adjusted to one litre to give a buffer of pH 8.6 and an ionic strength of 0.1. Stock agar was prepared by adding 3 g special Agar Noble (Difco) to 100 ml barbiturate buffer and heated on a boiling water bath until dissolved (distilled water was added to replace loss due to evaporation). When cool, the stock agar was kept at 4^{o} C until used.

E2 Preparation of anti-serum agar plate

Agar plates of dimensions 15 cm x 10 cm x 1 mm were used for the determination of α 1 - glycoprotein (α 1 GP) in animal serum and inflammatory exudate. For this, 7.5 g stock agar was weighed and 7.5 ml distilled water was added. This

mixture was melted on a boiling water bath and then allowed to cool to 60° C. Anti-serum to rat ∝ 1 GP (kindly supplied by Dr M Billingham, ICI Chemicals, Macclesfield, Cheshire) was then added to the agar mixture. The anti-serum was diluted to 1:600, ie, 0.25 ml in 15 ml. The temperature of the mixture was then kept at 60° C by the use of a temperature controlled water bath prior to pouring into the plastic dishes (Hyland). The agar/anti-serum mixture was pipetted into the 1 mm deep washed plastic dishes by the use of a hot Pasteur pipette, and allowed to set in the horizontal position for about twenty minutes.

E3 Assay of ∝ 1 - glycoprotein

A series of 3 mm wells were made in the agar plate using a gel cutter of 3 mm diameter, and the agar was sucked out under vacuum. 3 μ 1:20 dilution of the assay sample (in saline) was added to each well. Four standard sera of known ∝ 1 GP concentration were added to appropriate wells to construct a standard curve (fig. one) for calculating the unknowns. The standards supplied were 8, 4, 2, and 1 μg ∝ 1 GP/ml obtained from severely inflamed rats with arthritis. These were diluted 1:20 before use and 3 μl of the diluted standards and unknown were applied to the wells in the plates. The concentration of the protein is directly proportional to the (diameter^2) of the precipitation ring. The plates were left to reach equilibrium for at least 96 hours. The diameter of the precipitation ring was then measured using a magnifying eye-piece fitted with a millimeter scale divided into 0.2 mm divisions.

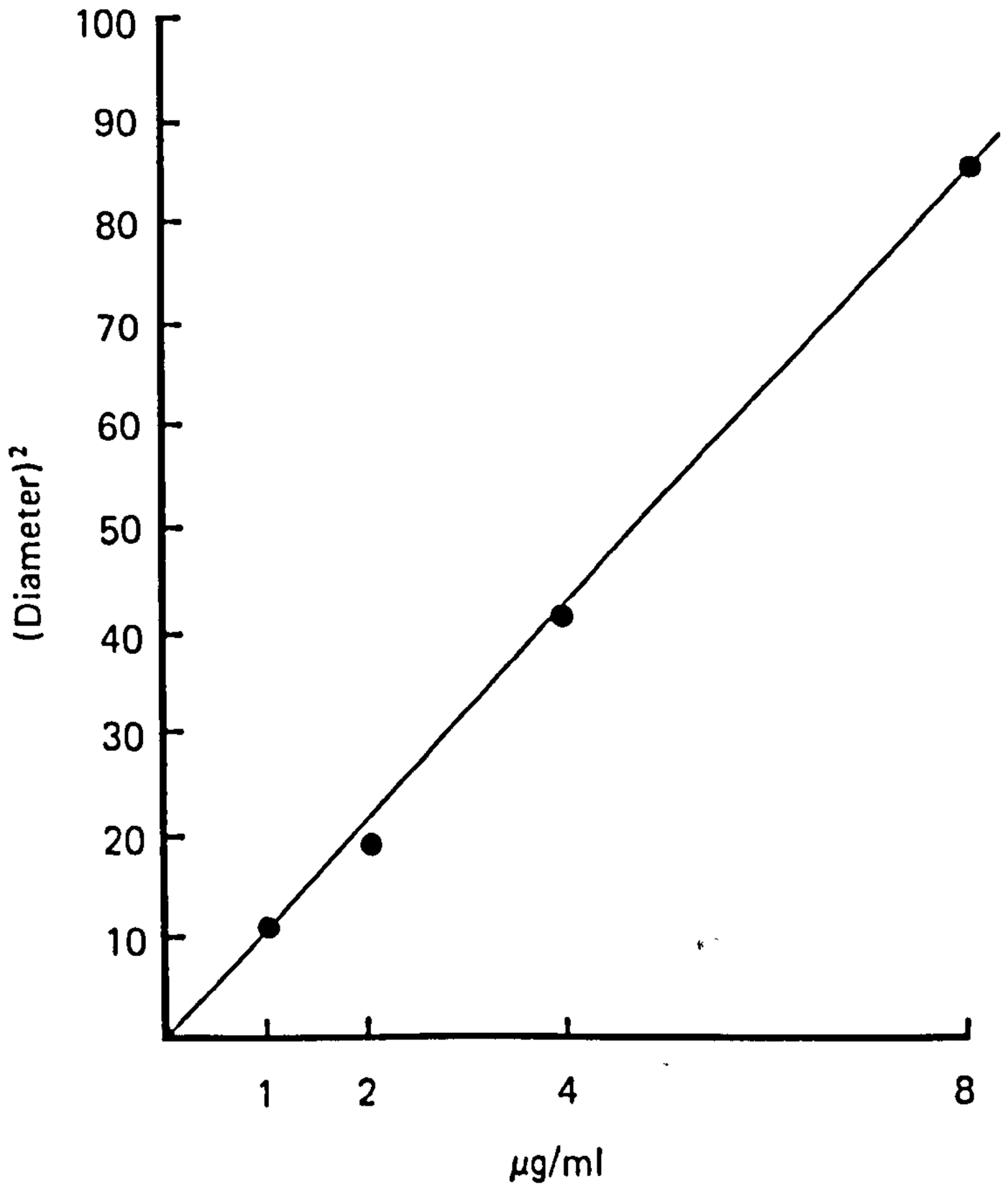

Fig.1

Standard curve for α-1-acid glycoprotein assay

F. PROTEOGLYCAN ASSAY

The amount of proteoglycan in the cartilage specimens was determined by the method of Farndale et al (1982) with a few minor modifications. This method is based on the metachromatic effect produced by binding acidic polymers to the basic dye, resulting in a shift of absorption maximum from 625 nm to 535 nm.

F1 Reagents

F1a Formate buffer solution

This solution was prepared as follows:- 2 g sodium formate (Sigma) and 2 ml formic acid (90%) were added together in a 1000 ml volumetric flask, and the volume completed to 1000 ml with distilled water. The solution was shaken well and the pH adjusted to 3.5 with a pH meter.

F1b 1, 9 Dimethylmethylene blue (DMB) stock solution

16 mg DMB (Serva Feinbiochemica, Heidelberg, Germany) was dissolved in a 100 ml volumetric flask with 5 ml ethyl alcohol. The volume was adjusted to 100 ml with formate buffer solution. The stock solution of DMB was shaken well and stored in a brown bottle at room temperature for up to one week.

F1c DMB working solution

The stock solution of DMB was diluted to 10-folds (1:9) with formate buffer and used on the same day. The A535 of this solution must be between 0.29 - 0.31 for a good result.

F1d Digest solution for cartilage sample

326 mg N-acetylcysteine and 744 mg EDTA (disodium) were dissolved in 100 ml 0.5 M phosphate buffer (pH 6.5) in a

1 litre volumetric flask. The volume of the solution was adjusted to 1000 ml with distilled water.

F2 Cartilage digestion

The rat cartilage samples were digested in 2 ml of the digestive solution with 20 µl papain (type III, Sigma Chemical Co Ltd, London) in a 10 ml Bijou bottle (Scientific Supplies Co Ltd, Vine Hill, London). The samples were incubated at 60° C for two hours. The amount of the digestive solution and the papain required for the mouse xiphisternal and femoral cartilage were 1 ml and 10 µl respectively.

F3 The spectrophotometric determination of the proteoglycan

40 µl of the rat cartilage digest was added to 2.5 ml DMB working solution in a suitable spectrophotometer cuvette and the contents were mixed by inversion several times. The spectrophotometer was zeroed against a blank sample of the DMB standard solution. Optical density was read at a wavelength of 535 nm on absorbance scale 1.0. The volume of the mouse xiphisternum digest was 250 µl and was 750 µl for the mouse femoral head.

F4 Standard curve

The A535 value was compared with a standard curve (fig 2) using the same volume of (40 µl) 0.5 M phosphate buffer (pH 6.5) containing 1 - 8 µg whale chondroitin 4-sulphate (Miles Laboratories Ltd, Slough, UK), mixed with the same volume of dye.

G. THERAPEUTIC AGENTS USED DURING THESE STUDIES

All drugs were suspended in water and the drug suspensions

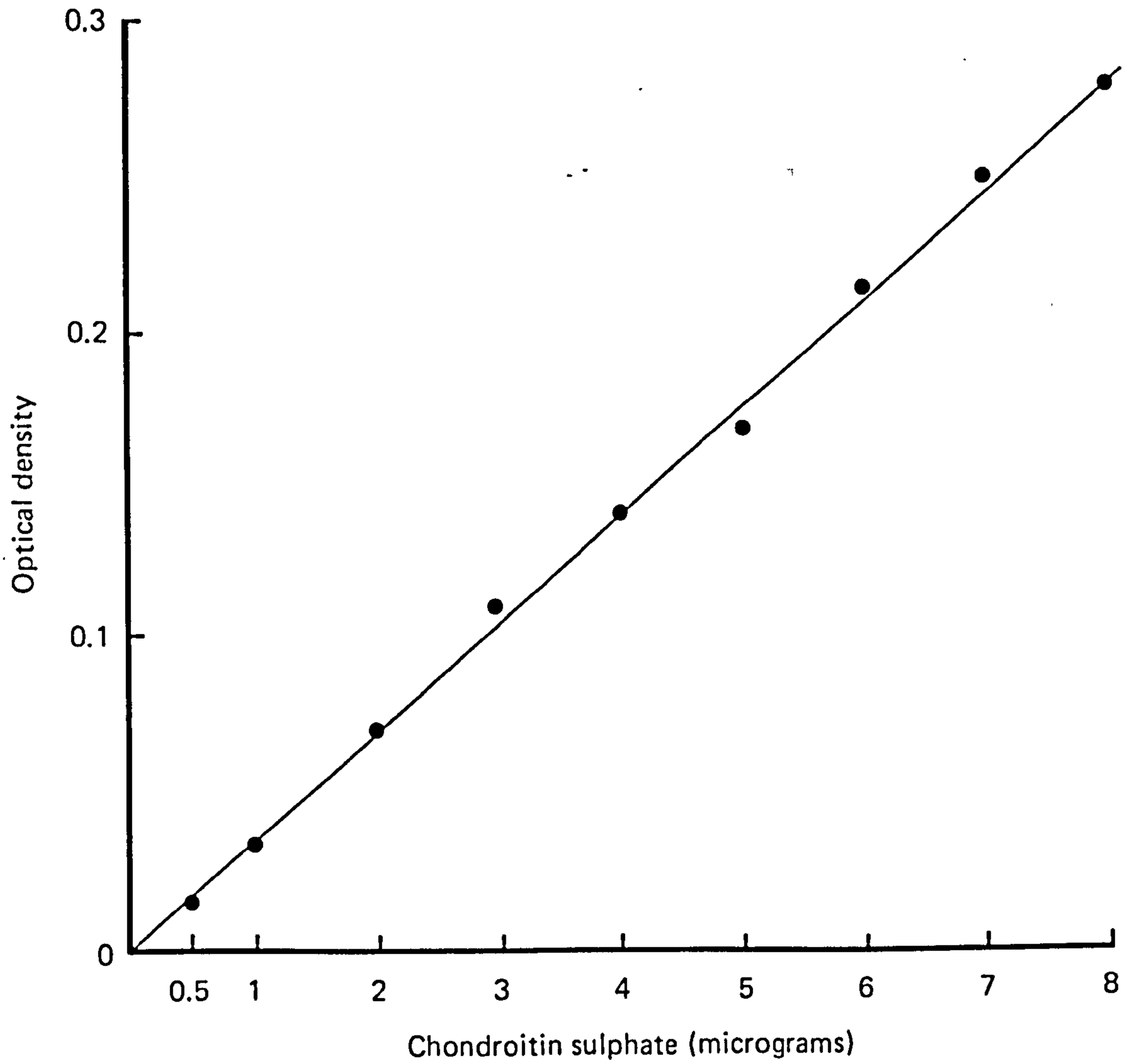

Fig. 2

Standard curve for proteoglycan assay

were freshly prepared before use. Rats received a single daily oral dose of drug for different time intervals, suspended in 1 ml water. Mice received the same dose for seven consecutive days in 0.2 ml water.

G1 Non-steroidal anti-inflammatory compounds

G1a Indomethacin

Indomethacin α - (1-P-Chlorobenzoyl-5-methoxy-2-methylindol-3-yl) - acetic acid, $C_{19}H_{16}ClNO_4$= 357.8. 25 mg white powder enclosed in capsule was obtained from Merck, Sharp and Dohm. A dose of 1.0 and 3.0 mg/kg was used throughout this study, suspended in water and administered orally.

G1b Aspirin

Aspirin, acetylsalicylic acid, $C_9H_8O_4$= 180.2. 300 mg tablets were obtained from Bayer, Germany. 100 mg/kg and 200 mg/kg doses of drug were suspended in water and administered orally.

G1c Benoxaprofen

Benoxaprofen (2, 4, Chloro-phenyl, alpha methyl 5 benzoxazole acetic acid) was kindly supplied by Lilly & Co, Ltd. The drug was suspended in water and administered orally at a dose of 10 mg/kg and 25 mg/kg.

G1d Piroxicam

Piroxicam, 4-hydroxy-2-methyl-3-(pyrid-2-ylcarbamyol)-2h-1, 2 - benzothiazine, 1,1 - dioxide, $C_{15}H_{13}N_3045$ = 331.3. This was obtained from Pfizer, USA, in 250 mg capsules. The drug was suspended in water and administered at a dose of 5 mg/kg and 10 mg/kg orally.

G2 Steroidal anti-inflammatory drugs

G2a Dexamethasone

Dexamethasone, 9 α - Fluoro - 16 methylprednisolone; $C_{22}H_{29}FO_5$ = 392.5. 2 mg tablets were obtained from Organon Pharmaceutical Co. Doses of 0.1 mg/kg and 0.2 mg/kg were suspended in water and administered orally.

G2b Hydrocortisone acetate

Hydrocortisone acetate, 21-acetoxy-11B, 17 α - dihydroxy-pregn -4-ene-3,20-dione; $C_{23}H_{32}O_6$ = 404.5. The drug was obtained from Organon Pharmaceutical Co., and suspended in medium 199. A dose of 1.0 and 5.0 mg/mouse was prepared and injected into the pouch lining tissue in a volume of 0.1 ml or injected directly into the air pouch in a volume of 1 ml.

G2c Hydrocortisone sodium succinate

Hydrocortisone 21 - sodium succinate, sodium 21 (B-Carboxy-propionyloxy)-11B, 17α-dihydroxypregn-4-ene-3,20 dione; $C_{25}H_{33}NaO_8$ = 484.5. The drug was obtained from Oganon Pharmaceutical Co., and dissolved in medium 199. A dose of 1.0 mg and 5.0 mg/mouse was prepared and injected in the same route used with hydrocortisone acetate.

G3 Anti-rheumatic drugs

G3a D-penicillamine

D-penicillamine, D-3-mercaptovaline; $C_5H_{11}NO_2S$ = 149.2. 250 mg tablets were obtained from Dista Pharmaceutical Co. A dose of 25 mg/kg and 50 mg/kg was suspended in water and administered orally.

G3b Levamisole

Levamisole ($C_{11}H_{12}N_2S$: (I) - 2, 3, 5, 6 - tetrahydro-6-phenyl-imidazo (2-1-b) thiazole hydrochloride = 240.8. This was kindly supplied by Janssen Pharmaceuticals Ltd. The drug was suspended in water and doses of 2.5 mg/kg and 5.0 mg/kg were administered orally.

H. ASSESSMENT OF GASTRIC IRRITANCY

H1 Macroscopic

Following the treatment of animals with non-steroidal anti-inflammatory agents, rats were assessed for evidence of gastric irritation. After killing the animals, the abdomen were cut open and the viscera was removed. The stomach, duodenum and ileum were placed in a beaker containing phosphate buffered saline (PBS). Portions of these tissues were cut and the contents removed by washing with a.pasteur pipette and PBS. The tissues were examined by putting them on clean glass plates. Each tissue was assessed for evidence of ulceration.

H2 Microscopic

Areas of stomach, duodenum and ileum were fixed in formal saline, embedded in paraffin wax, and the cut section was stained with haematoxylin and eosin. Sections were examined for microscopic evidence of ulceration.

I. STATISTICAL TESTS

Calculation of the mean, standard deviation and standard error of the mean.

I1 Mean

$(\bar{X}) = \frac{\sum X}{n}$ where $\sum X$ is the sum of X and n is the number of observations.

I2 Standard deviation

$$SD = \sqrt{\frac{\sum (\bar{X} - X)^2}{n - 1}}$$

I3 Standard error of the mean

$SEM = \frac{SD}{\sqrt{n}}$ this gives a measure of its precision.

I4 Significant tests between two sample means

The student t-test of significance was used.

$$t = \frac{\bar{X}1 - \bar{X}2}{\sqrt{SE^2 + SE_2^2}}$$ with n1 + n2 - 2 degree of freedom

By convention it is taken that a difference between sample and control mean with a probability of $P < 0.05$ is probably significant, $P < 0.01$ is significant and $P < 0.001$ is highly significant.

RESULTS

Autologous cartilage implantation in the six day pouch of the mouse

The mouse six day pouch was used as an *in vivo* model to study the mechanisms of cartilage degradation. In experiments where transplantation is studied, inbred or athymic animals should be used to avoid the problems of implant rejection. In the present study to overcome the rejection of cartilage, outbred mice were used as donorsand recipients. Xiphisternum was found to be a suitable source of autologous cartilage because it is easily removed and its removal causesrelatively small injury to the animal.

Mouse xiphisternum was carefully removed from the anaesthetised animal (see methods) and the different tissues attached to it were removed. Samples of removed cartilage were divided into two groups - alive and dead - and each group contained intact (whole) or minced samples. Care was taken throughout to ensure control cartilage was taken from animals of the same weight and age.

The autologous samples were implanted in the mice preformed six day pouches. Some of the air pouches were inflamed with 1 ml 1% carrageenan solution and the others were non-inflamed. At different time intervals after implantation, the implants were removed and their loss of proteoglycan analysed, both histologically and biochemically.

Location of implanted cartilage in the pouch

Cartilage, whether intact or finely minced, once introduced into the pouch, was randomly distributed on the lining

tissue. In non-inflamed pouches, the cartilage was found firmly attached to the lining tissue even one day after implantation. In contrast, when the cartilage was implanted into pouches previously inflamed with carrageenan, it remained free in the cavity throughout the experimental period.

Histological observations

Normal mouse xiphisternum

The concentration of proteoglycan in cartilage can be demonstrated by the intensity of staining with toluidine blue. Using this stain, it can be seen that the concentration is not evenly distributed throughout the tissue. In the thicker part, the matrix separating the chondrocytes has a relatively high amount of proteoglycan. In contrast, the matrix surrounding the cells further away from the sternum, and especially at the periphery of the tissue, has a lower concentration of proteoglycan. In cross section, the cartilage appears to have two distinct regions, a central region with well-defined lacunae and the peripheral perichondrium made up of blood vessels, adipose tissue and fibroblasts (fig 3).

Living intact cartilage

No major differences either in histological appearance or metachromatic reactivity were observed in intact cartilage obtained from mice 1, 3, 5, 7 or 14 days after implantation in normal air pouch. Loss of proteoglycan, as judged by weaker staining with toluidine blue, was seen at the area where the tissue had been separated from the sternum. Occasionally, small areas of depletion were also seen at the free ends and in areas which faced fibrous connective tissue.

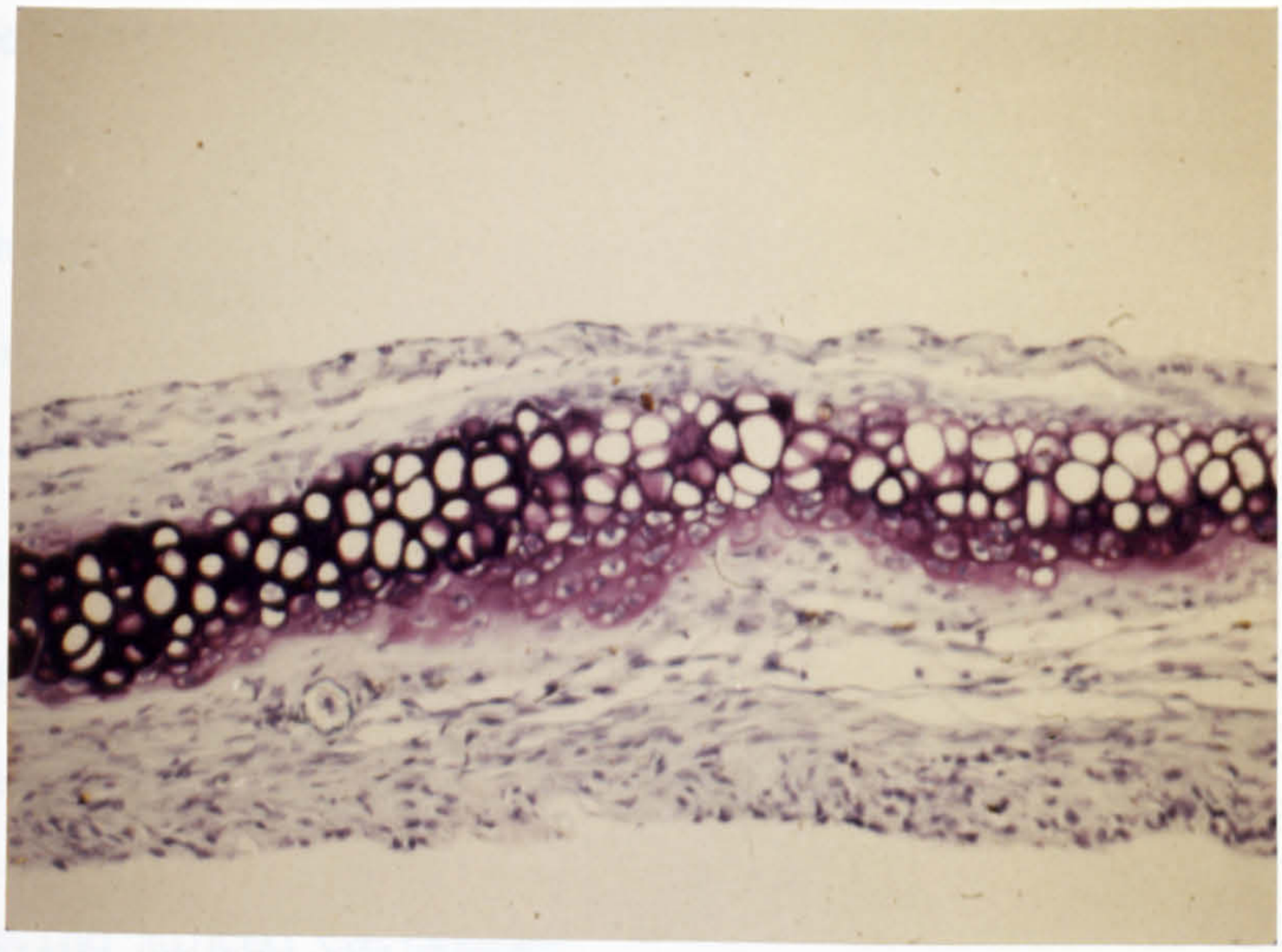

Fig.3 Normal mouse xiphisternum. Toluidine blue.
Magnification x 100.

Induction of inflammation in the air pouch, using carrageenan, was not found to change the intensity of toluidine blue staining in the implanted cartilage.

Dead intact cartilage

Dead intact cartilage implanted into inflamed or non-inflamed air pouches showed relatively greater and faster loss of proteoglycan than living intact cartilage. Furthermore, even in the absence of carrageenan, the majority of this tissue was seen to be surrounded by polymorphonuclear and mononuclear cells. Severe loss of proteoglycan could be detected in this tissue five days after implantation and by fourteen days, almost complete loss of metachromatic staining was observed.

Living minced cartilage

Living minced cartilage fragments implanted into air pouches showed an obvious loss of metachromatic staining, even three days after implantation. Complete loss of metachromasia could be seen in pieces obtained from the free ends of the xiphisternum, and frequently these cartilage fragments were found to be surrounded by polymorphonuclear and mononuclear cells, even in the absence of carrageenan. Cartilage obtained from animals five and seven days after implantation showed further loss of proteoglycan and by fourteen days, severe loss of staining was observed.

The presence of inflammatory cells surrounding the cartilage fragments suggests that the degradation of this tissue stimulates an inflammatory reaction. In general, those fragments implanted into carrageenan-inflamed pouches showed an accelerated loss of metachromasia, particularly in the areas

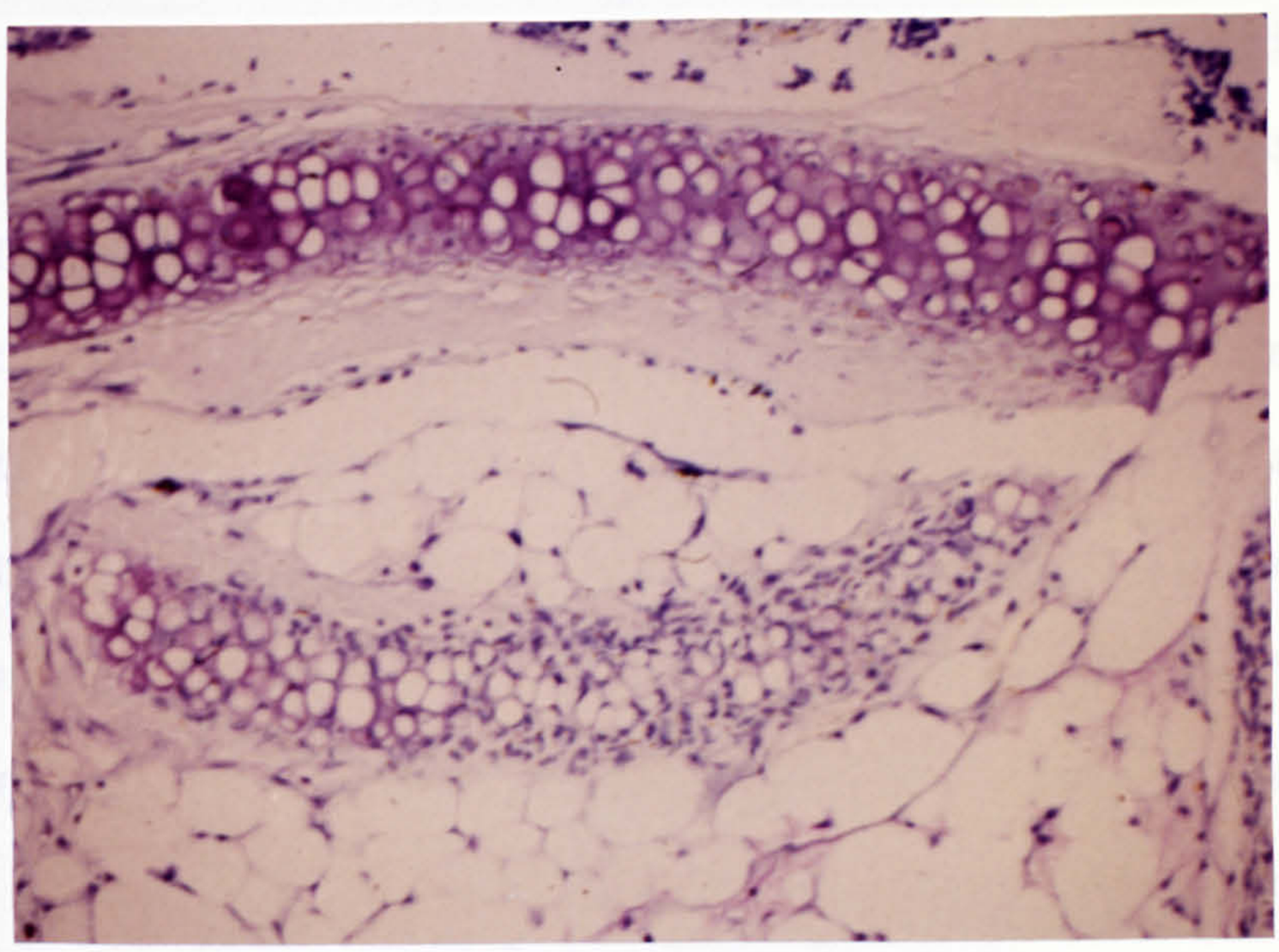

Fig. 4 Minced xiphisternum 6 days after implantation into a carrageenan air pouch. Toluidine blue. Magnification x 100.

close to inflammatory cells (fig 4).

Biochemical assessment of cartilage degradation

Cartilage implants were assayed for total proteoglycan. Biochemical analysis of samples showed a similar pattern of results to those found using histological techniques. Table (1) figure (5) show the amount of proteoglycan found in living intact and minced cartilage after implantation into normal and inflamed pouches. After seven and fourteen days of implantation, intact cartilage showed little change in levels of proteoglycan. The highest proteoglycan loss from intact implants was detected at fourteen days after implantation into carrageenan-inflamed pouches, and that was about 12%. At seven days of implantation, minced xiphisternal fragments showed a loss of about 24%, this was observed in normal pouches. Implantation into carrageenan-inflamed pouches for seven days caused a loss of 47%. Minced xiphisternal fragments implanted for fourteen days into normal and inflamed pouches lost about half its proteoglycan content . Proteoglycan determination of dead cartilage samples showed a rapid and massive loss of proteoglycan from implants. Table (2) figure (6) show a time course for the release of proteoglycan from dead intact xiphisternal implants. The loss of proteoglycan from dead intact samples implanted into normal or inflamed pouches was nearly the same. As the time of implantation increased, the loss also increased until it reached its maximum at fourteen days, where almost total loss could be detected.

Time / Experiment	7 days		14 days	
	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change
normal xiphisternum	13.12 ± 1.0	-	13.12 ± 1.0	-
intact xiphisternum in air pouch	13.4 ± 1.28	+2%	12.6 ± 1.2	-4%
intact xiphisternum in inflamed pouch	13.0 ± 1.0	-1%	11.56 ± 1.08	-12%
minced xiphisternum in air pouch	9.96 ± 0.92	-24%	6.84 ± 0.64	-48%
minced xiphisternum in inflamed pouch	7.48 ± 0.56	-47%	6.44 ± 0.6	-51%

Table (1) Amount of proteoglycan (µg) in living intact and minced xiphisternum at 7 and 14 days after implantation into 6 day old air pouch.

Mean Values ± S.E.M.

9 mice per group

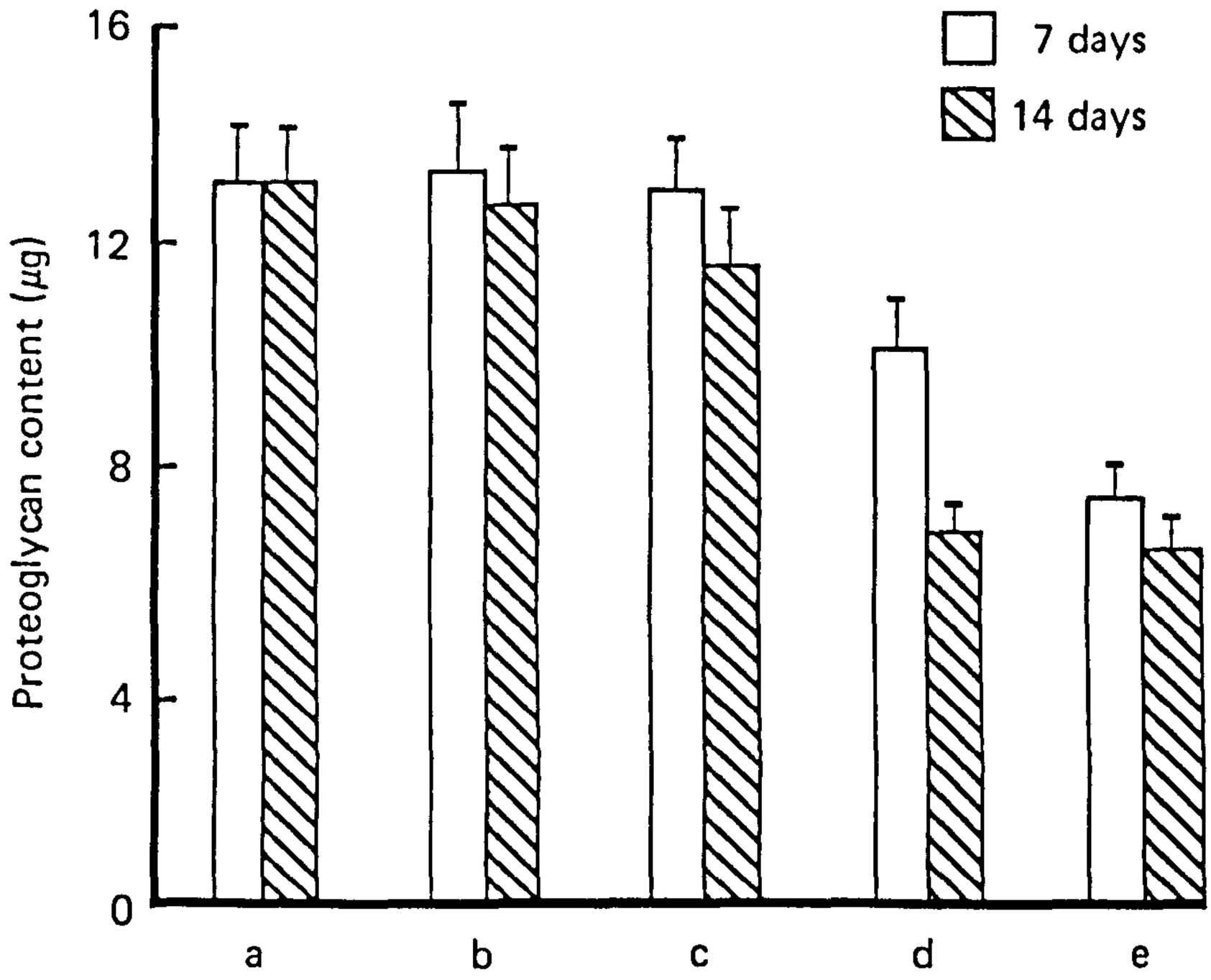

Fig. 5

Amount of proteoglycan in living intact and minced cartilage at 7 and 14 days after implantation into 6 day old air pouch. Groups: a — normal xiphisternum, b — intact cartilage in air pouch, c — intact cartilage in air pouch with carrageenan, d — minced cartilage in air pouch, e — minced cartilage in air pouch with carrageenan.

Time / Experiment	1 day		3 days		5 days		7 days		14 days	
	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change
normal xiphisternum	11.8 ± 0.96	-	11.8 ± 0.96	-	11.8 ± 0.96	-	11.8 ± 0.96	-	11.8 ± 0.96	-
intact cartilage in air pouch	10.96 ± 0.88	-7	9.08 ± 0.76	-23	6.48 ± 0.56	-45	3.8 ± 0.36	-68	0	-100
intact cartilage in inflamed pouch	10.72 ± 0.72	-9	9.32 ± 1.0	-21	6.84 ± 0.72	-42	3.32 ± 0.28	-72	0	-100

Table (2) Proteoglycan content (µg) of dead intact xiphisternum at 1, 3, 5, 7 and 14 days following implantation into normal and 1% carrageenan inflamed pouches.
Mean values ± S.E.M.
9 mice per group

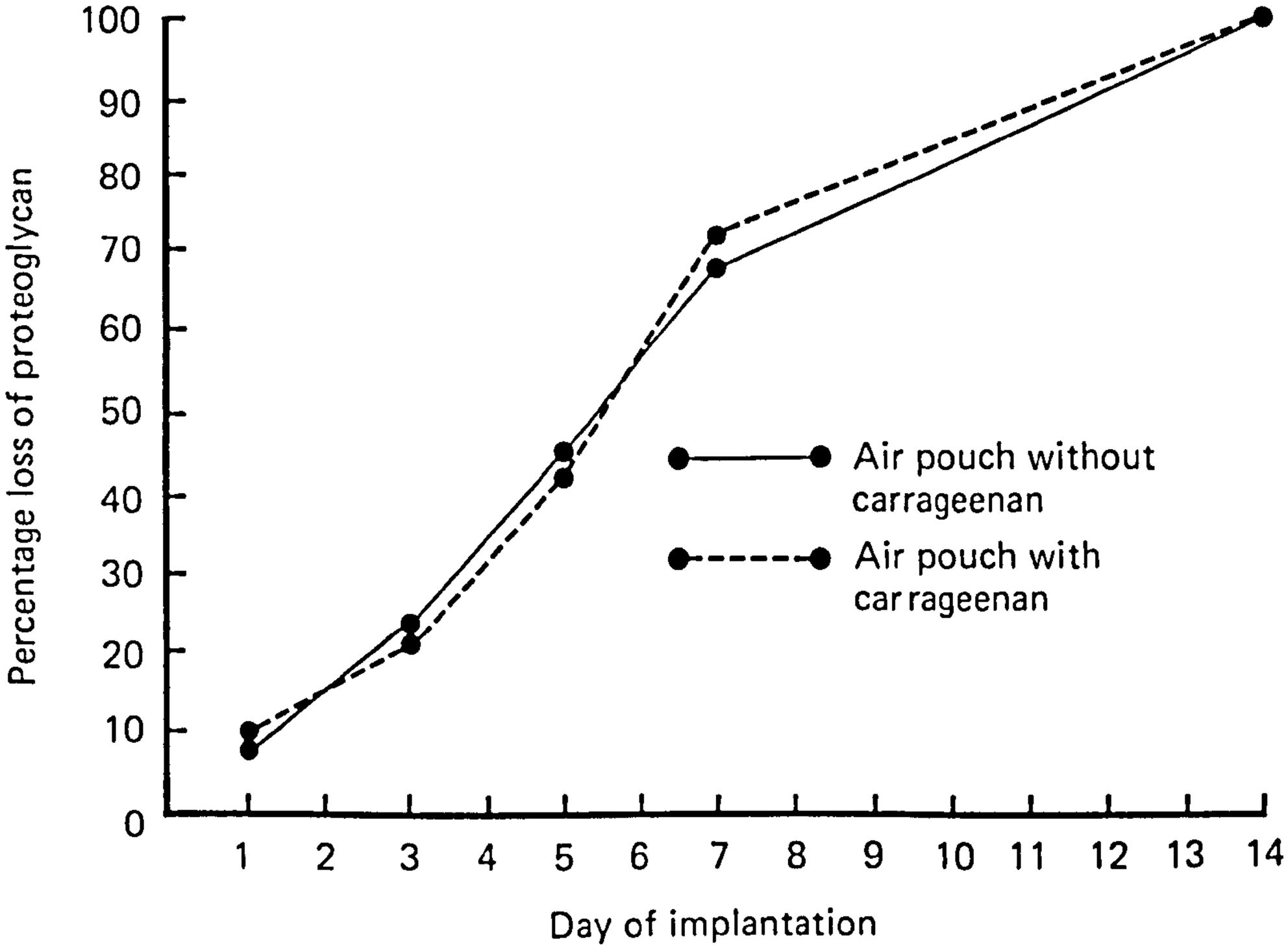

Fig. 6

Loss of proteoglycan in dead intact xiphisternum at different time intervals after implantation into 6 day old air pouch.

Millipore half chamber technique

Mouse xiphisternum is a tiny cartilaginous tissue and it is small both in size and weight. In experiments where minced xiphisternal fragments were used, the intact xiphisternum was cut into six pieces. Because of the difficulty in retrieving the small pieces of cartilage, a method was developed based on the millipore half chamber. This method allowed location of the cartilage fragments. Using this technique, there was 100% retrieval at the end of the implantation period. Implanting the millipore half chamber into the pouch could subject the implanted cartilage pieces to an additional inflammatory situation. This could be avoided by using the same procedure with the animals of the control group.

Effect of treatment with drugs on numbers of cells migrating into the mouse six day pouch and loss of proteoglycan from implanted xiphisternal fragments

Anti-inflammatory and anti-rheumatic drugs were tested on the migration of leucocytes and the loss of proteoglycan from xiphisternal fragments implanted in carrageen-inflamed six day pouch.

Non-steroidal anti-inflammatory drugs

Four non-steroidal anti-inflammatory drugs were tested and these were aspirin (100 and 200 mg/kg), indomethacin (1.0 and 3.0 mg/kg), benoxaprofen (10 and 25 mg/kg) and piroxicam (5 and 10 mg/kg). The drugs were given daily orally in a volume of 0.2 ml for seven days, commencing at

the time of carrageenan injection.

The effects of drugs on leucocyte migration and loss of proteoglycan from xiphisternal fragments are illustrated in table (3) and (4), figure (7). It was found that the system was not as reproducible as a similar system used in rats, fluctuations in cell numbers between individual mice producing high standard errors.

The total cell count in the control group was 5.5×10^6 after seven days of irritant injection. All the tested anti-inflammatory drugs were found to reduce the number of inflammatory cells migrating to the inflamed pouch. The significant effect was observed only with the high doses of aspirin (3×10^6), indomethacin (2.9×10^6) and benoxaprofen (2.8×10^6). Piroxicam showed a significant reduction with both the low (3×10^6) and the high (2.9×10^6) dose.

Results obtained from this study showed that there was no relationship between the anti-inflammatory action of the tested drugs and the loss of proteoglycan from the autologous implants. Implants obtained from pouches of control animals showed a loss in proteoglycan content of about 28.8%. Non-steroidal anti-inflammatories failed to reduce the loss of proteoglycan from the implants after seven days of daily treatment. Implants obtained from drug treated groups showed nearly the same profile of proteoglycan loss observed with the control group.

Anti-rheumatic drug

D-penicillamine was given in a dose of 25 mg/kg and 50 mg/kg orally daily for seven days, commencing at the time of irritant injection. D-penicillamine was found to have no

DRUG	Dose mg/kg	leucocyte number x 10^6
Carrageenan	1 ml of 1%	5.5 ± 1.1
Aspirin	200	3.0 ± 0.3**
	100	3.5 ± 0.7
Indomethacin	3	2.9 ± 0.6**
	1	3.5 ± 0.8
Benoxoprofen	25	2.8 ± 0.5**
	10	3.7 ± 1.0
Piroxicam	10	3.0 ± 0.9**
	5	2.9 ± 0.6
D-penicillamine	50	7.0 ± 0.1
	25	4.2 ± 0.1

Table (3) Effect of daily treatment for 7 days with a single oral dose of different drugs on the number of cells migrating into the inflamed 6 day pouch.

Mean values ± S.E.M.

10 mice per group

** $p < 0.01$

Drug	Dose mg/kg	Proteoglycan content (µg)	% change
Normal xiphisternum	-	11.8 ± 1.08	-
Carrageenan	1 ml of 1%	8.4 ± 0.44	-28.8
Aspirin	200	8.08 ± 0.6	-31.6
	100	8.48 ± 0.64	-28.2
Indomethacin	3	8.48 ± 0.44	-28.2
	1	8.52 ± 0.76	-27.9
Benoxaprofen	25	8.28 ± 0.84	-29.95
	10	8.76 ± 0.52	-25.9
Piroxicam	10	8.56 ± 0.72	-27.3
	5	8.52 ± 0.48	-27.9
D-penicillamine	50	10.92 ± 0.64**	-7.5
	25	9.16 ± 0.76	-22.4

Table (4) Effect of daily treatment for 7 days with a single oral dose of different drugs on the loss of proteoglycan from xiphisternal fragments implanted into carrageenan inflamed 6 day pouches.

Mean values ± S.E.M. 10 mice per group.

** $P < 0.01$

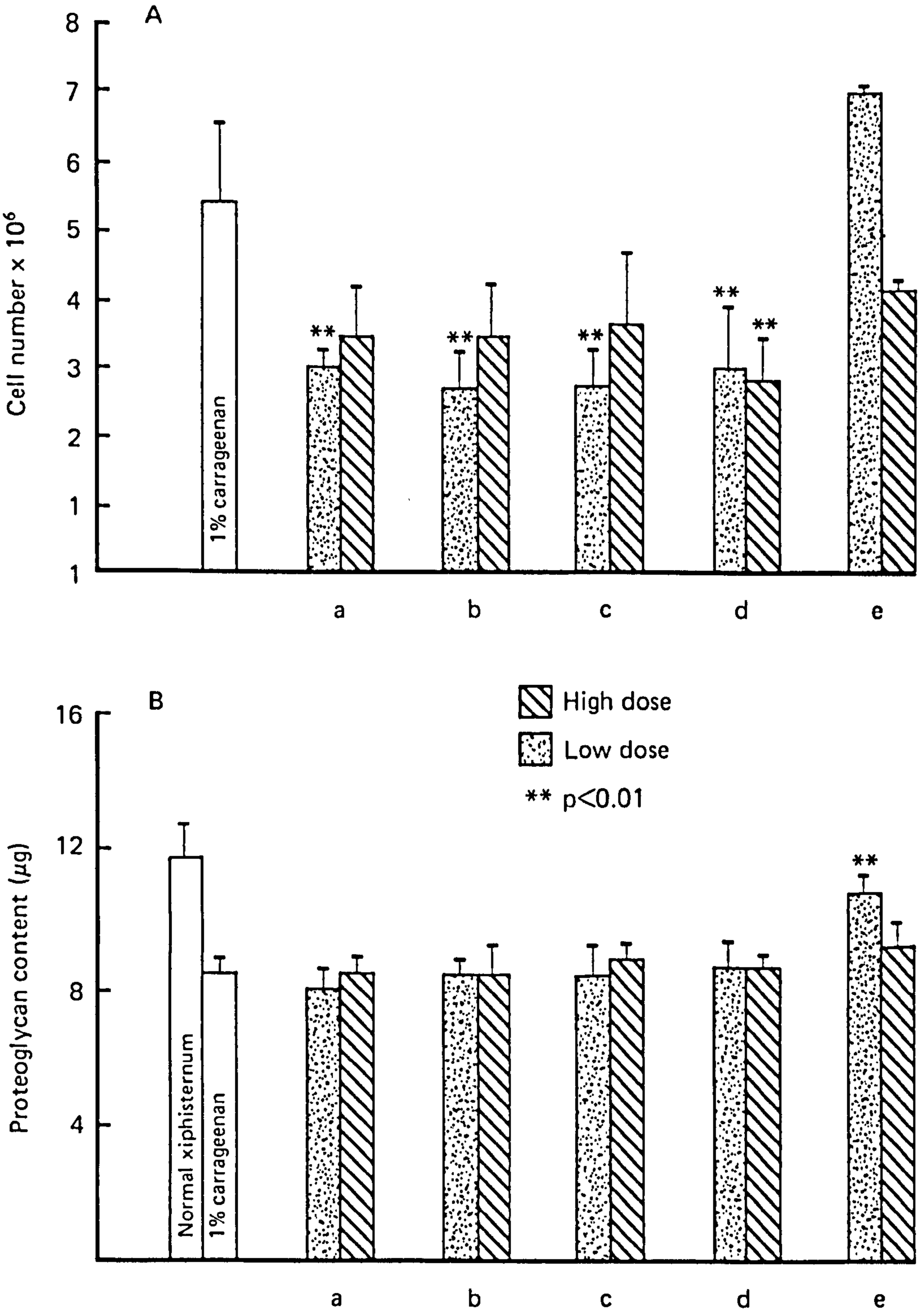

Fig. 7

Effect of daily treatment for 7 days with Aspirin (100, 200mg/kg); Indomethacin (1, 3mg/kg); Benoxaprofen (10, 25mg/kg); Piroxicam (5, 10mg/kg); D-Penicillamine (25, 50mg/kg); on number of cells and loss of proteoglycan from xiphisternal fragments implanted into carrageenan inflamed 6 day pouch of the mouse.

anti-inflammatory action, however, there was insignificant reduction in the number of migrating cells observed with 25 mg/kg treatment (4.2×10^6). A dose of 50 mg/kg D-penicillamine was found to increase the numbers of migrating leucocytes insignificantly (7×10^6). At this dose (50 mg/kg) D-penicillamine showed a significant ($P < 0.01$) protection to the implanted fragments and the proteoglycan loss was only 7.5%. However, 25 mg/kg D-penicillamine failed to protect the implanted cartilage significantly.

Effect of local administration of hydrocortisone salts on proteoglycan content of xiphisternal fragments implanted into six day pouch of the mouse

In this study, autologous xiphisternal fragments were introduced into millipore half chambers (see methods) and then implanted into carrageenan-inflamed pouches. Hydrocortisone sodium succinate (1 and 5 mg/animal) and hydrocortisone acetate (1 and 5 mg/animal) were diluted to appropriate concentrations with medium 199. These drugs were injected either directly into the air pouch or into the air pouch lining tissue, at the same time as cartilage implantation. Hyrdocortisone was prepared in a volume of 1 ml for direct pouch injection, or 0.1 ml for lining tissue injection. Seven days after implantation, the cartilage pieces were removed for proteoglycan analysis.

Table (5) figure (8) show the results for experiments in which the loss of proteoglycan from cartilage fragments of control animals was compared with that of animals treated with hydrocortisone salts. Minced autologous cartilage transplanted into control animals lost 39% of its

Treatment	Route	Dose mg/animal	micrograms of proteoglycan	% change
Normal control		-	12.84 ± 1.2	0
Carrageenan		1 ml of 1%	7.86 ± 0.76	-39
Hydrocortisone sodium succinate (soluble)	cavity	5	13.48 ± 1.28**	+5
	cavity	1	10.28 ± 0.88	-20
	lining	5	11.98 ± 0.95**	-8
	lining	1	9.76 ± 0.92	-24
Hydrocortisone acetate (insoluble)	cavity	5	6.56 ± 0.56	-49
	cavity	1	9.12 ± 0.95	-29
	lining	5	11.16 ± 0.88***	-13
	lining	1	10.8 ± 1.2	-16

Table (5) Effect of a single injection of hydrocortisone salts on proteoglycan loss from xiphisternal fragments implanted into inflamed 6 day air pouches for 7 days. Mean values ± S.E.M. 6 mice per group. ** $P < 0.01$ *** $P < 0.05$

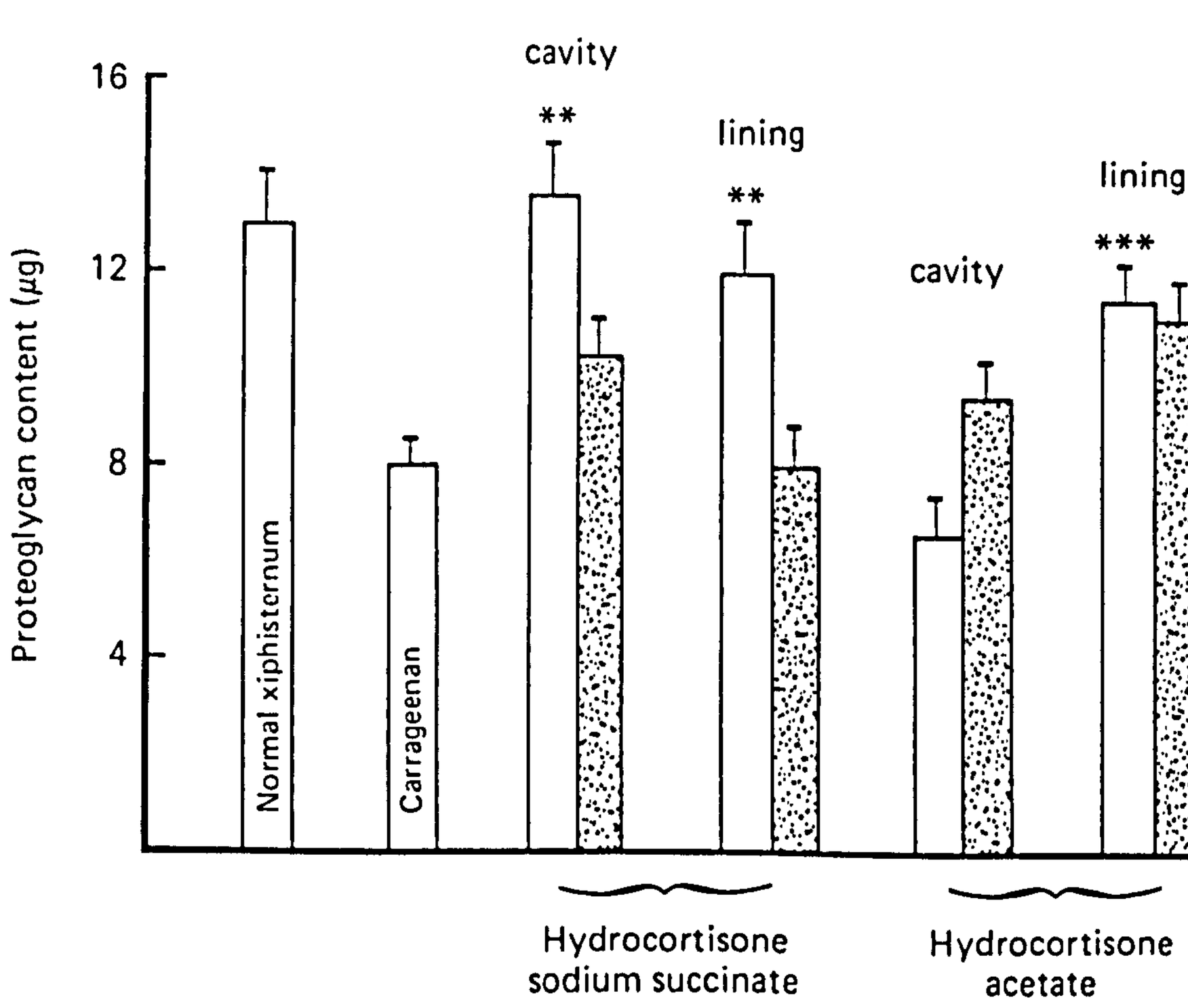

Fig. 8

Amount of proteoglycan in xiphisternal fragments after implantation into inflamed 6 day pouch for 7 days, and subjected to a single injection of hydrocortisone salts.

proteoglycan content. In comparison, cartilage transplanted into the soluble hydrocortisone sodium succinate-treated animals, showed decreased loss in proteoglycan. Results were given for drugs given either into the cavity or into the lining tissue. Proteoglycan loss was reduced in a dose dependent manner when this form of hydrocortisone was given by either route. Maximum protection was found with 5 mg hydrocortisone sodium succinate injected directly into the cavity.

When the insoluble hydrocortisone acetate was used, it was found that there was enhancement of the loss of proteoglycan from cartilage fragments when administered directly into the pouch at a dose of 5 mg/animal. As the concentration of drug was lowered, no enhanced loss of proteoglycan could be detected. When injected into the lining tissue, hydrocortisone acetate significantly reduced proteoglycan loss at a dose of 5 mg/animal.

The disadvantages of using the mouse as a source of cartilage

Mouse xiphisternum

The use of mouse xiphisternum as autologous implant into the six day pouch gave an indication of the mechanism (s) involved in cartilage degradation. However, the presence of perichondrium in this type of hyaline cartilage was always a major problem. Perichondrium could affect the process of cartilage degradation and also interfere with the effects of therapeutic agents on this process. In order to avoid the problems of perichondrium, cartilage samples were always minced before implantation.

Mouse articular cartilage

Cartilage degradation is always observed in diseases affecting the joints, so it is of great importance to use articular cartilage as implant. Articular cartilage is a modified type of hyaline cartilage, where there is no perichondrium in its structure (Barnett, 1968). Different sources of cartilage from the mouse were examined.

The cartilage cap of the femoral upper end was found to be most appropriate. However, the removal of the tiny cartilage cap from the bony head was technically impossible. This technical problem could affect the biochemical determination of proteoglycan of the cartilage cap by the interference of that present in the bony head.

Moreover, it was very difficult to detect the small amount of proteoglycan in the assayed samples, unless the assayed volume was increased massively (750 µl). Using such a large volume of the digest was found to affect the spectrophotometric assay by diluting the 2.5 ml dimethylmethylene blue working solution. These problems required the use of another source of articular cartilage.

Rat articular cartilage

Rat femoral head cartilage cap was found to be an ideal source for implantation. It is a reasonable size which enables easy removal from the bony head and allows the anatomical examination of degradation, ie, histological zonal loss of proteoglycan (fig 9). Also it contains enough sulphated glycosaminoglycans to be detected even with a small volume of sample (40 µl) affording a very reproducible system.

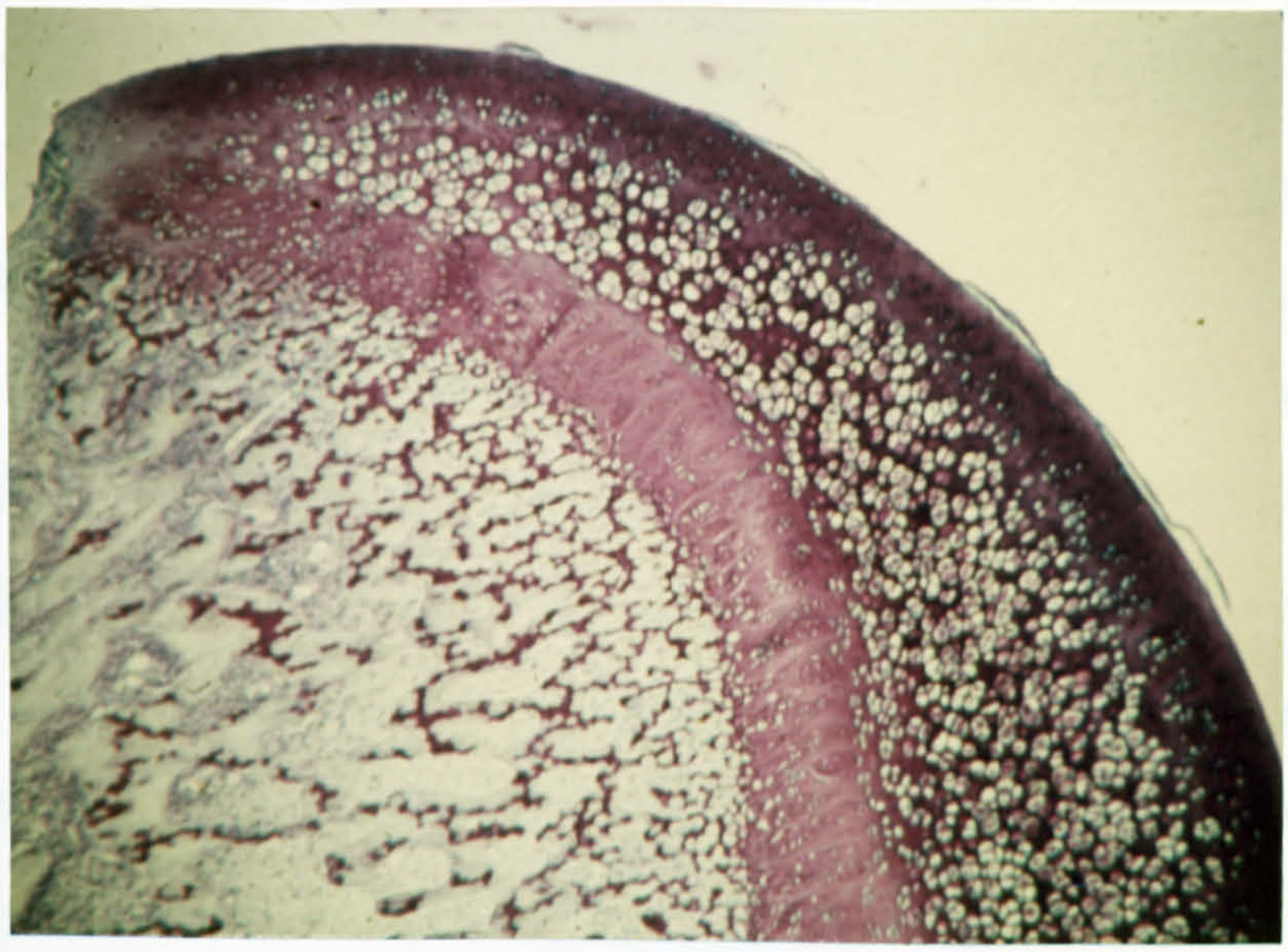

Fig. 9 Rat femoral head cartilage. Toluidine blue.
Magnification x 25.

The biochemical determination of the proteoglycan (sulphated glycosaminoglycans) of articular cartilage

Few alterations were introduced into the original method described by Farndale et al, 1982. The alterations were mainly applied to the procedures of cartilage digestion. The amount of digesting enzyme (papain) was increased from 10µl to 20 µl, and so was the time of incubation, which was increased from one to two hours. The temperature of incubation was lowered from 65° C to 60° C. Evaporation of solution from the digestive containers during incubation was a big problem, which could affect the whole assay, especially when the plastic coulter pots were used. The glass bijou bottles with their tight lids were found to prevent evaporation completely. Introducing the above mentioned improvements was a crucial factor in developing the assay.

Development of new model of prolonged inflammation in the rat six day pouch

Development of new models of prolonged inflammation was found to be very important to study the mechanism (s) of cartilage degradation from rat articular cartilage. To study these mechanisms, articular cartilage must be implanted into inflammatory and non-inflammatory situations. Our previous work showed that the mouse six day pouch was not a good inflammatory model when injected with carrageenan, ie, was not reproducible. Sedgwick et al, 1983, showed that the rat six day pouch responded in a "special" way to different inflammatory stimuli, however, these authors only examined the acute phase.

The viscarin type of carrageenan was found to induce a good inflammatory response in wistar rats. Previously, it has been shown to produce both acute (Capasso, Dunn, Yamamoto and Willoughby, 1975) and chronic (Robertson and Swartz, 1953) inflammatory response in the rat.

In this study, two different concentrations of carrageenan were used - 1% and 2%. Two concentrations were used to establish whether the inflammatory response produced was dependent on the quantity of carrageenan. Volumes of 2 ml physiological saline containing carrageenan were injected into pouches of different groups of animals, six days after the initial injection of air. At different time intervals following the injection of carrageenan, volumes of exudate were measured, total cell numbers were counted, exudate smears for differential counts were prepared and samples from normal and inflamed pouches were prepared for histology.

Results illustrated in table (6) figure (10) and (11) represent a comparison made at different time intervals, between the effects of injection of 2 ml of either 1% or 2% carrageenan solution on the type and total leucocyte number migrated to the pouch. Total cell numbers in the pouches injected with 2% carrageenan were greater than those obtained from 1% carrageenan treated pouches. The peak of the cellular response was seen two days after irritant injection, the total cell numbers being 311.8×10^6. 1% carrageenan gave a total cell count of 306.6×10^6, again peaking at two days. A significant ($P < 0.001$) increase in the total cell number was observed at 7, 14 and 21 days after injection of 2% solution. Total cell numbers were 187.7×10^6, 213.8×10^6 and 140.0×10^6 at 7, 14 and 21 days respectively.

time after carrageenan injection	1% carrageenan	2% carrageenan	polymorphs	mononuclears
1 day	188.7 ± 11.6	201.4 ± 16.9	96%	4%
2 days	306.6 ± 23.6	311.8 ± 17.1	89%	11%
7 days	109.3 ± 9.8	187.7 ± 13.8*	45%	55%
14 days	134.4 ± 8.2	213.8 ± 15.7*	24%	76%
21 days	108.8 ± 8.1	140.0 ± 10.0***	17%	83%

Table (6) Cell type and number ($x10^6$) observed in the 6 day pouch at different time intervals after injection of 1% or 2% carrageenan solutions.
Mean values ± S.E.M.
8 rats per group
* $P < 0.001$ *** $P < 0.05$

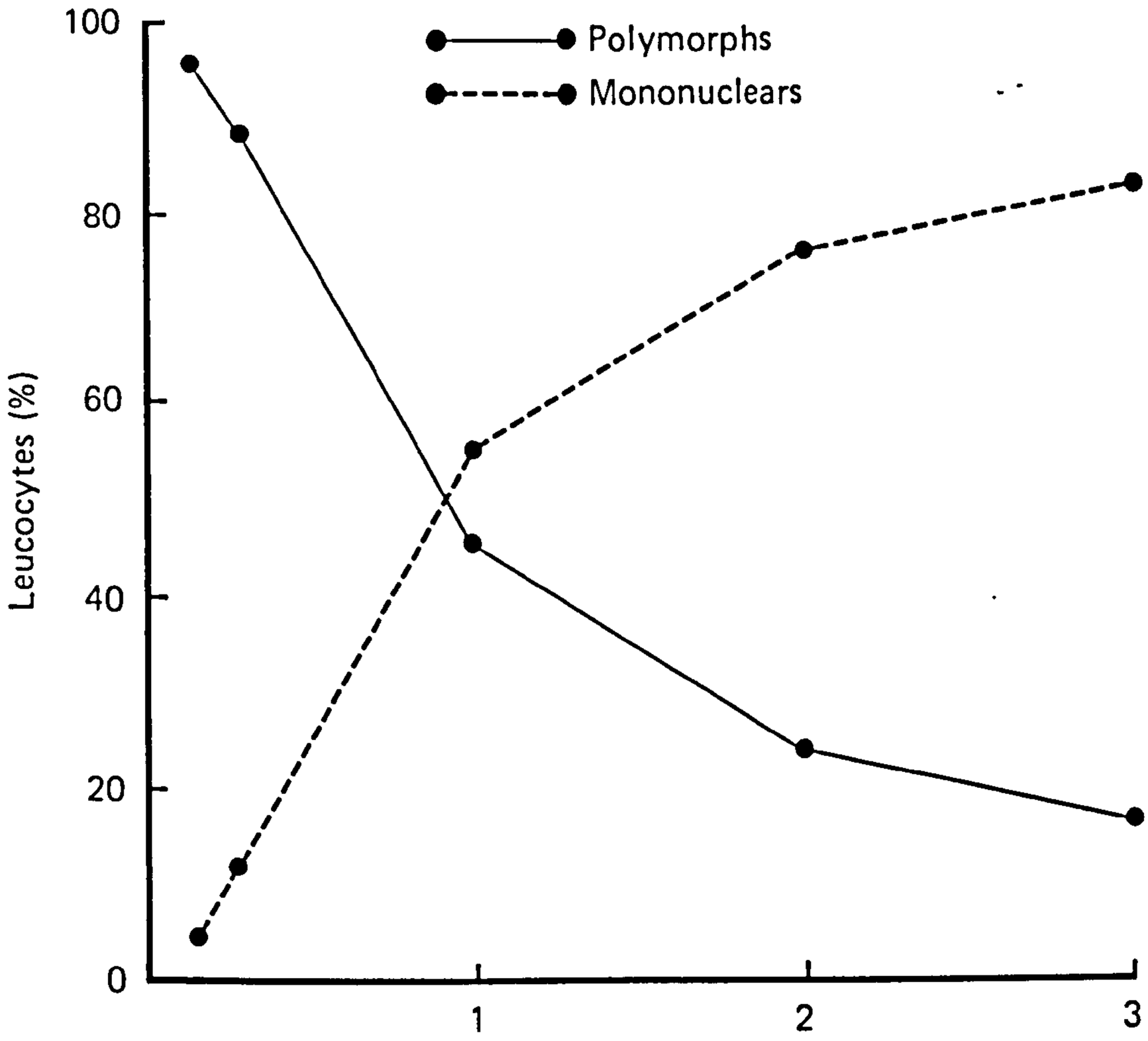

Fig. 10

Leucocyte type (%) observed in the six day pouch of rat at different time intervals following injection of 1 or 2% carrageenan solution (2ml).

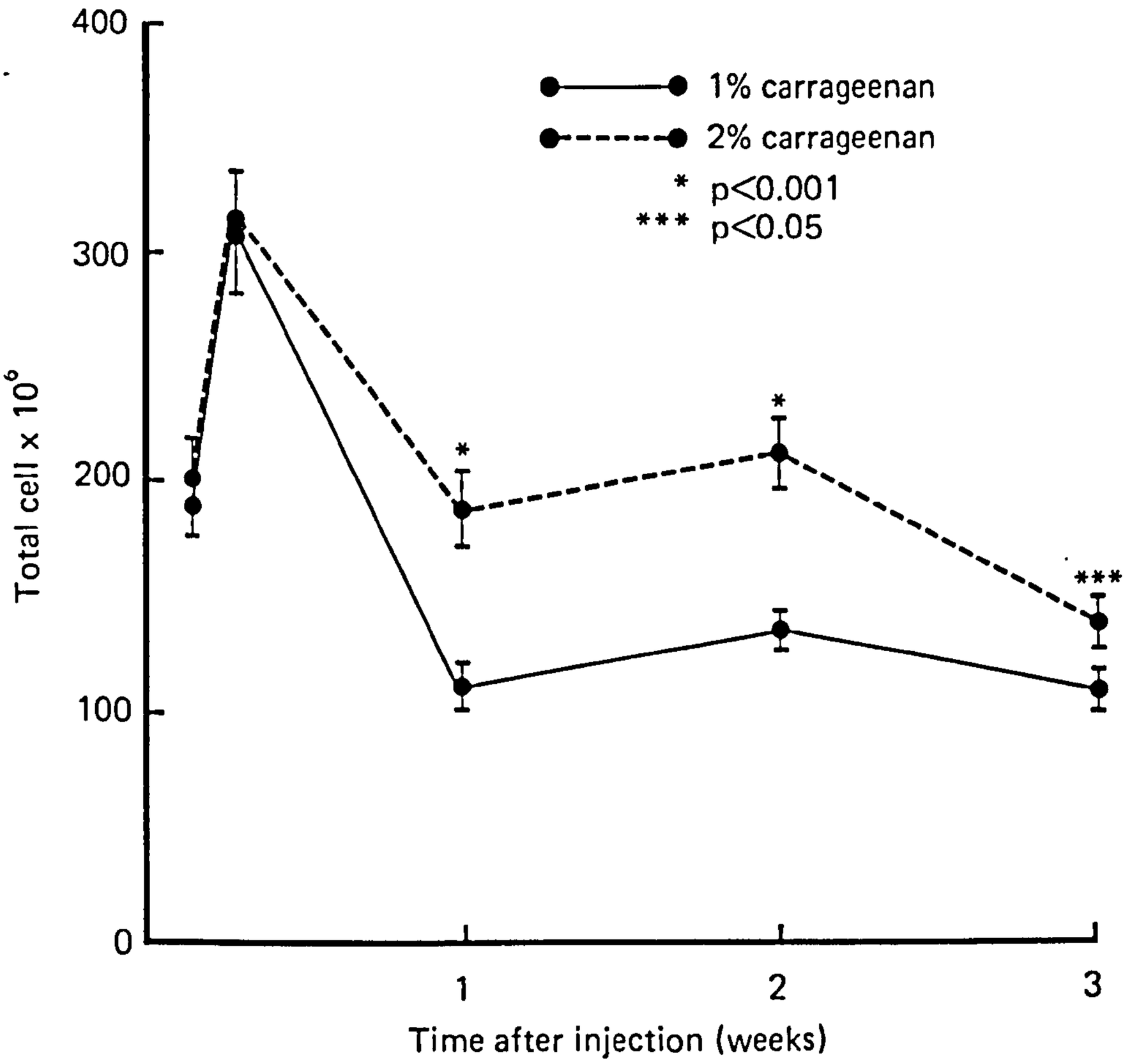

Fig. 11

Total leucocyte number (x 10^6) observed in the 6 day pouch at different time intervals after injection of 1 or 2% carrageenan solution.

Differential cell counts made on exudate smears showed a predominance of polymorphonuclear leucocytes (polymorphs) at the first and second days; the ratios of polymorphs to mononuclear leucocytes (mononuclears) at one and two days were96:4 and 89:11. One, two and three weeks after irritant injection, mononuclear leucocytes predominated, and the ratios were 45:55, 24:76 and 17:83 respectively. The differential cell counts on smears of exudate obtained from both concentrations of carrageenan showed no obvious difference. Trypan blue exclusion tests showed that more than 90% of the accumulating cells were viable.

The volumes of exudate obtained from the air pouches of different groups showed no significant differences. However, pouches treated with 2% solution were found to contain slightly larger exudate volumes than those treated with 1% solution. This was seen at 1, 2 and 7 days, while the reverse was observed at 14 and 21 days. Table (7) figure (12) represent a comparison between the effects of the two different concentrations of carrageenan on the volumes of exudate measured from pouches of different groups. The volume of exudate reached its maximum at 14 days following the injection, being 37.2 and 35.5 ml for 1% and 2% solutions.

Histological observations

Normal rat air pouch

Histological examination showed the non-inflamed six day air pouch tissue to have a lining layer of flattened mononuclear cells lying on a collagenous bed approximately 50 μm deep and orientated parallel to the pouch surface. Below this

Time after carrageenan injection	1% carrageenan	2% carrageenan
1 day	3.3 ± 0.18	4.6 ± 0.34
2 days	4.8 ± 0.34	6.5 ± 0.37
7 days	28.5 ± 2.04	31.0 ± 1.71
14 days	37.2 ± 2.3	35.5 ± 1.9
21 days	31.6 ± 2.41	29.4 ± 1.94

Table (7) Volume of exudate (ml) observed in the 6 day pouch at different time intervals after injection of 1% or 2% carrageenan solution.
Mean values ± S.E.M.
8 rats per group

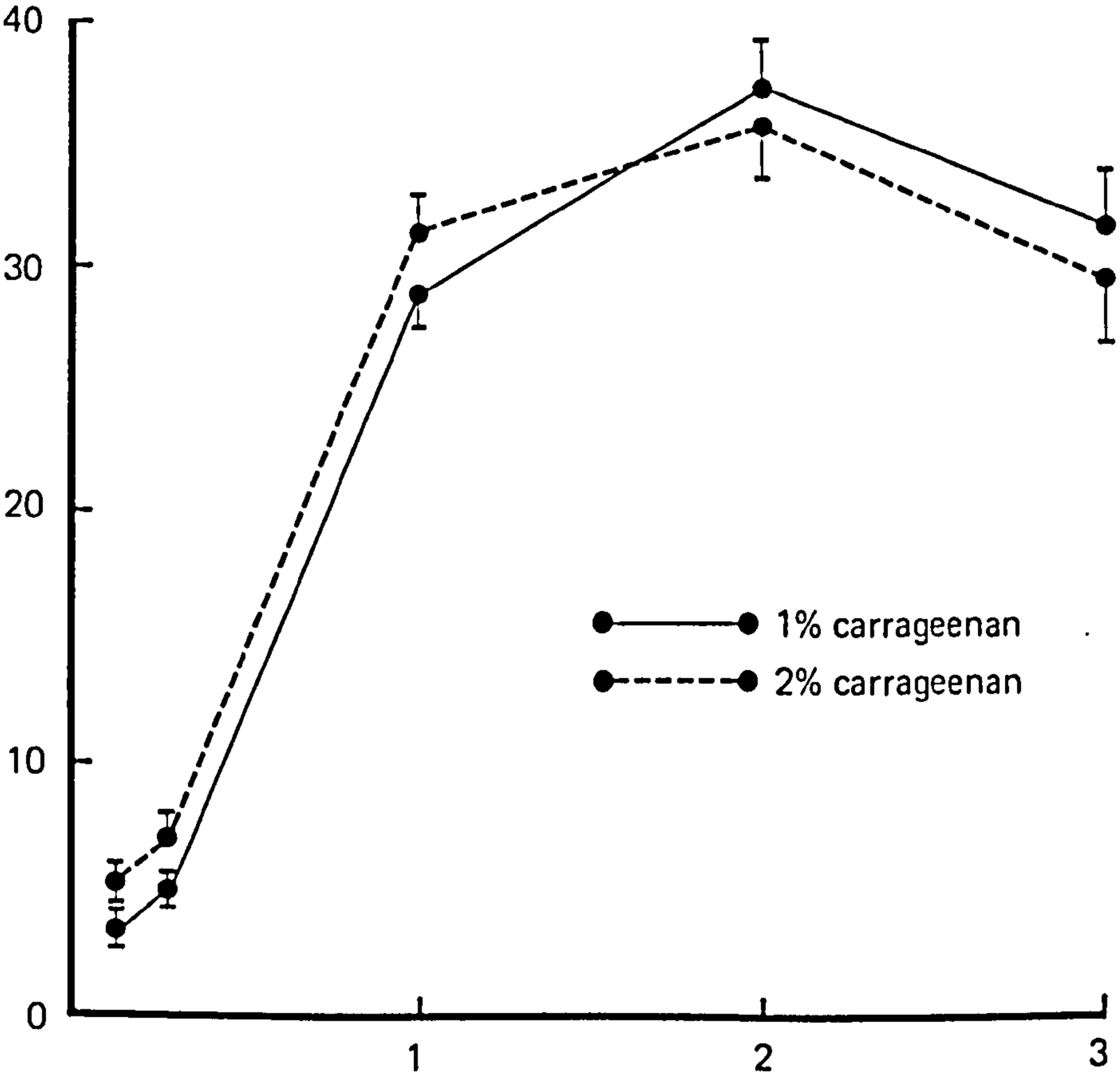

Fig. 12

Volume of exudate observed in the 6 day pouch at different time intervals after 2ml injection of 1 or 2% carrageenan solution.

was a region rich in blood vessels and lymphatics. A variable amount of adipose tissue was also present in this layer. Mast cells were found throughout the pouch lining; they were apparent on haematoxylin and eosin stained preparations, but were well demonstrated with Alcian blue. These cells were particularly associated with the vasculature. Deep to the vascular layer was loose connective tissue of relatively low cell density, again orientated parallel to the pouch surface. This layer of loose connective tissue was approximately twice the thickness of the upper layer and overlay subcutaneous muscle. Fibrin, as judged by MSB staining, was not a feature of the pouch lining. There was a suggestion that the pouch became more fibroblastic with time, but the general appearance was not found to change significantly throughout the three week period (figure 13).

Carrageenan-inflamed six day pouch

Air pouches inflamed with carrageenan showed polymorph infiltration of the lining one and three days after injection. Polymorphs were seen adhering to the blood vessel walls and migrating through the upper connective tissue to the pouch lining. There was little or no fibrin. After one week, there was dense cellular infiltrate superficial to the pouch lining, extending to a depth of approximately 100 μm. These cells were loosely arranged and several appeared stellate. Van Gieson staining showed an increase in collagen fibres in this superficial layer. At two weeks, a distinct surface layer of large eosinophilic cells from 1-3 cells thick was seen. This surface layer of cells was still present at three weeks. Increasing numbers of vacuolated mononuclear cells

Fig. 13 Normal rat air pouch 6 days after inflation.
Haematoxylin and eosin. Magnification x 200.

were seen in the connective tissue over the three week period. Up to seven days, these vacuolated cells were Alcian blue positive. The cells were morphologically distinct from mast cells and appeared to be macrophages. They were also Alcian blue positive. Later, by two to three weeks, some areas of vacuolated cells were seen to form homogenous masses, which stained poorly with Alcian blue and other histological reagents that have been used. These masses were both inside the lining and also seen as separate structures aherent to the lining surface. At two weeks, clusters of lymphatics and plasma cells were seen in association with the vacuolated cells (figure 14a & 14b).

Effect of drug treatment on carrageenan-induced prolonged inflammation

In this study, the effects of four different drugs on carrageenan-induced inflammation in the six day pouch were investigated. These compounds were administered to rats daily orally commencing at the time of carrageenan injection, for three, seven and fourteen days. Indomethacin was given at a dose of 1 mg/kg or 3 mg/kg, the steroid (dexamethasone) was given at a dose of 0.1 mg/kg or 0.2 mg/kg. D-penicillamine was given in a dose of 25 mg/kg and 50 mg/kg and finally levamisole was given in both 2.5 mg/kg or 5.0 mg/kg doses.

Indomethacin

The results illustrated in table (8) figure (15) are for experiments where the effect of indomethacin (1 or 3 mg/kg) on the exudate and cells migrating to the inflamed pouch was investigated. 3 mg/kg indomethacin

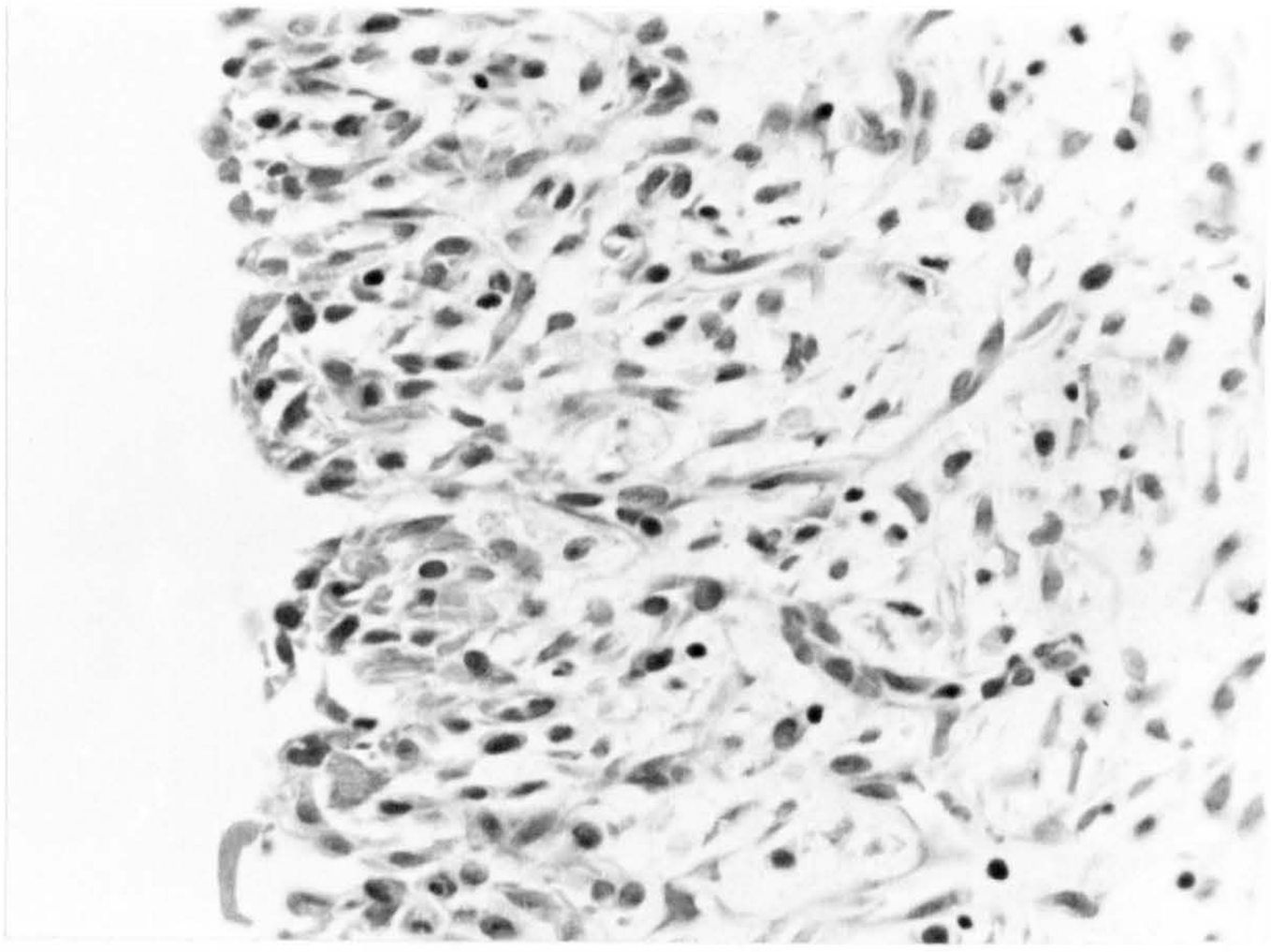

Fig.14a Rat air pouch 2 weeks after injection of carrageenan. Haematoxylin and eosin. Magnification x 200.

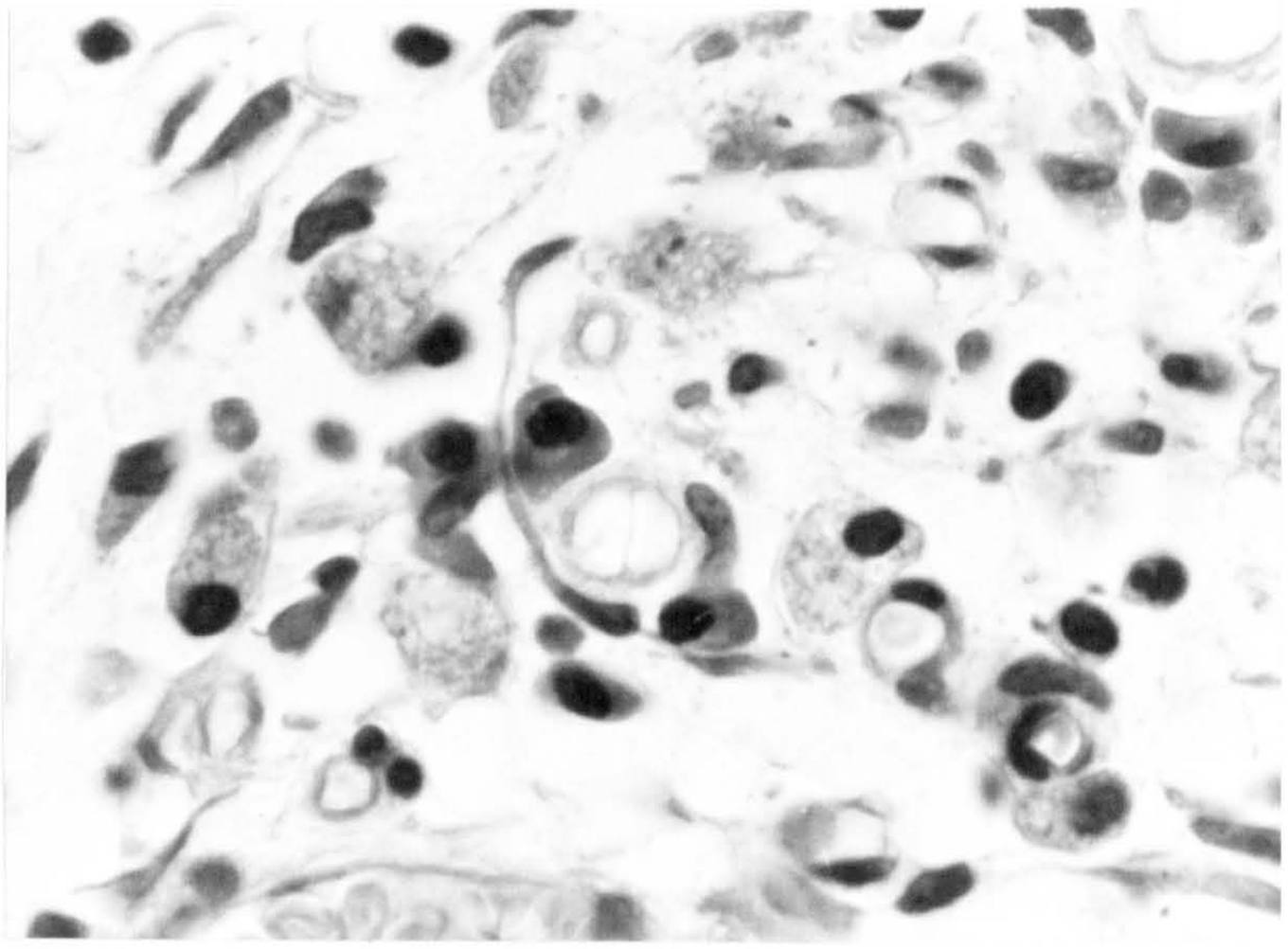

Fig.14b Rat air pouch 2 weeks after injection of carrageenan, showing plasma cells and foamy macrophages. Haematoxylin and eosin. Magnification x 800.

Experiment / Time	2.0 ml of 1% carrageenan		1.0 mg/kg indomethacin		3.0 mg/kg indomethacin	
	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6
3 days	9.07 ± 0.47	164.381 ± 10.15	4.45 ± 0.43*	135.96 ± 7.11	4.27 ± 0.3*	95.1 ± 10.3*
7 days	27.3 ± 0.71	138.58 ± 6.25	11.0 ± 1.20*	40.11 ± 2.85*	7.7 ± 0.39*	23.44 ± 1.28*
14 days	39.0 ± 9.3	125.71 ± 4.67	20.83 ± 2.2*	48.63 ± 1.51*	13.67 ± 1.5*	33.1 ± 3.1*

Table (8) Effect of daily oral administration of indomethacin (1 & 3 mg/kg) on carrageenan induced inflammation in the 6 day pouch.

Mean values ± S.E.M. 8 rats per group

* $P < 0.001$

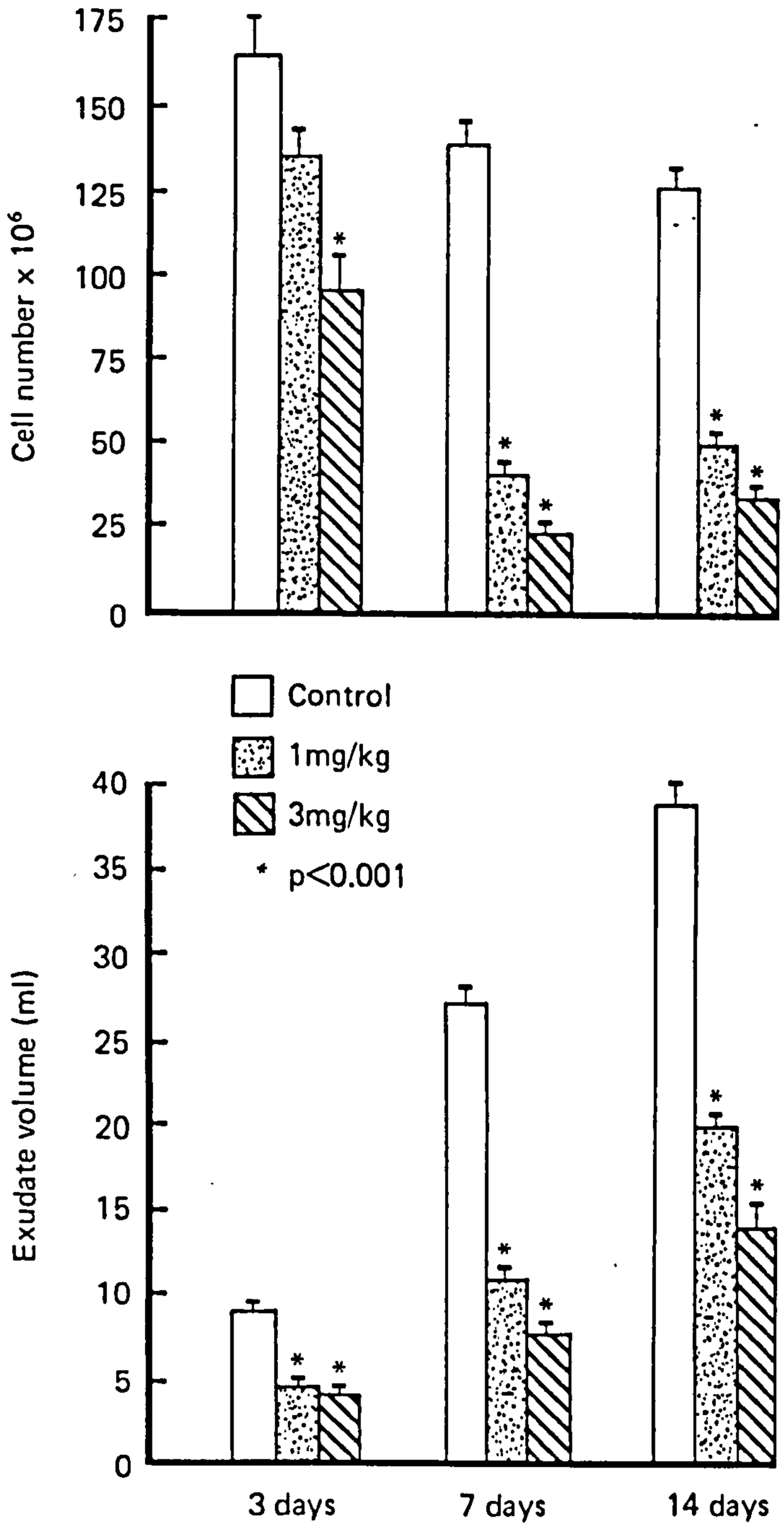

Fig. 15

Effect of daily oral administration of indomethacin (1 & 3mg/kg) on carrageenan (2ml of 1%) induced inflammation in the 6 day air pouch.

induced a significant ($P < 0.001$) inhibition in both the exudate volume (4.27 ml) and total cell number (95×10^6) accumulating in the pouch. The low dose of indomethacin (1 mg/kg) only reduced significantly ($P < 0.001$) the volume of exudate (4.45 ml) while there was a slight insignificant decrease in the total cell number. At 7 and 14 days of indomethacin treatment, both the tested doses had reduced the volume of exudate and total cell number significantly ($P < 0.001$). At the end of these experiments, the animals were killed and their gastro-intestinal tracts were examined for ulceration. No ulcers were detected either macroscopically or microscopically.

D-penicillamine

D-penicillamine was found to have a paradoxical effect on the inflammation induced by carrageenan in the six day pouch of the rat when tested throughout this study. Effects of D-penicillamine on inflammation induced by carrageenan are summarised in table (9) figure (16). Three days after continuous daily administration of a single dose of 25 mg/kg, the volume of exudate and total cell number were found to be reduced significantly ($P < 0.001$), while both cell numbers and exudate volumes were found to be reduced insignificantly after treatment with 50 mg/kg. Seven days after carrageenan injection, exudate volumes and total cell numbers (mean values) measured from pouches of control groups were 27 ml and 138×10^6.

Continuous administration of a single oral dose of either 25 mg/kg or 50 mg/kg for seven days, failed to reduce the inflammation. A slight increase in the volume of exudate (29.5 ml) and total cell number (144×10^6) was seen following

Experiment / Time	2.0 ml of 1% carrageenan		25.0 mg/kg D-penicillamine		50.0 mg/kg D-penicillamine	
	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6
3 days	9.1 ± 0.47	164.38 ± 10.15	4.73 ± 0.25*	102.61 ± 6.01*	8.0 ± 0.9	131.04 ± 14.95
7 days	27.3 ± 0.71	138.58 ± 6.25	23.7 ± 1.2	118.21 ± 12.4	29.5 ± 1.23	144.1 ± 5.51
14 days	39.0 ± 0.93	125.71 ± 4.67	41.33 ± 3.8	144.65 ± 5.28	48.67 ± 4.6*	214.36 ± 16.1*

Table (9) Effect of daily oral administration of D-penicillamine (25 & 50 mg/kg) on carrageenan induced inflammation in the 6 day pouch.
Mean values ± S.E.M. 8 rats per group

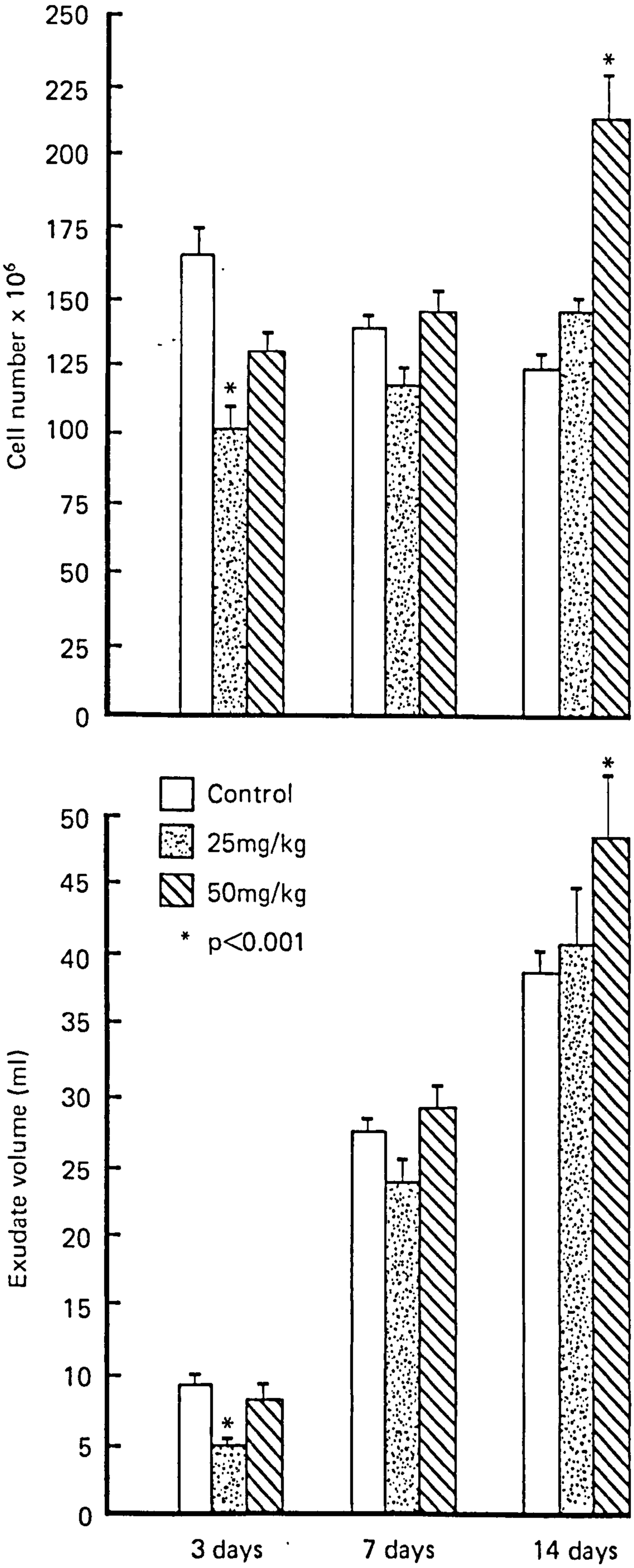

Fig. 16

Effect of daily oral administration of d-penicillamine (25 & 50mg/kg) on carrageenan (2ml of 1%) induced inflammation in the 6 day pouch.

the administration of 50 mg/kg. The significant ($P < 0.001$) increase was observed only with the high dose of D-penicillamine after fourteen days of daily treatment. Exudate volume increased from 39 ml in control groups to 48 ml in 50 mg/kg treated groups. The total cell number observed after fourteen days in the control group was 125×10^6, which became 214×10^6 after treatment with 50 mg/kg. The pro-inflammatory effect of D-penicillamine was also observed with 25 mg/kg dose after fourteen days of continuous treatment. However, this effect was insignificant.

Levamisole

Results illustrated in table (10) figure (17) are for experiments where a single dose of 2.5 or 5.0 mg/kg levamisole was administered daily orally for different periods to carrageenan treated rats. Three, seven and fourteen days following carrageenan injection, exudate volumes and total cell numbers were 8.2 ml and 166×10^6; 25.7 ml and 169×10^6; 33.9 ml and 158×10^6, respectively. Levamisole was found not to affect inflammation induced by carrageenan in the six day pouch of rats. Volumes of exudate and total leucocyte counts measured from pouches of animals treated with either 2.5 or 5.0 mg/kg levamisole, were found not to change significantly from values obtained from the control groups.

Dexamethasone

In this study, the steroidal anti-inflammatory dexamethasone was found to reduce the inflammation produced in the six day air pouch. The anti-inflammatory effect of dexamethasone was observed with both the tested doses of the

Experiment / Time	2.0 ml of 1% carrageenan		2.5 mg/kg levamisole		5.0 mg/kg levamisole	
	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6
3 days	8.2 ± 0.32	166.44 ± 8.49	7.73 ± 0.44	162.24 ± 4.96	7.83 ± 0.51	135.55 ± 13.71
7 days	25.7 ± 1.9	169.34 ± 5.95	28.9 ± 3.1	170.47 ± 18.84	23.1 ± 1.69	152.77 ± 17.1
14 days	33.9 ± 1.7	158.73 ± 5.56	36.7 ± 1.86	163.8 ± 17.9	30.8 ± 3.2	150.31 ± 8.4

Table (10) Effect of daily oral administration of levamisole (2.5 & 5.0 mg/kg) on carrageenan induced inflammation in the 6 day pouch.
Mean values ± S.E.M. 8 rats per group.

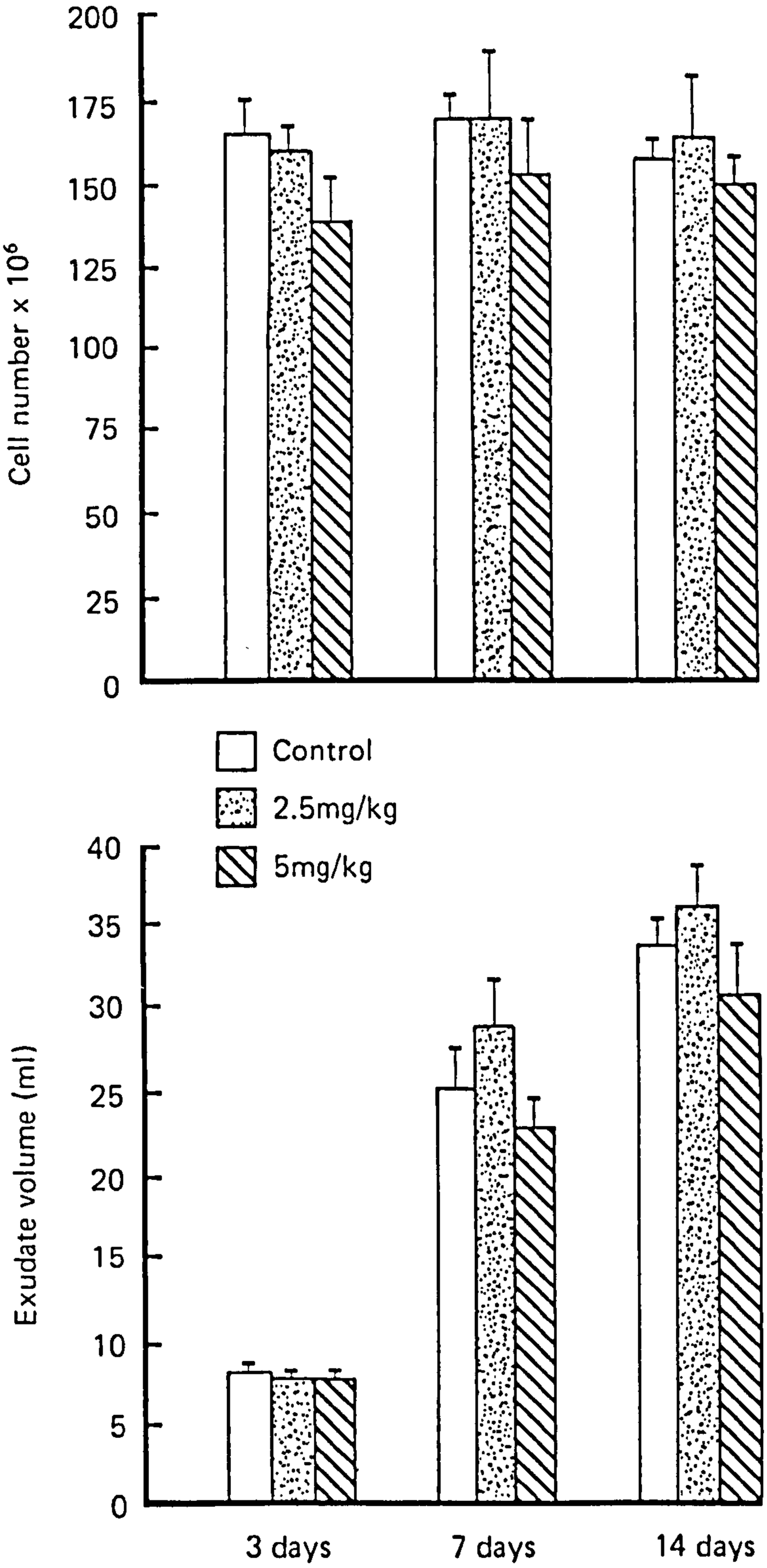

Fig. 17

Effect of daily oral administration of levamisole (2.5 & 5mg/kg) on carrageenan (2ml of 1%) induced inflammation in the 6 day pouch.

drug (0.1 and 0.2 mg/kg). The effect induced by this drug was strong enough to block the detection of any measurable inflammatory exudate. Moreover, the external pouch tissue was thin, when compared with normal or carrageenan treated pouches.

Leucocytes differential counts

A comparison was made in table (11) to show the effect of the drugs on the ratio of polymorphs to mononuclears observed in the carrageenan inflamed pouches. The ratios of polymorphs to mononuclears at three, seven and fourteen days in the control group were 94:6, 43:57 and 26:74. The predominance of polymorphs at three days remained nearly unchanged, and this was observed with all the tested drugs. At seven days, differential counts made on exudate smears obtained from control groups showed that mononuclears were the predominant cells (57%). Indomethacin in both 1 mg and 3 mg/kg at seven days, turned the dominance towards polymorphs (71% and 76%) and so did levamisole in both 2.5 mg and 5.0 mk/kg (83% and 81%). D-penicillamine at seven days was found to increase the number of mononuclear cells migrating to the inflamed pouch. This effect was dose-dependent. Fourteen days after carrageenan injection, mononuclears constituted about 74% of the migrated leucocytes in the pouches of the control animals. Indomethacin in both 1 mg and 3 mg/kg, levamisole (2.5 and 5.0 mg//kg) and both doses of D-penicillamine (25 mg and 50 mg/kg) were all found to change the ratio towards polymorphs.

Time / Drug	3 days		7 days		14 days	
	PMN	MN	PMN	MN	PMN	MN
carrageenan	94	6	43	57	26	74
1.0 mg/kg indomethacin	97	3	71	29	57	43
3.0 mg/kg indomethacin	98	2	76	24	69	31
25.0 mg/kg D-penicillamine	87	13	34	66	92	8
50.0 mg/kg D-penicillamine	93	7	29	71	52	48
2.5 mg/kg levamisole	87	13	83	17	77	23
5.0 mg/kg levamisole	85	15	81	19	88	12

Table (11)

Effect of daily administration of drugs on the ratio of polymorphonuclears to mononuclear leucocytes at different time periods, observed in carrageenan inflamed 6 day pouches.

Implantation of articular cartilage

Rat femoral cartilage caps were obtained from animals according to the technique described earlier. Groups of samples were subjected to a process of repeated freezing and thawing, in order to prepare dead samples. Other groups were cut into six pieces for experiments where minced cartilage was needed. The rest of the samples remained intact (whole) with no further killing (freezing and thawing) or mincing.

Implantation of living cartilage

Results illustrated in table (12) figure (18) represent the change in proteoglycan contents of minced and whole living samples, implanted into different situations for different times. The proteoglycan contents of control samples (mean values) were 196.5 µg (one week), 159.5 µg (two weeks) and 207.0 µg (three weeks).

Cartilage viability

One of the problems with studying implanted cartilage was to make sure that the cartilage was still viable after removal from pouches. Cartilage was examined for histological changes. Also, autoradiographic studies with $^{35}SO_4$ incorporation showed silver grains overlying nests of cells in the hyaline cartilage and matrix components immediately adjacent to labelled cells. The cells of the epiphyseal disc were well labelled but few grains were associated with the hypertrophoid chondrocytes in their calcified matrix. Intensity of labelling was found to vary among the cells of

Implantation period		1 week		2 weeks		3 weeks	
Experiment		proteoglycan content (µg)	% change	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change
normal cartilage	whole	196.5 ± 18.0	-	159.6 ± 6.0	-	207.0 ± 13.5	-
implantation into subcutaneous tissue	minced	191.5 ± 11.5	-2.54	57.0 ± 4.0*	-64.26	74.5 ± 7.5*	-64.01
	whole	213.5 ± 16.0	+8.65	94.5 ± 8.5*	-40.75	110.5 ± 10.0*	-46.62
implantation into normal air pouch	minced	185.5 ± 14.0	-5.6	58.5 ± 5.0*	-63.64	55.0 ± 4.0*	-73.43
	whole	216.0 ± 12.0	+9.92	80.5 ± 5.0	-49.53	59.5 ± 5.0*	-71.26

Table (12) Proteoglycan content (µg) of living femoral head cartilage, whole or minced, following implantation for 1, 2, and 3 weeks into normal 6 day pouch, subcutaneous tissue, 1% or 2% carrageenan inflamed pouch (see next page for carrageenan results). continued on next page (table

Mean values ± S.E.M.

6 rats per group. * $P < 0.001$

Implantation period		1 week		2 weeks		3 weeks	
Experiment		proteoglycan content (μg)	% change	proteoglycan content (μg)	% change	proteoglycan content (μg)	% change
implantation into inflamed pouch (1%)	minced	179.5 ± 15.0	-8.65	73.0 ± 8.0*	-54.23	123.0 ± 11.5*	-40.58
	whole	186.0 ± 14.5	-5.85	106.5 ± 5.5*	-33.23	124.5 ± 14.5*	-39.86
implantation into inflamed pouch (2%)	minced	152.5 ± 11.0	-22.39	32.5 ± 4.5*	-79.62	124.0 ± 9.0*	-40.10
	whole	196.0 ± 18.5	-0.25	67.5 ± 8.5*	-57.68	136.5 ± 11.5*	-34.06

Table (12) Continued from previous page

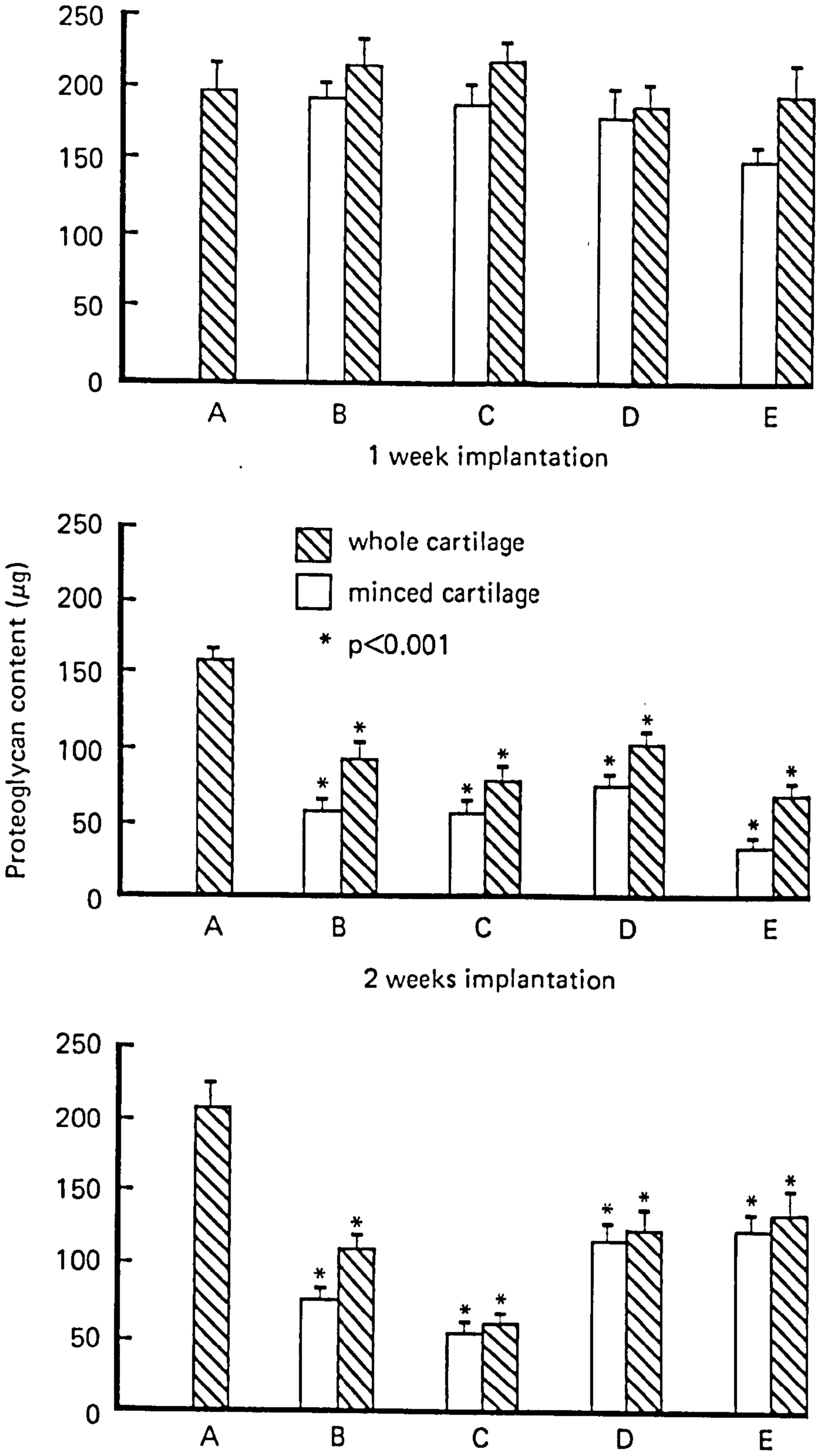

Fig. 18

Amount of proteoglycan (μg) in living whole and minced rat femoral head cartilage at 1, 2 and 3 weeks following implantation into subcutaneous tissue (B), non-inflamed 6 day pouch (C), 1 and 2% carrageenan inflamed pouch (D, E), Group (A) represents normal rat cartilage.

non-implanted control cartilage and also to vary to a similar degree in implanted cartilage. Incorporation of labelled sulphate did not appear to diminish with increasing time of implantation (figure 19).

Implantation into normal air pouch

Minced living cartilage was implanted into groups of normal six day pouches. Another group was implanted with whole (intact) living samples. At one week minced fragments lost about 5.6% of proteoglycan content and the loss was about 63% and 73% after two and three weeks of implantation. After one week the whole implanted samples showed an increase in proteoglycan content of about 9.9%. After two and three weeks the implanted cartilage lost about 49.5% and 71% of proteoglycan content.

Implantation into subcutaneous tissue

were

Groups of animals/treated with 2 ml 1% carrageenan solution, injected into their pouches five days after the initial air injection, and other groups were treated with the same volume of 2% solution. Pieces of whole and minced living cartilage were implanted into different groups of carrageenan treated pouches one day after irritant injection. One week after implantation, minced samples obtained from 1% carrageenan solution treated pouches lost about 8.6% of their proteoglycan content. The loss was about 22% from those samples obtained from pouches treated with 2% carrageenan solution. The reverse was observed with whole samples, recovered after one week of implantation, where

Fig.19 Autoradiograph of articular cartilage removed from a 2 week carrageenan air pouch. Labelled sulphate incubation. Magnification x 800.

there was nearly no loss observed in 2% carrageenan treated pouches (0.25%), while those samples recovered from 1% solution treated pouches lost about 5.8% of their contents.

Two weeks following implantation into 1% carrageenan solution treated pouches, minced and whole samples lost about 54% and 33% of proteoglycan contents. The loss of proteoglycan observed with samples recovered from 2% solution treated pouches was about 79% for minced and 57% for whole samples. The loss of proteoglycan from minced and whole cartilage recovered from 1% and 2% solution treated pouches after three weeks' implantation, was nearly the same, suggesting that the inflammatory response was not too small to induce proteoglycan loss.

To conclude, it was found that after two and three weeks of implantation into normal and inflamed situations, minced and whole living cartilage lost large amounts of their proteoglycan contents, and that the loss was significant ($P < 0.001$).

Implantation of dead cartilage

Results summarised in table (13) figure (20) are for experiments where the proteoglycan contents of dead whole samples implanted into different situations for different times, were compared with those of normal non-implanted cartilage. Dead implanted samples showed significant loss throughout the implantation periods (one, two and three weeks). The highest rates of loss were observed at three weeks with samples implanted into subcutaneous tissues and normal air pouches. These were 79% and 76% respectively. After three

Implantation period	1 week		2 weeks		3 weeks	
cartilage / Experiment	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change	proteoglycan content (µg)	% change
normal cartilage	217.0 ± 11.0	-	210.5 ± 12.5	-	226.5 ± 14.0	-
implantation into subcutaneous tissue	132.0 ± 11.0*	-39.13	67.5 ± 6.0*	-67.94	46.5 ± 4.5*	-79.47
implantation into normal air pouch	135.0 ± 5.5*	-37.79	109.0 ± 9.0*	-48.22	52.5 ± 4.0*	-76.82
implantation into inflamed pouch (1%)	182.0 ± 6.5***	-16.13	128.5 ± 8.5*	-38.95	139.0 ± 6.5*	-38.63
implantation into inflamed pouch (2%)	179.0 ± 12.0***	-17.51	103.5 ± 10.5*	-50.83	114.0 ± 8.0*	-49.67

Table (13) Proteoglycan content (µg) of dead whole femoral head cartilage, following implantation for 1,2 and 3 weeks into normal 6 day pouch, subcutaneous tissue, 1% or 2% carrageenan inflamed pouch.
Mean values ± S.E.M. 6 rats per group.
* $P < 0.001$ *** $P < 0.05$

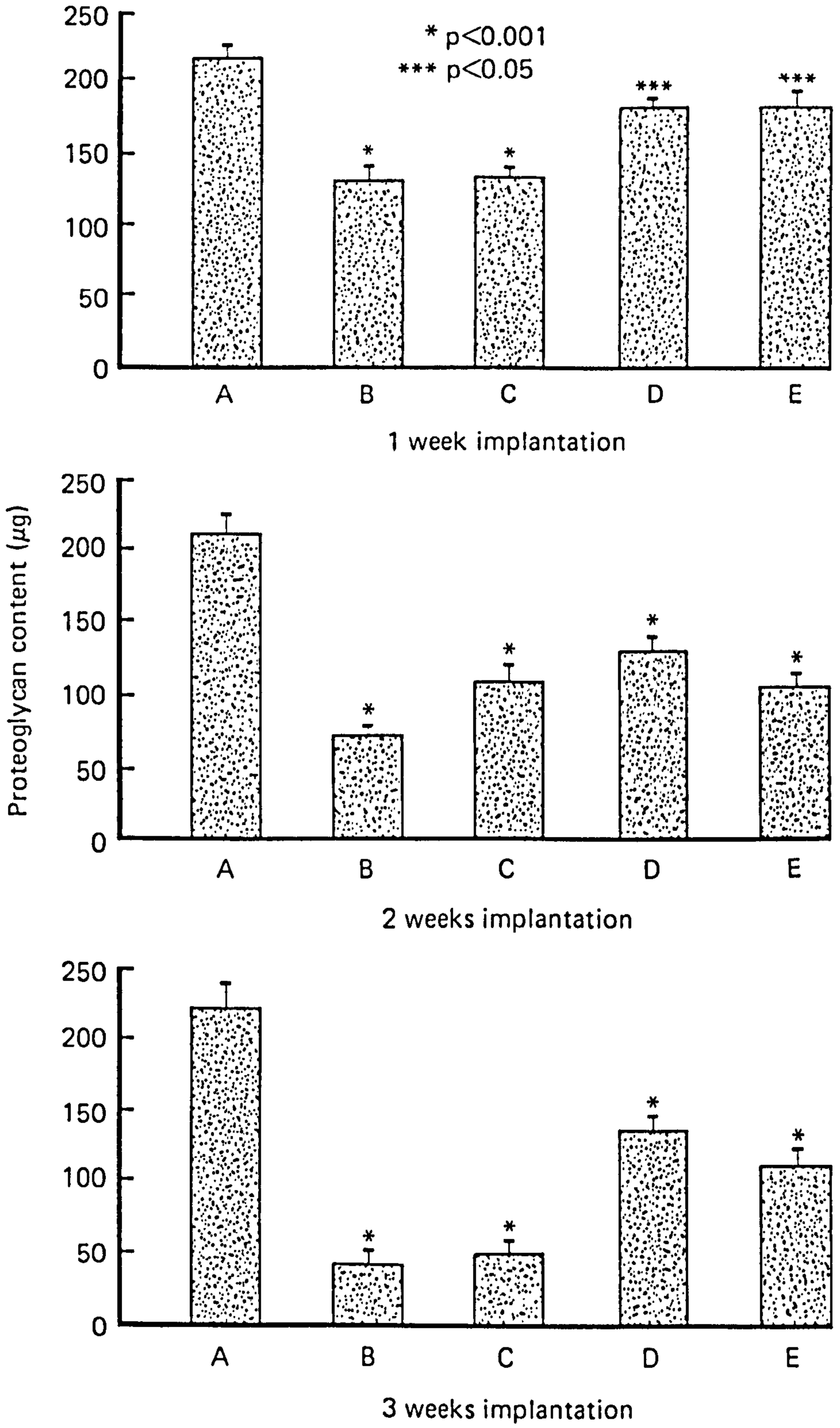

Fig. 20

Amount of proteoglycan (μg) in dead whole rat femoral head cartilage at 1, 2 and 3 weeks following implantation into subcutaneous (B), non-inflamed 6 day pouch (C), 1 and 2% carrageenan inflamed pouch (D,E). Group A represents normal rat cartilage.

weeks, samples recovered from 1% and 2% carrageenan treated pouches showed a loss of 38% and 49%. However, that loss was smaller than that observed with samples recovered from a non-inflamed situation.

Studies into the immunogenicity of cartilage

Femoral upper end cartilage caps obtained from wistar rats were finely minced and suspended in sterile saline. The cartilage suspension was mixed with an equal volume of Freund's adjuvant and then homogenised until a good emulsion was formed. Groups of wistar rats were immunised for three and four weeks with cartilage/adjuvant emulsion. Samples of whole cartilage caps were implanted for two and three weeks into preformed six day pouches of animals immunised for three weeks. Another group of animals immunised for four weeks were implanted with cartilage samples in the air pouches. Table (14) figure (21) show a comparison of the loss of proteoglycan from cartilage implanted for two and three weeks into air pouches of normal and immunised animals.

After two and three weeks of implantation, implanted samples showed a significant loss in their proteoglycan content. It was found that the loss of proteoglycan from cartilage obtained from normal and immunised animals did not differ significantly. However, the proteoglycan loss observed after implantation into a normal situation was slightly larger. Cartilage implanted into normal pouches lost about 42% of proteoglycan content after two weeks and the loss was about 58% at three weeks. It was also found that the length of the period of immunisation was not an important factor.

Time	Experiment	proteoglycan content (μg)	% change
implantation for 2 weeks	Normal cartilage	309.0 ± 9.0	---
	Normal air pouch	178.0 ± 10.5*	-42.4
	Normal pouch of 3 weeks immunised rat	199.0 ± 25.0**	-35.6
	Normal pouch of 4 weeks immunised rat	189.5 ± 14.5*	-38.68
implantation for 3 weeks	Normal cartilage	309.0 ± 9.0	--
	Normal air pouch	127.0 ± 13.0*	-58.9
	Normal pouch of 3 weeks immunised rat	148.5 ± 10.0*	-51.94
	Normal pouch of 4 weeks immunised rat	160.0 ± 18.0*	-48.22

Table (14) Comparison of the loss of proteoglycan by whole femoral head cartilage following implantation for 2 and 3 weeks into normal 6 day pouches of cartilage immunised and non-immunised animals.
Mean values ± S.E.M. 6 rats per group
* $P < 0.001$ ** $P < 0.01$

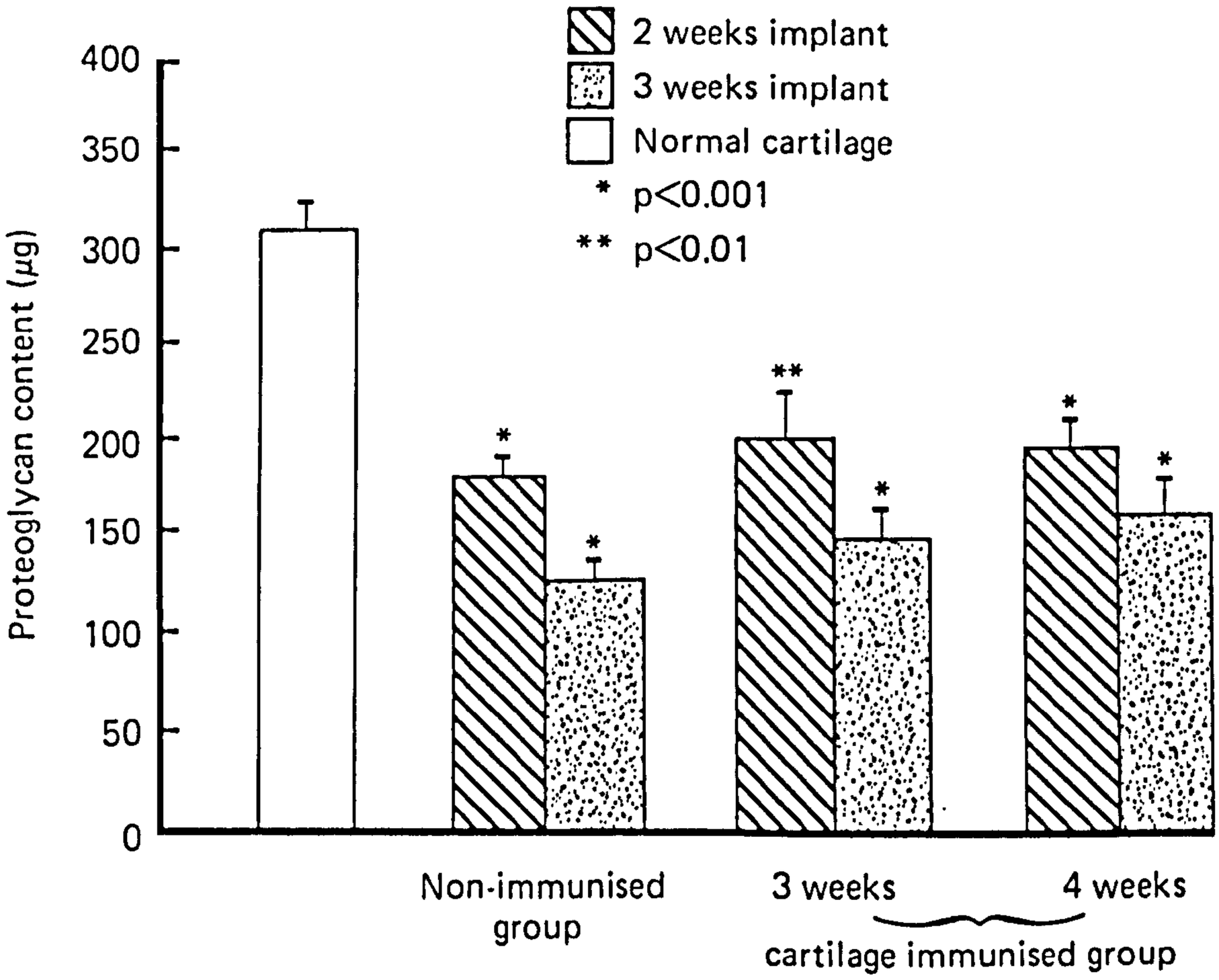

Fig. 21

Comparison of the loss of proteoglycan by whole femoral head cartilage following implantation for 2 and 3 weeks into normal 6 day air pouches induced on cartilage immunised and non-immunised animals.

The loss observed with implants recovered after two weeks from three week immunised animals was 51% and 48% from four week immunised animals.

Studies into allograft and isograft implantation

Whole and minced living articular cartilage obtained from inbred Fisher rats were implanted into six day pouches of rats from the same strain. Some of the air pouches were normal and the others were treated with 1% carrageenan solution. The loss of proteoglycan from inbred Fisher implants was compared with that from outbred wistars. The comparison is made in table (15) figure (22). In both the tested groups, minced cartilages showed greater loss than the whole ones. Whole and minced implants recovered from both groups showed a significant loss ($P < 0.001$) when compared with normal cartilage. Implants recovered from outbred animals showed slightly greater insignificant loss over the inbred implants.

Whole implants recovered from normal pouches of inbred and outbred animals lost about 45% and 49% of proteoglycan content. After implantation for two weeks into inflamed pouches of inbred and outbred animals, the loss was 30% and 33% respectively, suggesting that inflammation was not the only factor affecting cartilage degradation.

Implantation of articular cartilage after one week of irritant injection

Groups of rats with preformed six day pouches were injected with 2 ml 1% carrageenan solution, six days after

strain	Experiment	Normal inbred cartilage	Normal outbred cartilage	Implantation into normal air pouch		Implantation into inflamed pouch (1%)	
	cartilage	W	W	M	W	M	W
inbred Fisher	proteoglycan content (μg)	190.5 ± 19.0	-	78.5 ± 7.0*	103 ± 8.0*	83.5 ± 8.0*	132.0 ± 7.5*
	% change	-	-	-58.8	-45.94	-56.17	-30.71
outbred Wistar	proteoglycan content (μg)	-	159.5 ± 6.0	58.0 ± 6.0*	80.5 ± 5.0*	73.0 ± 8.0*	106.5 ± 5.5*
	% change	-	-	-63.64	-49.53	-54.23	-33.23

Table (15) Proteoglycan content (μg) of femoral head cartilage, whole (W) or minced (M) of inbred Fisher and outbred Wistar rats, following implantation for 2 weeks into normal 6 day air pouch or 1% carrageenan inflamed pouch.

Mean values ± S.E.M. 6 rats per group

* $P < 0.001$

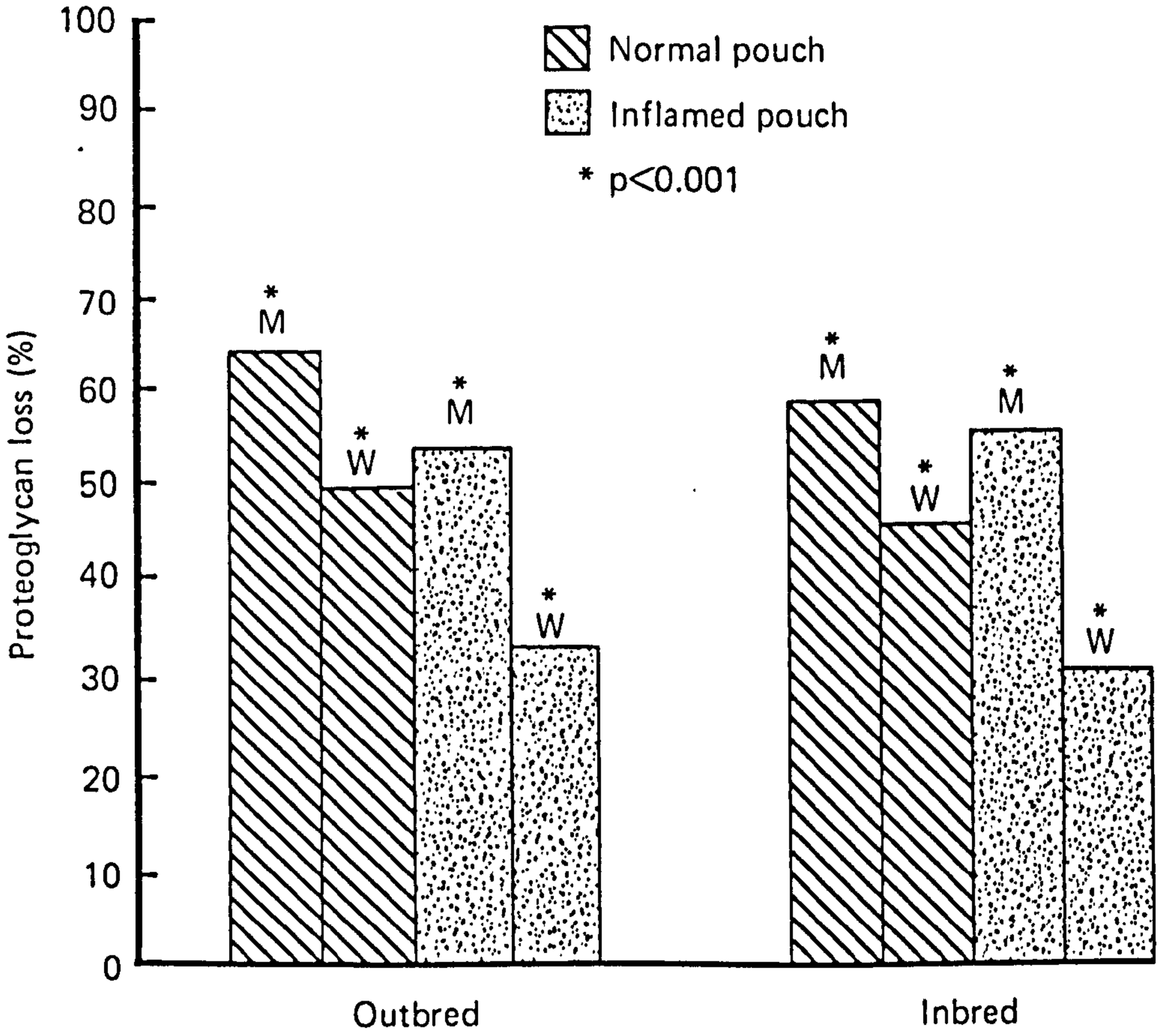

Fig. 22

Proteoglycan loss (%) from femoral head cartilage, whole (W) or minced (M) of inbred Fisher and outbred Wistar rats following implantation for 2 weeks into normal 6 day air pouch or 1% carrageenan inflamed pouch.

the initial air injection. Seven days following carrageenan injection, groups of living whole femoral head cartilage were implanted into the inflamed pouches for one and two weeks. The changes in the proteoglycan content of the implanted cartilage after one and two weeks in that situation were summarised in table (16) figure (23). Biochemical assessment of the proteoglycan content of the implants showed that there was nearly no change (+ 0.25%) at one week of implantation. At two weeks, implants lost about 15.7% of proteoglycan content, however, this loss waS insignificant. It seems that implantation seven days after irritant injection affords the implanted cartilage some protection, where the loss was about 15.7%. From the previous experiments, it was found that implantation of cartilage for two weeks, 24 hours after irritant injection, caused a proteoglycan loss of about 33%.

Histological observations of articular cartilage implanted into different situations

Normal mouse femoral head cartilage

The femoral head cartilage was hyaline in character and approximately four cells thick. As the cartilage examined had been obtained from adult animals, there had been fusion of the epiphyseal discs so that cartilage abutted directly onto bone. The nuclei were round and well stained with haematoxylin and they were approximately 6 μm in diameter. Shrinkage of the matrix around the chondrocytes (presumably due to tissue processing) caused the articular surface to appear bumpy.

cartilage / experiment	proteoglycan content (µg)	% change
normal cartilage	3.82 ± 0.11	-
implantation for one week	3.83 ± 0.23	+0.26
implantation for two weeks	3.22 ± 0.29	-15.71

Table (16) Proteoglycan content (µg) of femoral head cartilage following implantation for one and two weeks into six day pouches one week after carrageenan injection.

Mean values ± S.E.M.

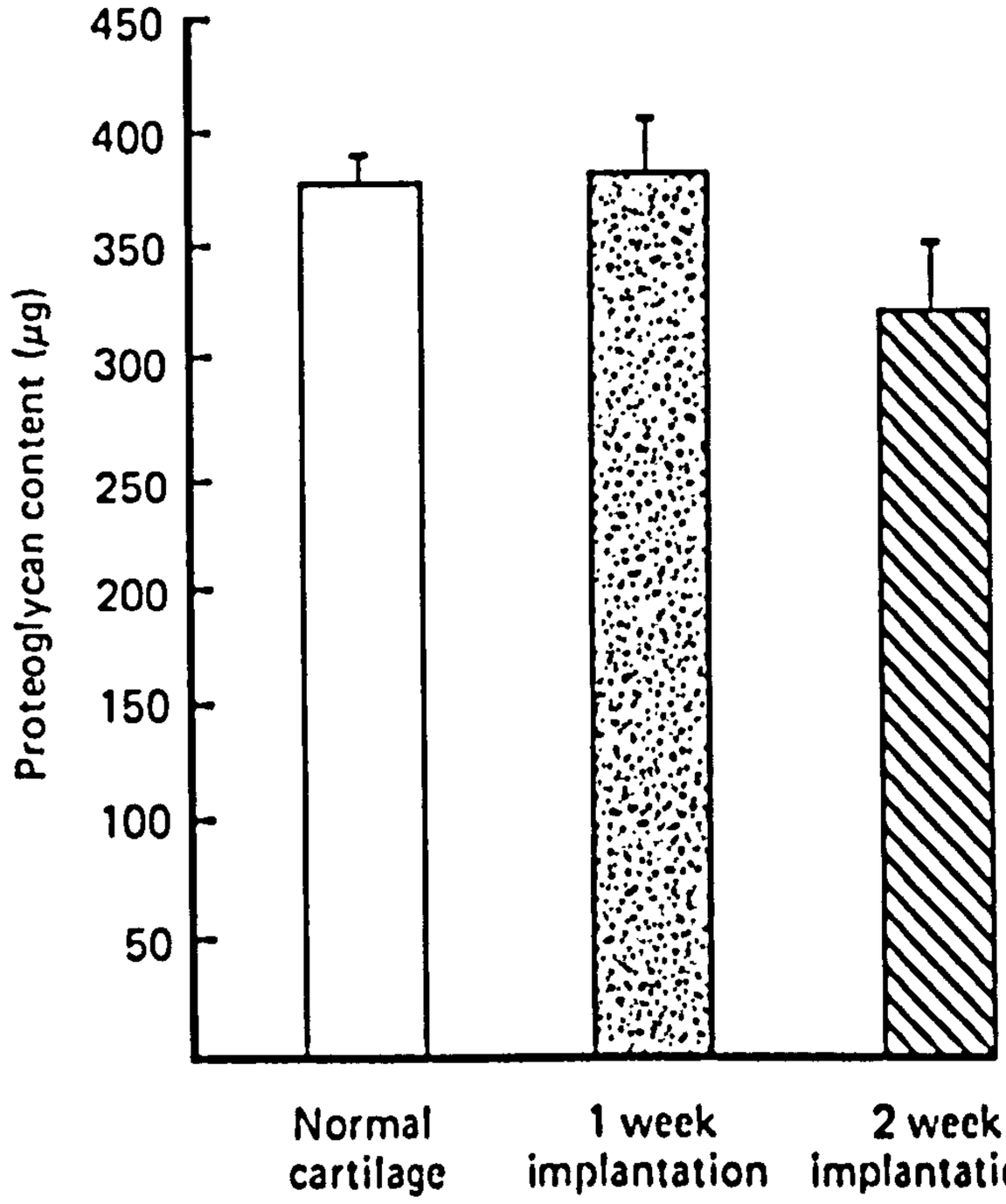

Fig. 23

Proteoglycan content (μg) of femoral head cartilage following implantation for 1 & 2 weeks into 6 day pouches after one week of carrageenan injection.

Normal rat femoral head cartilage

The rat femoral head cap consisted of hemisphere of cartilage with three main layers - a superficial layer of hyaline cartilage, a layer of hypertrophied chondrocytes in a calcified matrix, and the epiphyseal disc. The hyaline cartilage was approximately 8-12 cells thick, the cells being typically arranged in nests of two or four cells sitting in a lacunae. The nuclei were round, approximately 8 µm in diameter and the cytoplasm was indistinct. Metachromatic staining with toluidine blue was particularly strong at the edges of lacunae and fairly uniform over the rest of the matrix. At two sites there was a change in chondrocyte morphology towards a more fibroblastic appearance and a similar graded loss of metachromatic staining. These sites were at the insertion of Teres' ligament and at the cartilage soft tissue junction at the base of the hemisphere. Below the cartilage was the layer of hypertrophied chondrocytes occupying greatly enlarged calcified lacunae, with little intervening matrix. Both cell nuclei and cytoplasm were indistinct. This layer formed the bulk of the cartilage hemisphere and showed strong metachromatic staining. Below this layer were the cells of the epiphyseal disc, which consisted of columns of tightly packed cells with round nuclei approximately 8 µm diameter and with basophilic cytoplasm (figure 9).

Implantation into non-inflamed situations

The histological observations of implants obtained from subcutaneous tissues or non-inflamed air pouches were

essentially the same. At seven days, a fibrous granulation tissue formed around the implant, approximately 50 μm thick, consisting of capillaries, fibroblasts and some pigment-containing macrophages (probably containing haemosiderin). The cells of the hyaline cartilage appeared to be mainly healthy. Erosion of cartilage was not a feature of the articular surface but erosion of the subchondral plate was seen and was more apparent with time. Loss of metachromatic staining did not correspond with gross biochemical loss, presumably as enough proteoglycan still remained for a maximal staining reaction. Where there appeared to be damage to the articular surface (eg, during initial cartilage removal, when Teres' ligament was torn away) there was subtantial erosion. Loss of metachromatic staining was sometimes seen adjacent to the damaged areas.

When present, Teres' ligament became incorporated into the surrounding granulation tissue, and sometimes contained cartilage from the acetabulum. Where this cartilage was seen to be over the femoral cartilage, the cells of the intervening granulation tissue appeared to take some chondrocyte characteristics.

When dead cartilage was implanted, there was a lack of staining of the cell nuclei with haematoxylin. The erosion of the cartilage by soft tissue showed the same profile as with living cartilage. In particular it was noted that the articular surface was free from erosion unless there was some obvious damage.

Minced cartilage fragments showed increased erosion in comparison with whole cartilage. The increased erosion

occurred where the cut surface exposed the calcified layer of hypertrophied chondrocytes. Subchondral erosion was much the same as with whole cartilage. The articular surface again appeared to be protected from substantial erosion. Some gradation from chondrocytes to fibroblast-like cells occurred at the cut surface of the hyaline cartilage, accompanied by a similar graded loss of metachromatic staining. The chondrocytes at all time points appeared healthy. When two fragments were sufficiently close that granulation tissue was adherent between them, some of these cells appeared to take on chondrocyte characteristics.

Implantation into inflamed pouches

Cartilage implanted into carrageenan inflamed pouches was nearly always found encapsulated within a large mass of what appeared to be degenerating macrophages and carrageenan, which may or may not be adherent to the pouch wall. The morphology of chondrocytes in cartilage removed from inflamed animals was no different to normal chondrocyte morphology. Erosion of the implanted cartilage was similar in profile to the erosion seen in non-inflamed animals, but appeared less extensive at most time points. Again, the articular surface appeared to be protected from erosion unless Teres' ligament was absent or there was some other surface damage. Autoradiography showed that cartilage implanted into inflamed air pouches incorporated $^{35}SO_4$ at all time points. The histological profile of minced cartilage implanted into inflamed pouches was not significantly different to the profile seen in non-inflamed pouches.

Effect of drug treatment on the proteoglycan content of implanted articular cartilage

Rat femoral head cartilage was implanted into carrageenan inflamed six day pouches of rats for two weeks. The effects of some anti-inflammatory and anti-arthritic drugs were tested on this model. The anti-inflammatory drugs were the non-steroidal indomethacin and the steroidal dexamethasone. The anti-arthritic drugs used were D-penicillamine and levamisole.

Drug treatment was started on the same day as cartilage implantation and lasted throughout the implantation period. A single oral dose of each drug was given daily for fourteen days.

Indomethacin

Two doses of indomethacin have been used, 1 mg/kg and 3 mg/kg. Table (17) figure (24) show the results of experiments where the effects of treatment with indomethacin on the loss of proteoglycan from implanted cartilage was studied. It was found that when indomethacin was used at 1 mg/kg, there was no protection to the implants and the loss was about 30%. 3 mg/kg showed a significant ($P < 0.01$) protection to the implanted cartilage, which lost about 12.7% proteoglycan. At the end of these experiments, examination of the gastro-intestinal tract showed that there were no ulcers.

D-penicillamine

D-penicillamine at both 25 mg/kg and 50 mg/kg showed

Experiment	proteoglycan content (μG)	% change
Normal cartilage	192.0 ± 8.5	--
2 ml of 1% carrageenan	121.0 ± 4.5	-37
1 mg/kg Indomethacin	134.0 ± 5.0	-30.21
3 mg/kg Indomethacin	167.5 ± 13.0	-12.76**

Table (17)

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into inflamed 6 day pouches of animals treated daily with an oral dose of either 1 mg or 3 mg/kg indomethacin throughout implantation period.

Mean values ± S.E.M.

8 rats per group

** $P < 0.01$

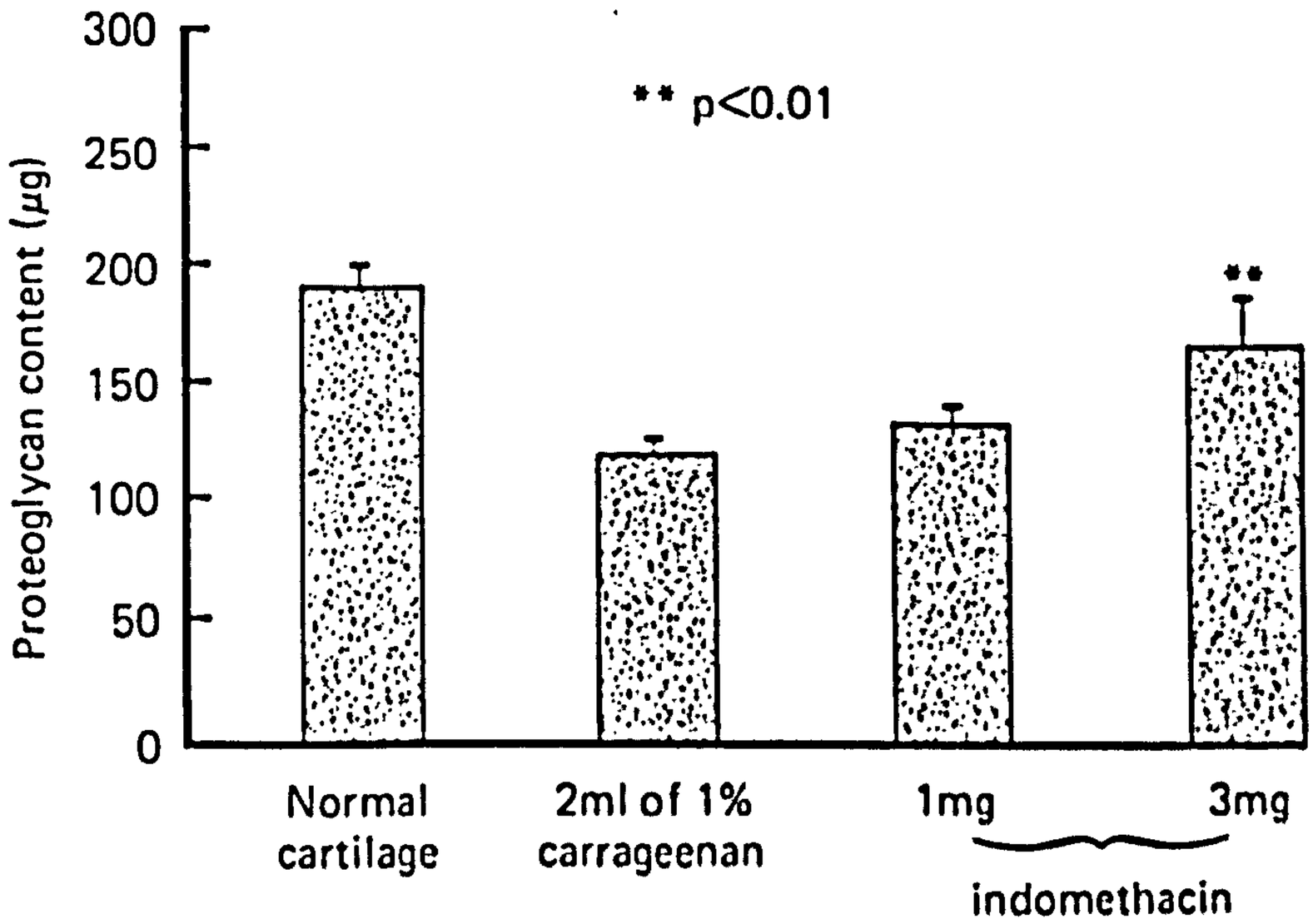

Fig. 24

Proteoglycan content (µg) of femoral head cartilage implanted for 2 weeks into carrageenan inflamed 6 day pouches of animals treated with a daily oral dose of either 1 or 3mg/kg indomethacin throughout implantation period.

a significant protection to the implanted cartilage (table 18, figure 25). Implants obtained from the 25 mg/kg treated group lost only 15.6% of protcoglycan content, compared with a loss of 37% observed in the control group. The 50 mg/kg treated implants showed a loss of 10.6% proteoglycan content.

Dexamethasone

A significant ($P < 0.001$) protection to the implanted cartilage was achieved with the two tested doses of dexamethasone. There was only a 17.4% loss of proteoglycan content observed in 0.1 mg/kg treated group, compared with a loss of about 46% observed in the control group. When 0.2 mg/kg was used, the loss was only 10% and this showed that the protection observed was dose-dependent (table 19 figure 26).

Levamisole

Levamisole was used in both 2.5 mg/kg and 5.0 mg/kg doses. A maximum significant ($P < 0.001$) protection was obtained with 5.0 mg/kg and the proteoglycan loss from the implanted cartilage was less than 1% (table 20, figure 27). However, a significant ($P < 0.01$) protection was also obtained with 2.5 mg/kg treatment, but the loss was about 18.8% which was greater than that observed with 5.0 mg/kg.

Experiment	proteoglycan content (µg)	% change
Normal cartilage	192.0 ± 8.5	--
2 ml of 1% carrageenan	121.0 ± 4.5	-37
25 mg/kg D-penicillamine	162.0 ± 6.5	-15.63*
50 mg/kg D-penicillamine	171.5 ± 15.0	-10.68**

Table (18)

Proteoglycan content (µg) of femoral head cartilage implanted for 2 weeks into inflamed 6 day pouches of animals treated daily orally with a dose of either 25 or 50 mg/kg D-penicillamine throughout implantation period.

Mean values ± S.E.M.

8 rats per group

* $P < 0.001$ ** $P < 0.01$

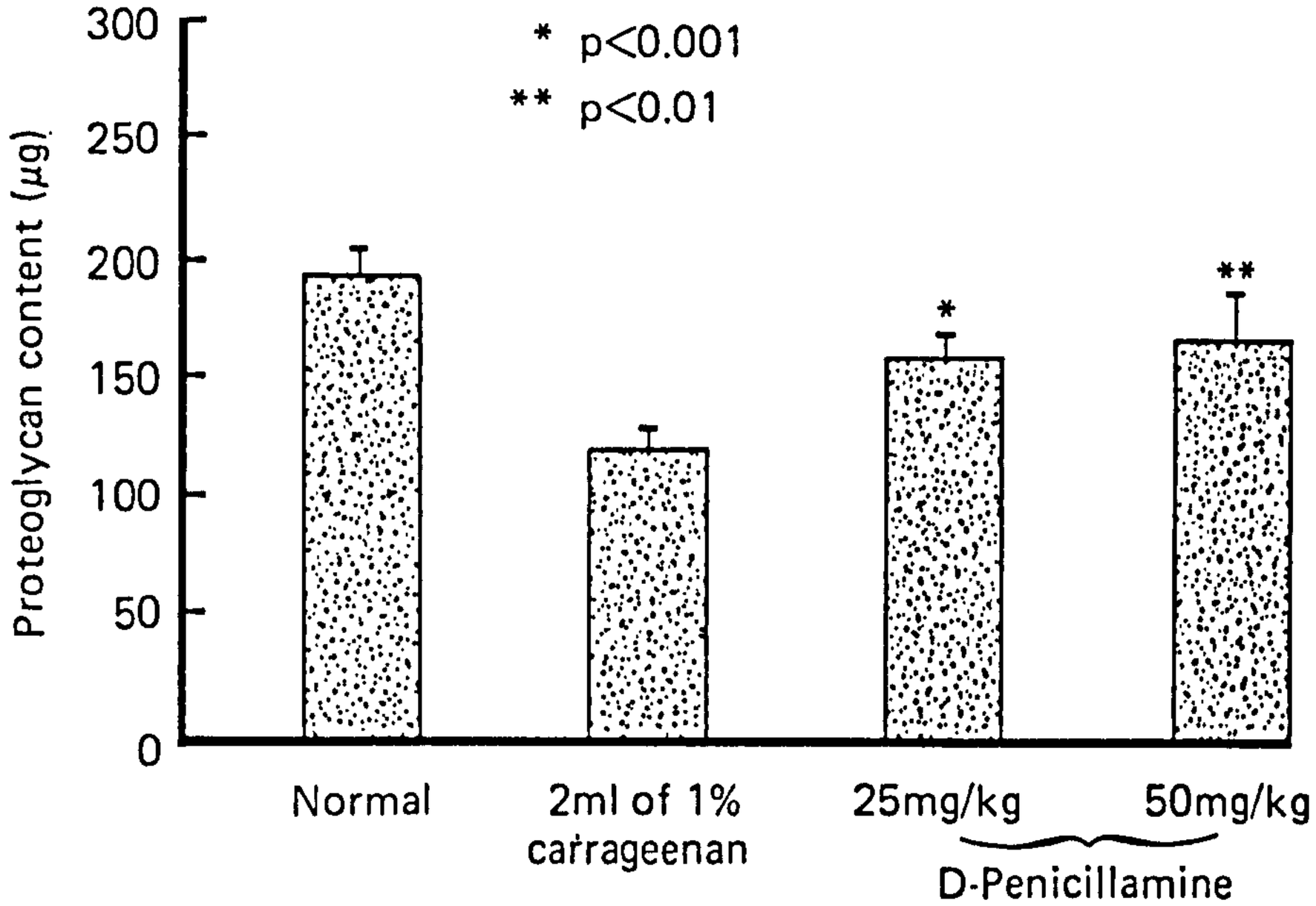

Fig. 25

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into carrageenan inflamed 6 day pouches of animals treated with a daily oral dose of either 25 or 50mg D-Penicillamine throughout implantation period.

Experiment	proteoglycan content (µg)	% change
Normal cartilage	261.0 ± 7.0	---
2.0 ml of 1% carrageenan	140.5 ± 4.0	-46.17
0.1 mg/kg dexamethasone	215.5 ± 10.5	-17.44*
0.2 mg/kg dexamethasone	234.0 ± 14.0	-10.35*

Table (19)

Proteoglycan content (µg) of femoral head cartilage implanted for 2 weeks into inflamed 6 day pouches of animals who received an oral daily dose of either 0.1 or 0.2 mg/kg dexamethasone throughout implantation period.

Mean values ± S.E.M.

8 rats per group

* $P < 0.001$

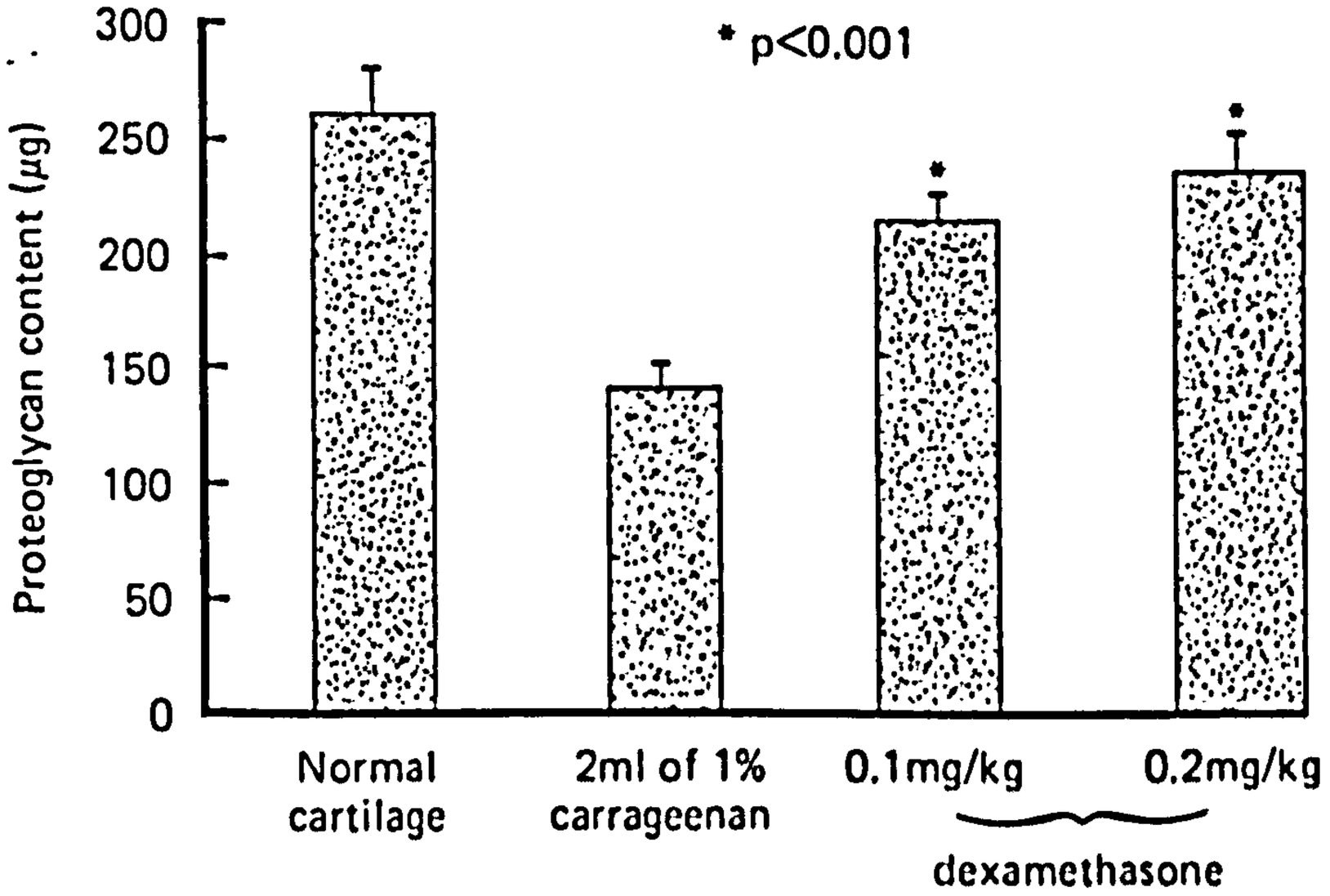

Fig. 26

Proteoglycan content (µg) of femoral head cartilage implanted for 2 weeks into carrageenan inflamed 6 day pouches of animals treated daily orally with 0.1 or 0.2mg/kg dexamethasone throughout implantation period.

Experiment	proteoglycan content (µg)	% change
Normal cartilage	284.5 ± 17.5	—
2.0 ml of 1% carrageenan	189.0 ± 9.0	-33.57
2.5 mg/kg levamisole	231.0 ± 8.0	-18.81**
5.0 mg/kg levamisole	282.0 ± 19.5	-0.88*

Table (20)

Proteoglycan content (µg) of femoral head cartilage implanted for 2 weeks into inflamed 6 day pouches of animals treated with a daily oral dose of either 2.5 or 5.0 mg/kg levamisole throughout implantation period.

Mean values ± S.E.M.

8 rats per group

* $P < 0.001$ ** $P < 0.01$

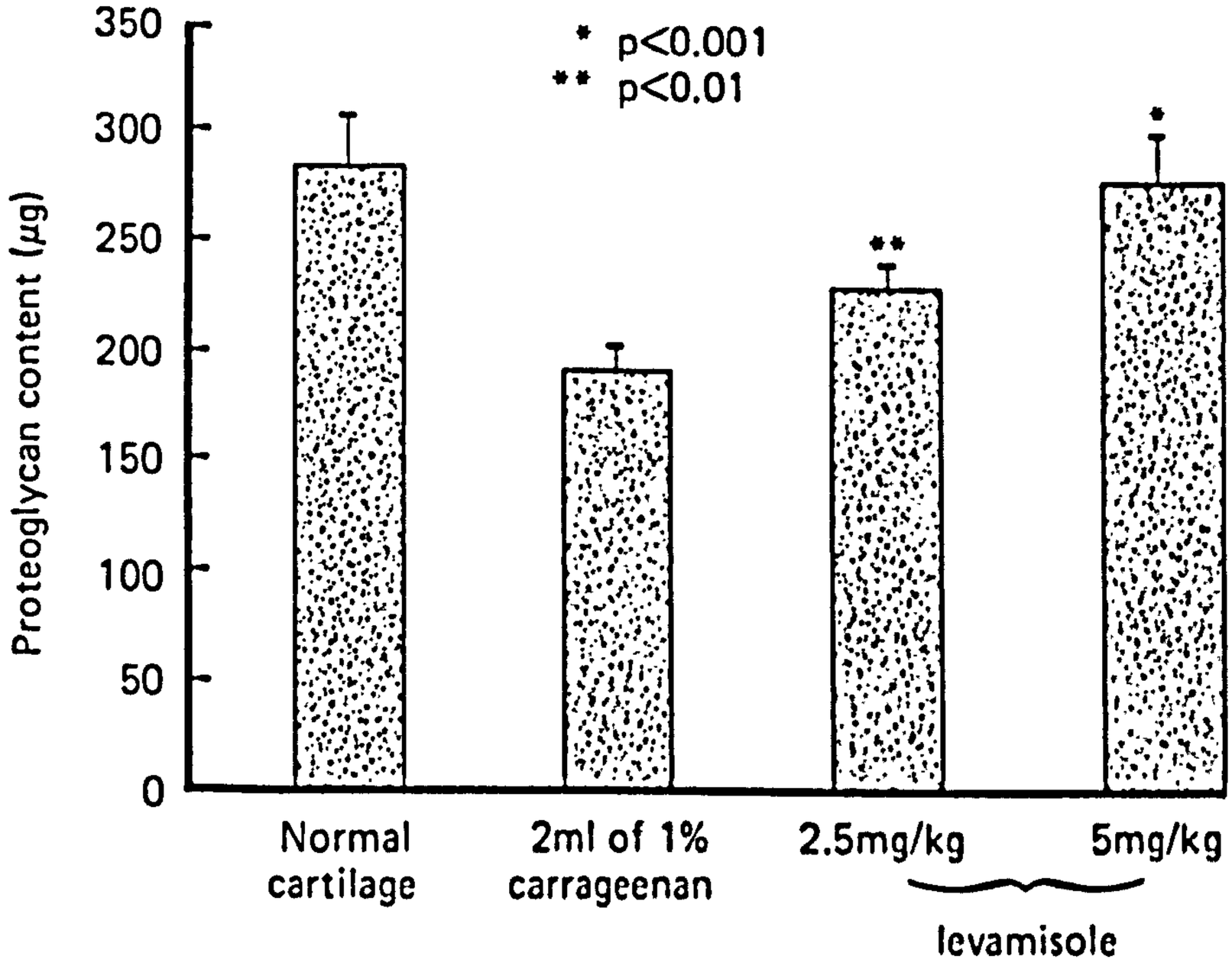

Fig. 27

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into carrageenan inflamed 6 day pouches of animals treated with a daily oral dose of either 2.5 or 5mg/kg levamisole throughout implantation period.

Effect of drug treatment on proteoglycan content of articular cartilage implanted in subcutaneous tissue

The effects of indomethacin, D-penicillamine, dexamethasone and levamisole were tested on the loss of proteoglycan from femoral head cartilage caps implanted into subcutaneous tissue of rats. Effects of daily oral administration of single doses of the drugs on the loss of proteoglycan from cartilage implanted into subcutaneous tissue were illustrated in table (21) figure (28). Cartilage implanted into subcutaneous tissues of non-treated animals lost about 37.8% of proteoglycan content.

Implants obtained from 3 mg/kg indomethacin treated animals lost nearly the same amount of proteoglycan as was observed in the non-treated group (37.2%). D-penicillamine (50 mg/kg) also failed to protect the implanted cartilage and the loss was about 35.8%. The loss of proteoglycan from implants obtained from levamisole treated group (5 mg/kg) was found to be greater than that observed with the non-treated group (43%). Dexamethasone (0.2 mg/kg) however, showed a protective activity towards the implanted cartilage. The loss of proteoglycan from implants obtained from dexamethasone treated animals was only 11.9%, compared with 37.8% in the control group. The protective effect of dexamethasone on the implants was significant ($P < 0.001$).

Histological observations following drug treatment

Histological examination of air pouch samples and implanted cartilage obtained from animals treated for two weeks, showed no significant changes from the control samples

Experiment	proteoglycan content (µg)	% change
Normal cartilage	243.5 ± 17.5	--
subcutaneous tissue	151.5 ± 12.5	-37.8
3.0 mg/kg indomethacin	153.0 ± 16.0	-37.2
50.0 mg/kg D-penicillamine	156.5 ± 14.5	-35.8
0.2 mg/kg dexamethasone	217.0 ± 10.0*	-11.9
5.0 mg/kg levamisole	139.0 ± 10.5	-43.0

Table (21) Effect of a daily drug treatment for 14 days on the proteoglycan content (µg) of femoral head cartilage implanted for 14 days into subcutaneous tissue of the rat.

Mean values ± S.E.M.

6 rats per group

** $P < 0.01$

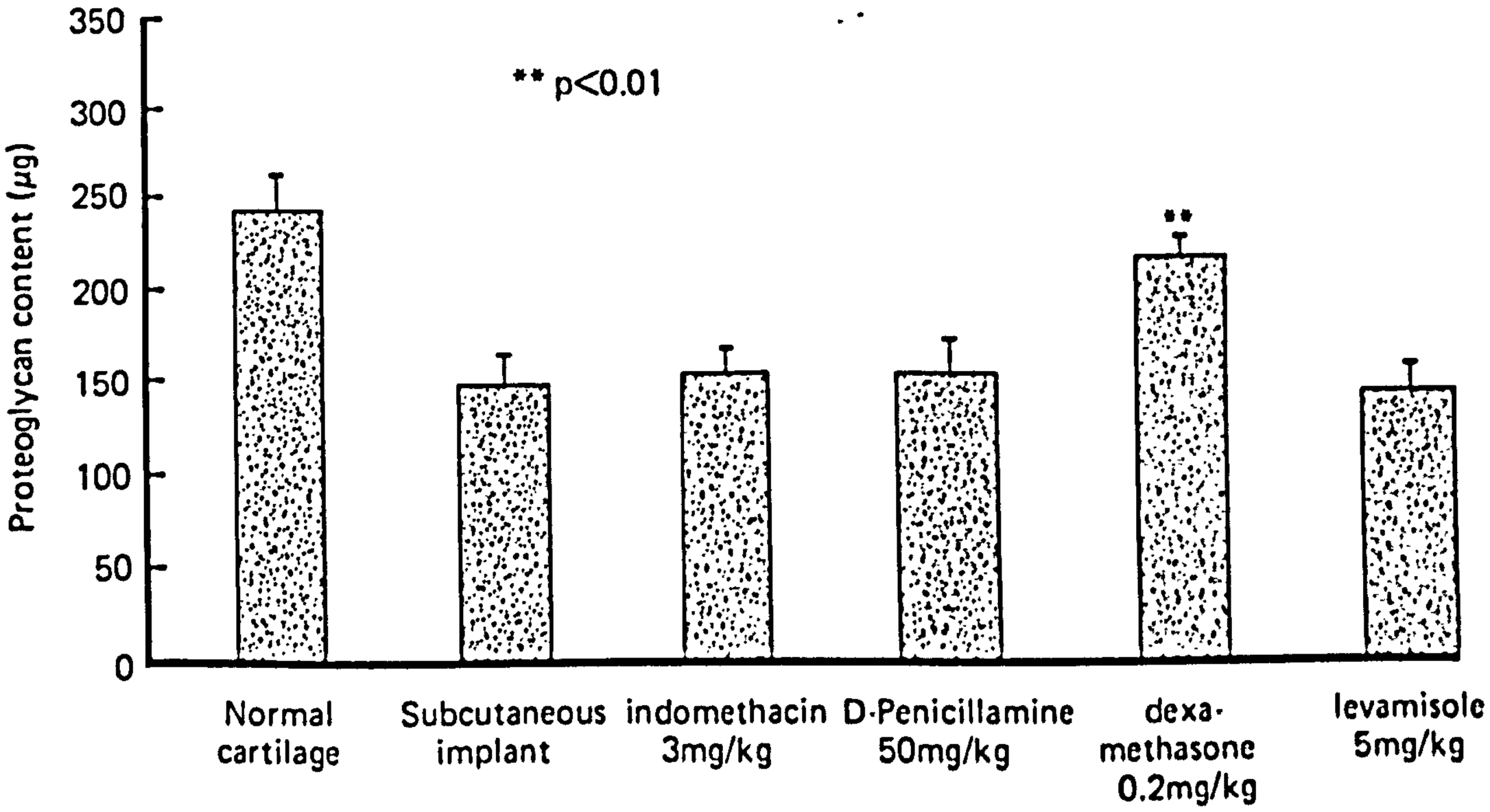

Fig. 28

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into subcutaneous tissues of rats treated daily orally with different therapeutic agents.

obtained from indomethacin , D-penicillamine and levamisole treated animals. The only changes observed were with dexamethasone treated animals. Pouches obtained from 0.1 mg/kg and 0.2 mg/kg dexamethasone treated animals were macroscopically thinner than those from control animals. This finding was confirmed microscopically, where it was found that the pouch showed less fibrous connective tissue in the more superficial layer and was considerably less infiltrated by imflammatory cells.

Immune inflammation in the rat air pouch

Active synovitis of the joints is one of the main features of rheumatoid arthritis. In 1980, Ziff proposed that immune complexes localise in the joint cavities or across blood vessel walls, inducing inflammation. The present study has examined whether the increased reactivity of mature synovial-like lining tissue of the air pouch occurs when antigen-antibody complexes are the inflammatory stimuli.

Active Arthus reaction

After sensitisation of rats with 5 mg BSA and formation of one and six day pouches, animals were challenged with 1 mg BSA. Groups of animals were killed at four hours, 3, 5, 7, 9 and 14 days following antigen challenge, and the volumes of the inflammatory exudate and the total leucocyte numbers in the air pouches were measured. Table (22) shows the results of experiments where the total numbers

air pouch / time	one day pouch		six day pouch	
	cell number $\times 10^6$	volume of exudate (ml)	cell number $\times 10^6$	volume of exudate (ml)
4 hours	1.8 ± 0.19	0.8 ± 0.07	7.5 ± 0.68	3.6 ± 0.29
3 days	2.1 ± 0.2	0.9 ± 0.1	3.1 ± 0.3	1.9 ± 0.18
5 days	-	-	-	-
7 days	-	-	-	-
9 days	-	-	-	-
14 days	-	-	-	-

Table (22) Total cell number ($\times 10^6$) and exudate volume (ml) observed in one and six day pouhces of sensitised animals at different times after antigen challenge.

Mean values ± S.E.M.

6 rats per group

inflamed pouches were measured at different times following antigen challenge. It can be seen that one day pouches at 4 and 72 hours responded with only a low number of infiltrating leucocytes and a small volume of inflammatory exudate, and the figures were 1.8×10^6, 0.8 ml at four hours and 2.1×10^6, 0.9 ml at 72 hours. In contrast, six day pouches became more reactive to the inflammatory stimuli. The maximum cell number and exudate volume was observed at four hours after antigen challenge and the figures were 7.5×10^6, 3.6 ml. At 72 hours, exudate volume and total cell number were 3.1×10^6, 1.9 ml.

The small inflammatory reaction at 72 hours indicates that response to the antigen is an immediate type hypersensitivity. Examination of different pouches at 5, 7, 9 and 14 days showed that there was no obvious inflammatory response. Differential cell counts of exudate smears showed that the increase in leucocytes observed at four hours was due to an increase in influx of polymorphonuclear cells (90% PMN).

Reverse passive Arthus reaction

Twenty minutes after intravenous injection of 5 mg BSA, animals were challenged with anti-BSA by injection into their six day pouches. Six hours after initial injection, the animals were killed and the air pouches were opened to assess the inflammatory reaction. Examination of the air pouches showed that there was no obvious inflammatory reaction following those procedures. There are many possibilities to explain this unexpected reaction. This was

probably because the amounts of injected antigen or antibodies were not ideal enough to induce immediate immunological reaction, ie, either too high or too low. The time between the two injections of antigen and its antibody may also have been insufficient to trigger the reaction.

Prolonged immune inflammation in the rat six day pouch

It was found that a prolonged immune inflammation could be produced by injecting the six day pouches of pertussis sensitised rats with pertussis vaccine. Tables (23) and (24) and figure (29) show volumes of exudate and total cell numbers observed in one and six day pouches of sensitised and non-sensitised rats at different times following challenge with pertussis vaccine.

Six day pouches of non-sensitised rats failed to respond to the injected antigen, whereas there was no inflammatory reaction detected throughout the experiment. Assessment of inflammation in one day pouches of sensitised rats showed that there was a weak inflammatory response and the volume of exudate reached its peak after six days of antigen challenge. At six days the volume of exudate was 1.1 ml and the total leucocyte number also reached its peak at six days, when it was 8.6×10^6. Detection of inflammation in the one day pouches of sensitised rats showed that these pouches failed to produce measurable inflammation at periods over six days.

Six day pouches of sensitised animals showed a steady

Time after challenge (days)	6 day pouch in sensitised animal	1 day pouch in sensitised animal	6 day pouch in non-sensitised animal
1 day	1.3 ± 0.1	0	0
2 days	3.7 ± 0.26	0.6 ± 0.05	0
3 days	6.1 ± 0.59	0.74 ± 0.04	0
6 days	9.6 ± 0.82	1.1 ± 0.1	0
9 days	11.7 ± 1.1	0	0
13 days	6.7 ± 0.6	0	0
16 days	6.3 ± 0.5	0	0
21 days	1.1 ± 0.1	0	0
30 days	0	0	0

Table (23) Volume of exudate (ml) in different aged air pouches in sensitised and non-sensitised animals observed at different time intervals following challenge with Bordetella pertussis organisms.
Mean values ± S.E.M.
8 rats per group

Time after challenge (days)	6 day pouch in sensitised animal	1 day pouch in sensitised animal	6 day pouch in non-sensitised animal
1 day	10.3 ± 0.98	0	0
2 days	22.4 ± 2.1	3.61 ± 0.29	0
3 days	29.2 ± 2.5	6.88 ± 0.66	0
6 days	130.7 + 12.6	8.6 ± 0.81	0
9 days	96.3 ± 8.4	0	0
13 days	37.8 ± 3.1	0	0
16 days	13.8 ± 1.2	0	0
21 days	6.9 ± 0.5	0	0
30 days	0	0	0

Table (24) Total leucocyte number (x 10^6) in different aged air pouches in sensitised and non-sensitised animals observed at different time intervals following challenge with Bordetella pertussis organisms.

Mean values ± S.E.M.

8 rats per group

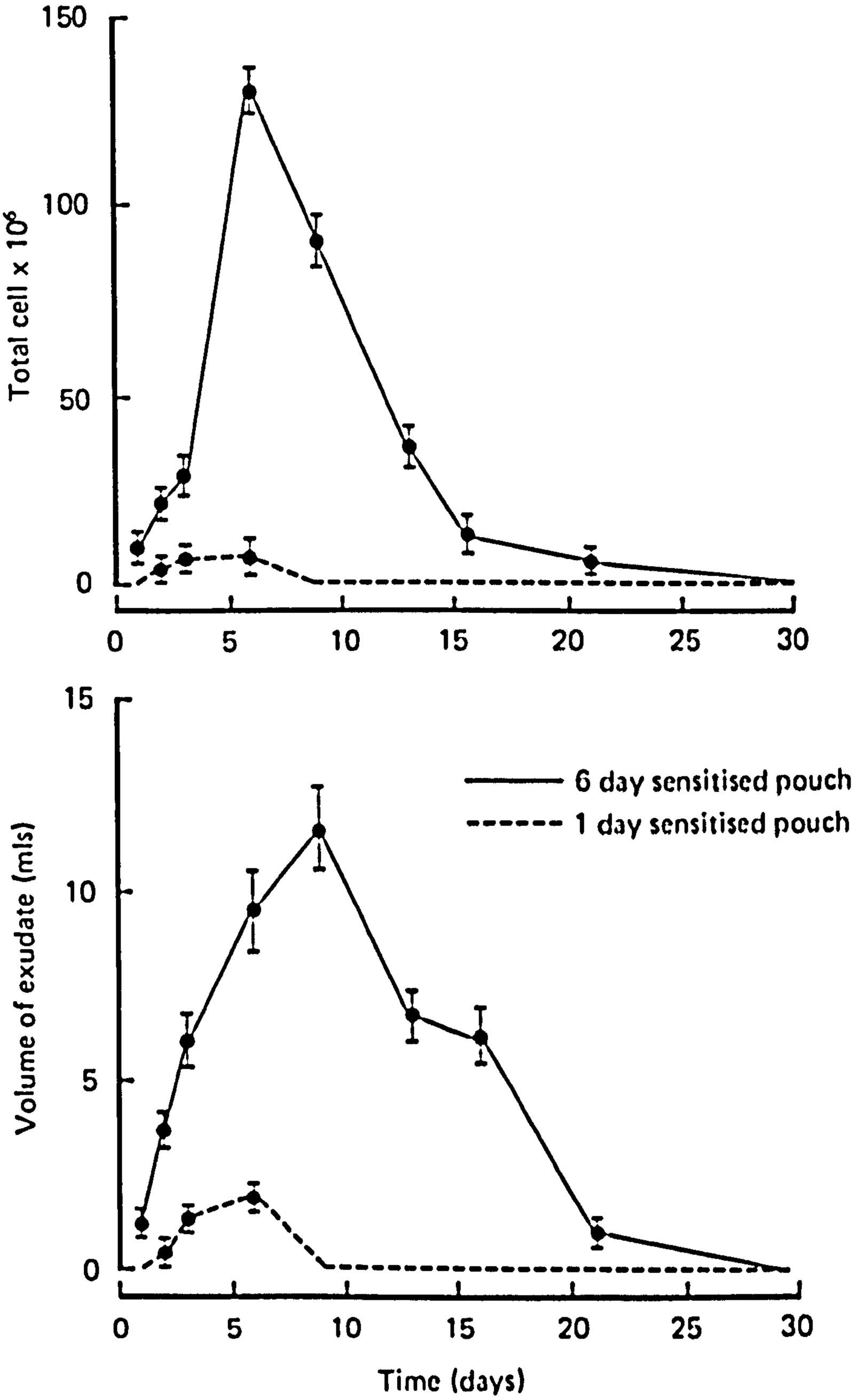

Fig. 29

Volume of exudate (ml) and total cell count ($x10^6$) in 1 and 6 day air pouches in sensitised animals observed at different time intervals following challenge with Bordetella Pertussis organisms.

increase in exudate volumes up to nine days after antigen challenge, where the volume of exudate was 11.7 ml. On the other hand, total leucocyte number reached its maximum at six days, where it was 130.7 x 10^6. At thirteen and sixteen days, there was still a good inflammatory reaction where the volumes of exudate and total cell numbers were 6.7 ml and 37.8 x 10^6 (13 days) and 6.3 ml and 13.8 x 10^6 (16 days). The inflammatory reaction persisted for up to twenty-one days after challenge with pertussis vaccine, however, this was a weak inflammatory reaction (1.1 ml and 6.9 x 10^6). At thirty days after the challenge, no inflammatory exudate could be detected in the six day pouches of sensitised animals.

Differential cell counts made on exudate smears (stained with May-Grünwald-Giemsa) showed a predominance of polymorphonuclear leucocytes (polymorphs) up to three days after the challenge (table 25 , figure 30). One day after challenge, the ratio of polymorphs to mononuclears was 86:14 and was nearly unchanged (87:13) at two days. The predominance of polymorphs remained up to the sixth day after the challenge, where the ratio was 54:46. At the ninth day, the predominance reversed towards the mononuclears, where it became 46:54. At the thirteenth day, there was an unexpected phenomenon where the polymorphs dominated again and the ratio was 67:33. At sixteen days, the ratio was 10:90 and a complete dominance of mononuclears (100%) was observed at twenty-one days. Trypan blue exclusion tests showed that more than 90% of the accumulating cells were viable.

Time after challenge (days)	polymorphs (%)	mononuclears (%)
1 day	86	14
2 days	87	13
3 days	81	19
6 days	54	46
9 days	46	54
13 days	67	33
16 days	10	90
21 days	0	100
30	0	0

Table (25) Type of leucocyte (%) in the 6 day old pouch of sensitised animals observed at different time intervals following challenge with Bordetella pertussis organisms.

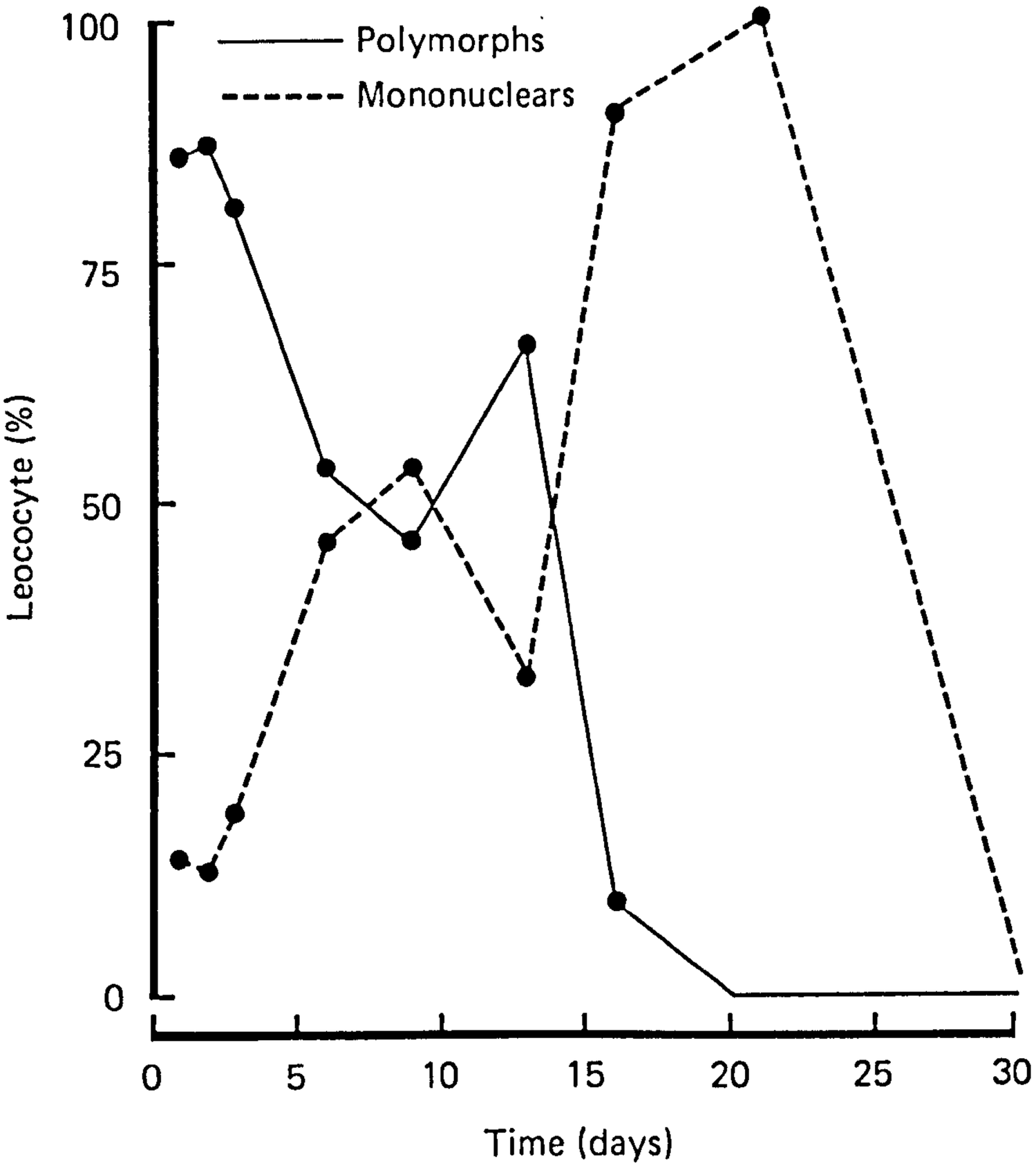

Fig. 30

Type of leucocyte (%) in the 6 day old pouch of sensitised animals observed at different time intervals following challenge with Bordetella pertussis organisms.

Histology observations

Air pouch histology

Sections of air pouch tissue taken from sensitised animals 24 hours after challenge, showed infiltration of the superficial connective tissue by migrating polymorphonuclear leucocytes with some clumping at the pouch surface. Migration of polymorphonuclear cells through the superficial layer at 48-72 hours was much reduced, but the pouch surface showed a mass of degenerating cells. These cells were intimately associated with dense fibrin deposits, as judged by MSB staining. At six days, polymorphonuclear cells had largely disappeared from the tissue surface, leaving an unorganised lining of mononuclear cells. By nine days, a distinct surface layer of large pigmented cells was seen. These had abundant cytoplasm with a variable number of branching processes. Haematoxylin and eosin staining showed the cells to be more basophilic than the lining cells in carrageenan inflammation and the majority of the cells were pyronin positive. This layer appeared approximately 1-3 cells thick,on sectioning at right angles to the surface, and some areas appeared hyperplastic - again, reminiscent of chronic synovitis (figure 31). The lining cells retained this character until thirty days. From six days, a zone of lympho-cyte and plasma cells were seen in clusters around the vascular layer.

Histological examination of air pouch tissue from non-sensitised animals showed an inflammatory reaction, which was mild in comparison to the reaction in sensitised animals.

Fig.31a Air pouch 48 hrs after challenge with B. pertussis into sensitised rats. Haematoxylin and eosin. Magnification x 200.

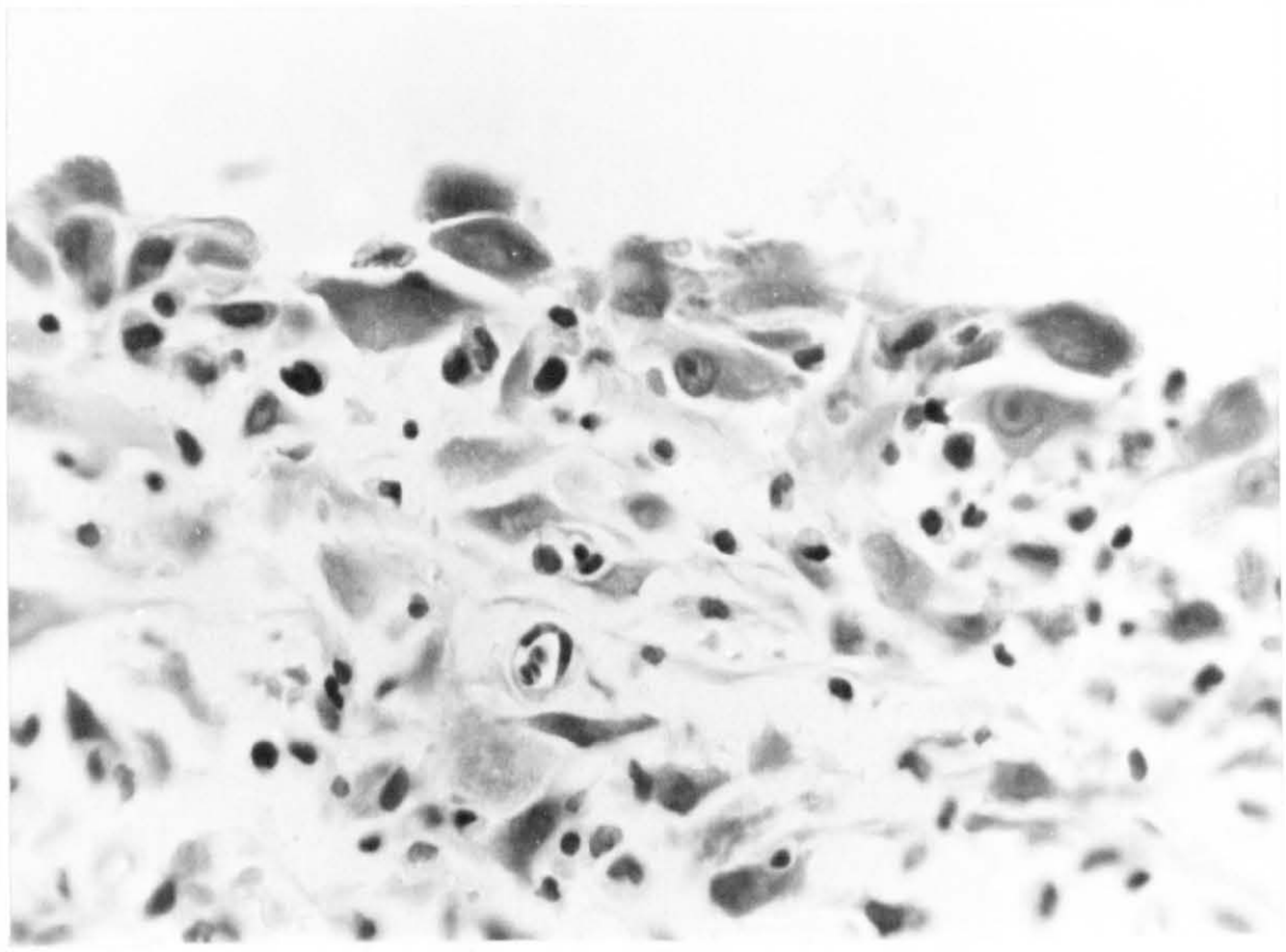

Fig.31b Air pouch 2 weeks after challenge with B. pertussis into sensitised rats. Haematoxylin and eosin. Magnification x 200.

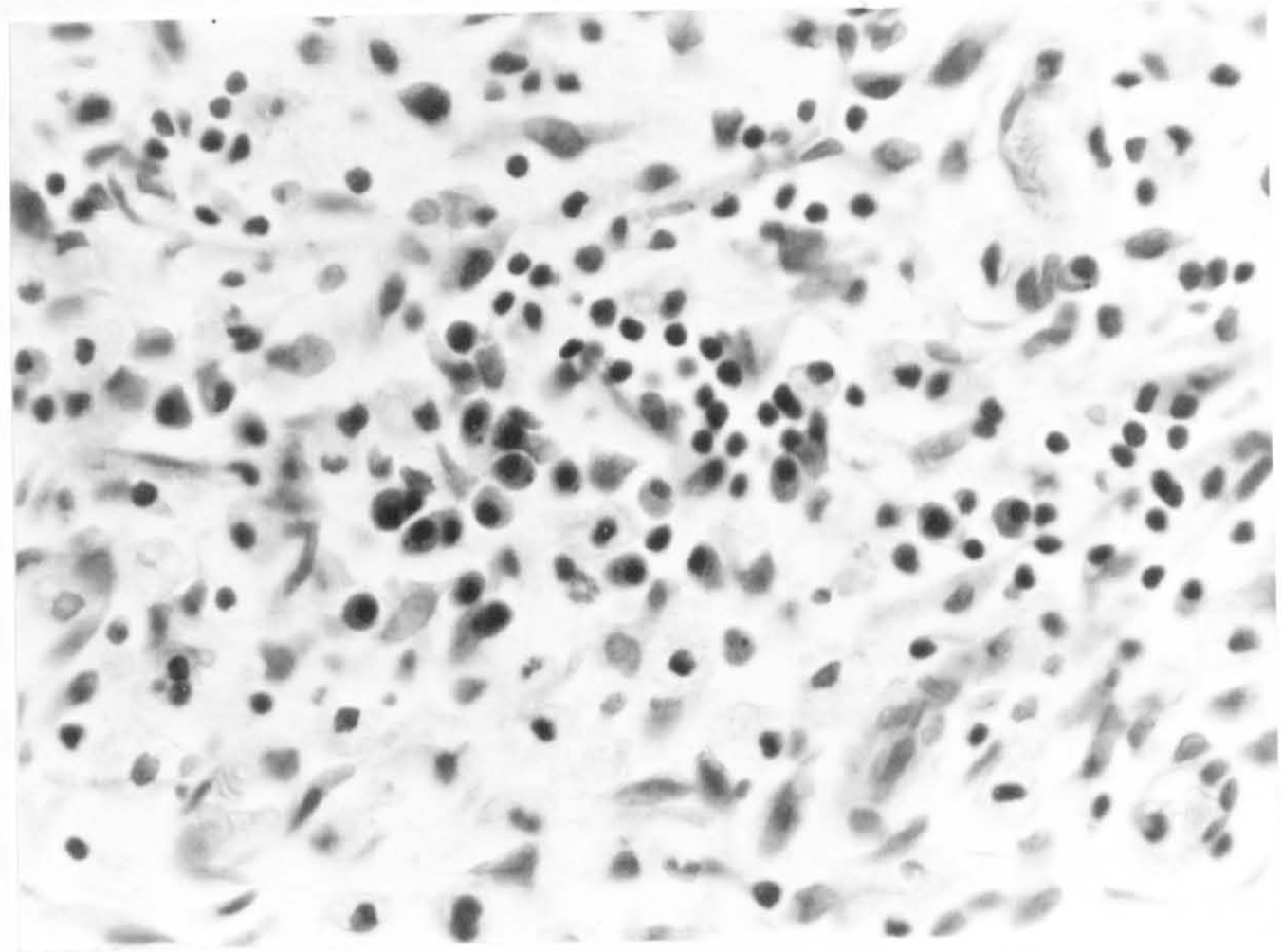

Fig 31 c Air pouch 2 weeks after challenge with B. pertussis into sensitised rats, showing lymphocytes and plasma cells. Haematoxylin and eosin. Magnification x 200.

Lymphoid tissues

Rat spleen was found to be difficult to assess for pathology because of its dual lymphoid and haemopoietic roles. However, no obvious histological changes were seen over the thirty days. Lymph nodes showed medullary and paracortical expansion. Granulomas were a feature of the lymph nodes at all time points. These cells had a large oval vesicular nucleus, usually with one or more prominent nucleoli. Their dimensions were approximately 15 x 8 μm (as measured by stage micrometry). The cytoplasm was abundant and foamy and the cell borders were indistinct. The total dimensions of the cell were approximately 25 x 10 μm. They were arranged in nests of cells with a pavementing character. Mitotic figures were frequently observed. Central necrosis was a feature of many of these granulomas at 1-3 days. In some specimens, the necrosis was so extensive as to occupy the bulk of the lymph node. By six days necrosis was no longer an obvious feature.

Effect of drug treatment on pertussis induced prolonged inflammation

Bordetella pertussis vaccine was found to produce a marked inflammatory reaction when injected into six day pouches of previously sensitised rats. In this study, the effects of four different drugs on pertussis induced inflammation in the six day pouch were investigated. These drugs were administered to rats daily orally, commencing at the time of antigen challenge for three, seven and fourteen days. Indomethacin was given at a dose of 1 mg or 3 mg/kg,

dexamethasone was given at a dose of 0.1 mg or 0.2 mg/kg and D-penicillamine was given in a dose of 25 mg or 50 mg/kg. Levamisole was also tested in both 2.5 mg or 5.0 mg/kg.

Inflammation observed in the pouches of control animals reached its maximum at seven days after antigen challenge. The mean values of exudate volumes and total cell numbers at three, seven and fourteen days in the control groups were 3.95 ml, 33.5 x 10^6; 5.74 ml, 99 x 10^6; and 6.2 ml, 31.2 x 10^6, respectively. The tested drugs had similar effects on this system to those seen on the model following carrageenan.

Indomethacin

When indomethacin was tested in both 1 mg and 3 mg/kg, it reduced both the volume of exudate and the total cell number significantly ($P < 0.001$). The significant anti-inflammatory effect was observed at all the tested times. Results summarised in table (26) figure (32) are for experiments where the inflammatory reactions observed in the indomethacin treated groups were compared with those in control groups. At seven days after antigen challenge, where the inflammatory response reached its maximum, the volume of exudate dropped from 5.74 ml to 3.2 ml in 1 mg/kg treated group, and to 2.0 ml in the 3 mg/kg treated group. Total cell number was 99.6 x 10^6 at seven days in the control group and dropped to 57 x 10^6 and 40 x 10^6 in 1 mg and 3 mg/kg treated groups. At the end of these experiments, the animals were killed and their gastro-intestinal tracts were examined for ulceration. No ulcers were detected either macroscopically

Experiment / Time	Bordetella pertussis		1.0 mg/kg indomethacin		3.0 mg/kg indomethacin	
	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6
3 days	3.95 ± 0.31	33.51 ± 3.4	1.61 ± 0.15*	14.16 ± 1.5*	1.25 ± 0.13*	13.82 ± 0.62*
7 days	5.74 ± 0.39	99.61 ± 4.9	3.21 ± 0.33*	57.11 ± 3.22*	2.01 ± 0.14*	40.1 ± 1.13*
14 days	6.2 ± 0.33	31.24 ± 2.7	2.9 ± 0.29*	34.2 ± 3.6	1.29 ± 0.11*	14.45 ± 1.35*

Table (26) Effect of daily oral administration of indomethacin (1 & 3 mg/kg) on pertussis induced inflammation in the 6 day pouch. Mean values ± S.E.M. 8 rats per group.

* $P < 0.001$

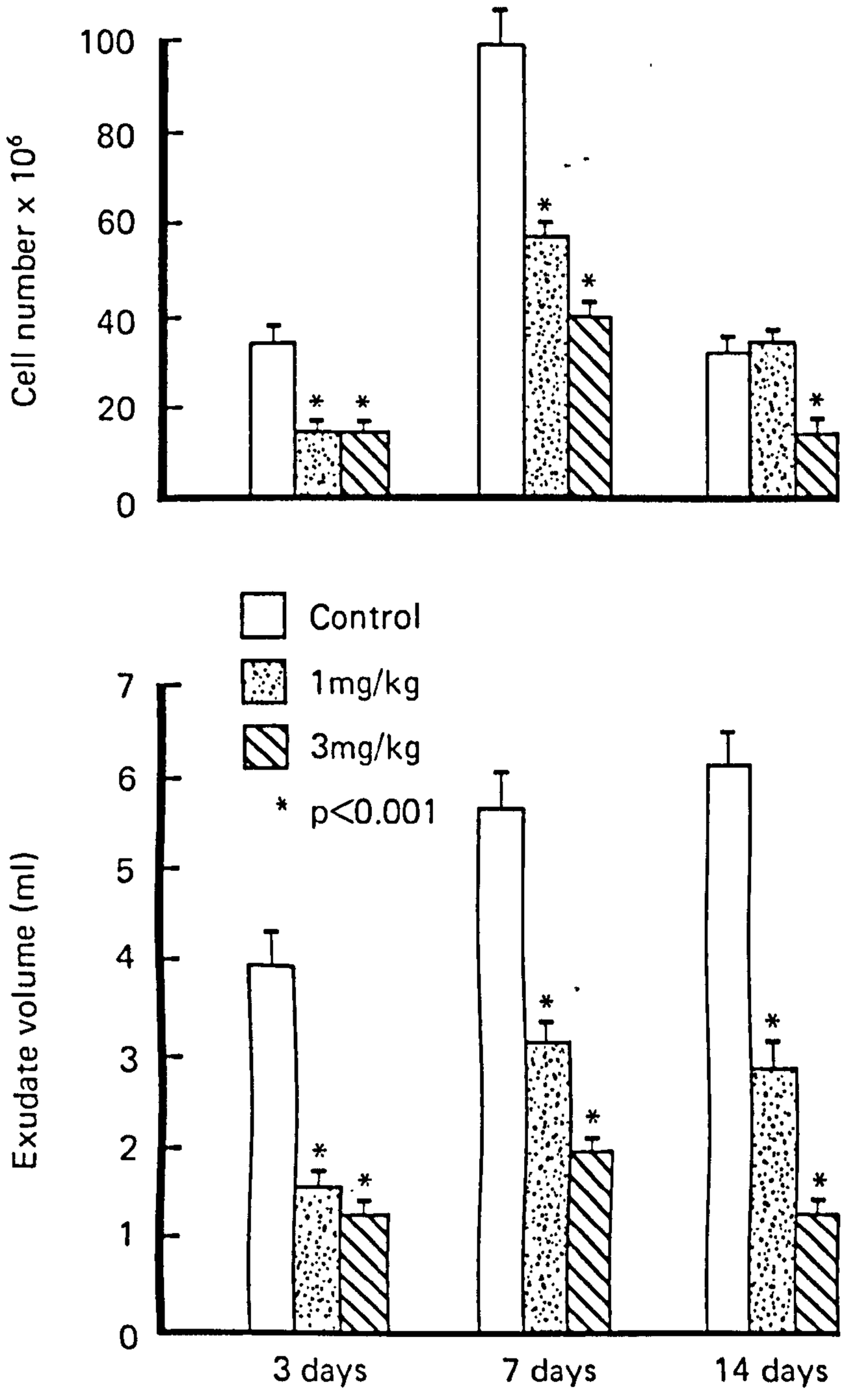

Fig. 32

Effect of daily oral administration of indomethacin (1 & 3mg/kg) on pertussis induced inflammation in the 6 day pouch.

or microscopically.

D-penicillamine

D-penicillamine was found not to affect the inflammation produced in the six day pouches by pertussis antigen. There was significant ($P < 0.01$) increase in the number of inflammatory cells accumulating in the pouch at seven days in the 50 mg/kg treated group. Results obtained from experiments where 25 mg and 50 mg/kg D-penicillamine were used, were summarised in table (27) figure (33). At three days both the tested doses of penicillamine reduced the volume of exudate and the total cell number. However, that reduction was insignificant. Seven days after antigen challenge, the tested doses of D-penicillamine were found to increase the inflammatory reaction insignificantly. The only significant ($P < 0.01$) action observed was with 50 mg/kg on the total cell number. At two weeks, D-penicillamine seems to have no effect on the produced inflammatory reaction.

Levamisole

Table (28) figure (34) represent a comparison between the inflammation observed in levamisole treated groups and that observed in non-treated control groups. At seven days following the antigen challenge, inflammation reached its maximum in the control group. Volume of exudate was 5.87 ml and the total cell number was 96×10^6. Levamisole was unable to alter exudate volumes or total cell numbers in all the tested groups, and this indicates that levamisole has no anti-inflammatory action when tested in this particular

Experiment / Time	Bordetella pertussis		25.0 mg/kg D-penicillamine		50.0 mg/kg D-penicillamine	
	exudate volume (mls)	cell number $x10^6$	exudate volume (mls)	cell number $x\ 10^6$	exudate volume (mls)	cell number $x\ 10^6$
3 days	3.95 ± 0.31	33.51 ± 3.4	3.6 ± 0.16	25.26 ± 2.16	3.66 ± 0.38	27.04 ± 2.11
7 days	5.74 ± 0.39	99.61 ± 4.9	6.5 ± 0.29	121.61 ± 3.23	7.3 ± 0.39	125.7 ± 5.33**
14 days	6.2 ± 0.33	31.24 ± 2.7	5.76 ± 0.61	33.1 ± 3.44	6.1 ± 0.44	30.69 ± 2.79

Table (27) Effect of daily oral administration of D-penicillamine (25 & 50 mg/kg) on pertussis induced inflammation in the 6 day pouch.

Mean values ± S.E.M. 8 rats per group

** $P < 0.01$

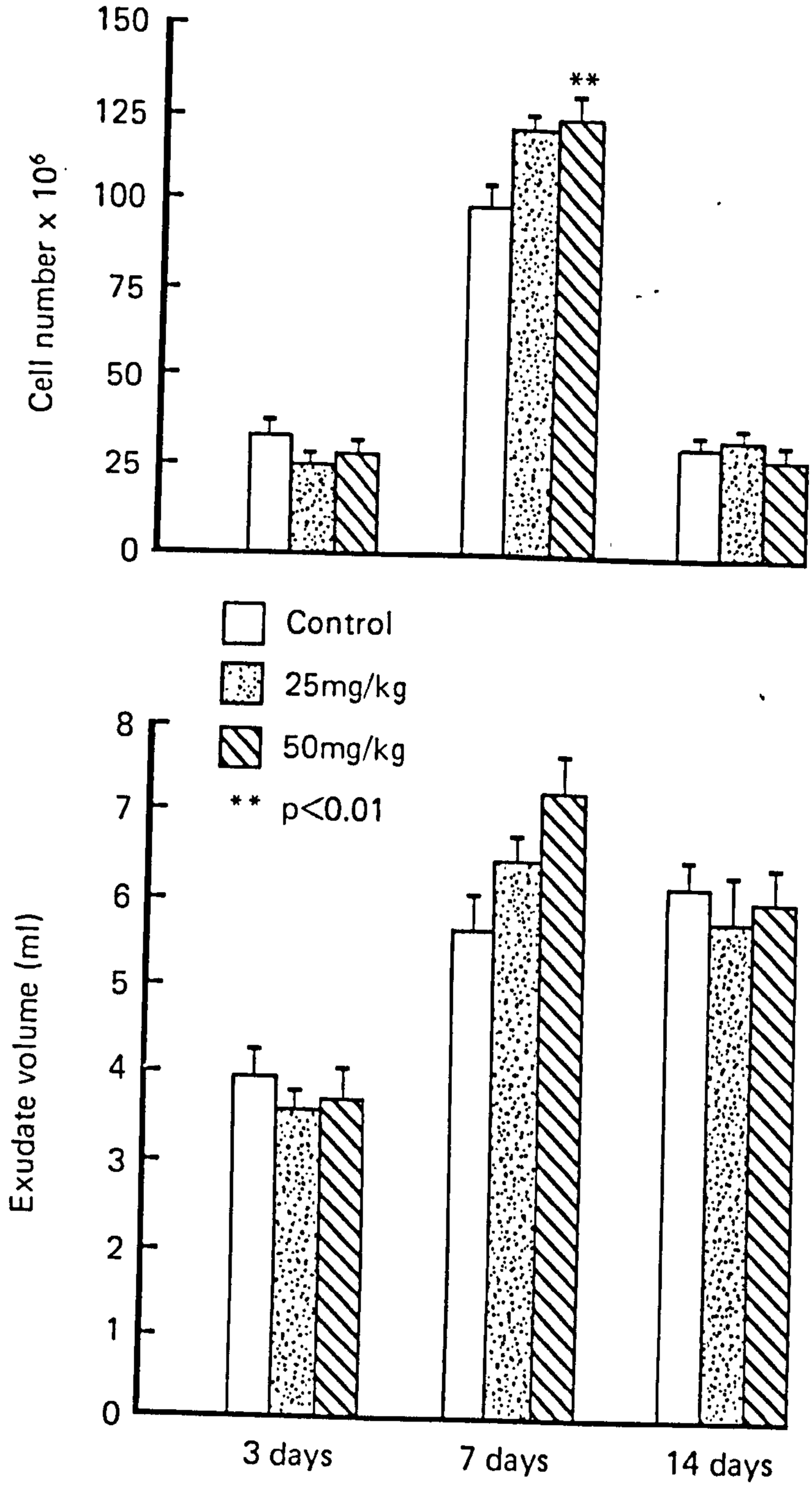

Fig. 33

Effect of daily oral administration of d-penicillamine (25 & 50mg/kg) on pertussis induced inflammation in the 6 day pouch.

Experiment / Time	Bordetella pertussis		2.5 mg/kg levamisole		5.0 mg/kg levamisole	
	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6	exudate volume (mls)	cell number x 10^6
3 days	3.6 $\pm$ 0.35	26.73 $\pm$ 1.52	3.4 $\pm$ 0.25	25.53 $\pm$ 1.1	3.33 $\pm$ 0.34	25.48 $\pm$ 0.76
7 days	5.87 $\pm$ 0.45	96.03 $\pm$ 4.3	5.35 $\pm$ 0.52	96.36 $\pm$ 5.9	6.25 $\pm$ 0.53	83.43 $\pm$ 9.1
14 days	5.8 $\pm$ 0.28	32.45 $\pm$ 3.4	5.6 $\pm$ 0.62	29.75 $\pm$ 3.2	6.03 $\pm$ 0.29	33.03 $\pm$ 2.63

Table (28) Effect of daily oral administration of levamisole (2.5 & 5.0 mg/kg) on pertussis induced inflammation in the 6 day pouch.

Mean values $\pm$ S.E.M. 8 rats per group

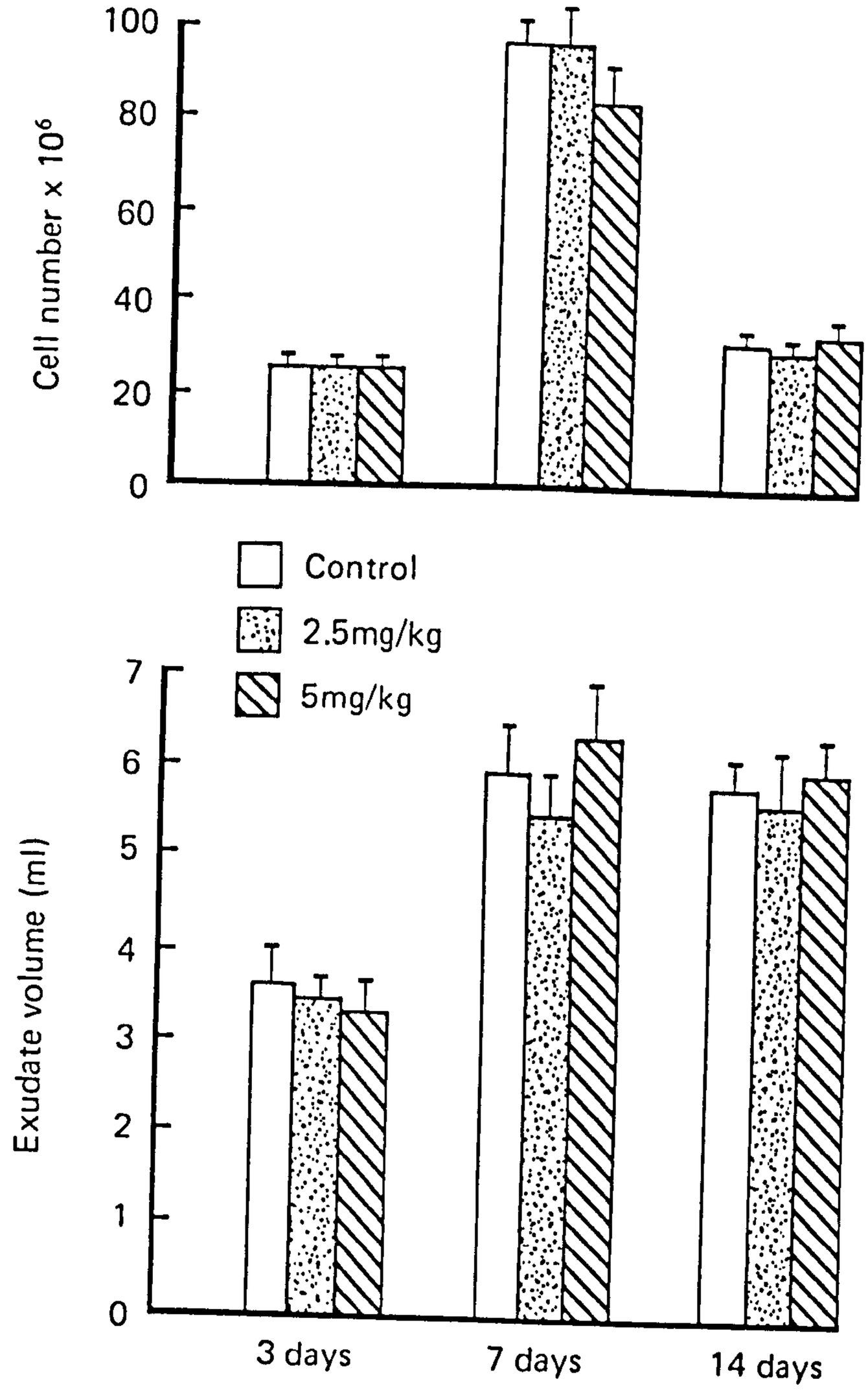

Fig. 34

Effect of daily oral administration of levamisole (2.5 & 5mg/kg) on pertussis induced inflammation in the 6 day pouch.

inflammatory model.

Dexamethasone

Dexamethasone again proved to be a potent anti-inflammatory agent when tested in this model of immune inflammation. At different times following the antigen challenge and drug treatment in both 0.1 mg and 0.2 mg/kg, animals were killed and their pouches were opened. In general, all the animals of the dexamethasone treated groups showed weight loss, especially at seven and fourteen days of treatment. The tissues of their air pouches were thin when compared with those of control groups, or even compared with those of other drug treated groups. The pouches contained no exudate at all and hence it was impossible to count inflammatory cells.

Leucocytes differential counts

Differential counts made on exudate smears obtained from control groups (non-treated), showed that there was a polymorphonuclear leucocyte dominance at all the tested time points. The ratio of polymorphs to mononuclears at three, seven and fourteen days was 93:7, 54:46 and 67:33. The dominance of polymorphs remained at three days after treatment with the different drugs (table 29). At seven days, polymorphs still dominated after drug treatment, except with 2.5 mg/kg levamisole, where there was a mononuclear dominance (59%). At fourteen days, in the control group the ratio was 67:33 and this was totally changed towards mononuclear dominance, except with 3 mg/kg indomethacin, where the ratio was in favour of polymorphs (66:34).

Time / Drug	3 days		7 days		14 days	
	PMN	MN	PMN	MN	PMN	MN
Pertussis group	93	7	54	46	67	33
1.0 mg/kg indomethacin	92	8	76	24	27	73
3.0 mg/kg indomethacin	89	11	65	35	66	34
25.0 mg/kg D-penicillamine	91	9	84	16	16	84
50.0 mg/kg D-penicillamine	90	10	78	22	10	90
2.5 mg/kg levamisole	99	1	41	59	36	64
5.0 mg/kg levamisole	84	16	55	45	48	52

Table (29) Effect of daily administration of drugs on the ratio of polymorphonuclears to mononuclear leucocytes at different time periods, observed in pertussis inflamed 6 day pouches.

Implantation of articular cartilage into pertussis inflamed six day pouch

Groups of animals were sensitised with emulsion containing equal volumes of bordetella pertussis vaccine and incomplete Freund's adjuvant. Seven days later, animals were injected with 20 ml air in the subcutaneous tissue of their backs. Three days following initial air injection, the animals were re-injected with 10 ml air to keep their pouches open. Twelve days following sensitisation with emulsion, animals were challenged with undiluted bordetella pertussis vaccine. On the thirteenth day, groups of animals of nearly the same weights and ages were killed and their femoral cartilage caps were removed and implanted into the preformed pouches for different time intervals.

The rate of proteoglycan loss from the implanted cartilage was found to be directly proportional to the period of implantation, ie, the longer the time of implantation, the greater the proteoglycan loss. Analysis of the results in table (30) figure (35) shows that there was a slight insignificant loss of proteoglycan from implants recovered after three and seven days of implantation. Implantation for three days caused a loss in proteoglycan content of about 3% and the loss at seven days was 5%. A significant ($P < 0.001$) decrease in the proteoglycan content was only observed at fourteen days and twenty-one days. Following fourteen days of implantation, the implants lost about 48% proteoglycan content. A massive loss was observed after twenty-one days and the implanted cartilage lost about 61% proteoglycan content.

Time after implantation	normal cartilage	cartilage in inflamed pouch	
	proteoglycan (μg)	proteoglycan (μg)	% change
3 days	238.5 ± 15.5	230.5 ± 12.0	-3.35
7 days	238.5 ± 15.5	225.5 ± 15.0	-5.45
14 days	238.5 ± 15.5	124.5 ± 11.0*	-47.8
21 days	238.5 ± 15.5	93.5 ± 8.0*	-60.8

Table (30) Amount of proteoglycan (μg) in rat femoral head cartilage at 3, 7, 21 and 14 days following implantation into 6 day pouch of pertussis sensitised animals.
Mean values ± S.E.M.
8 rats per group
* $P < 0.001$

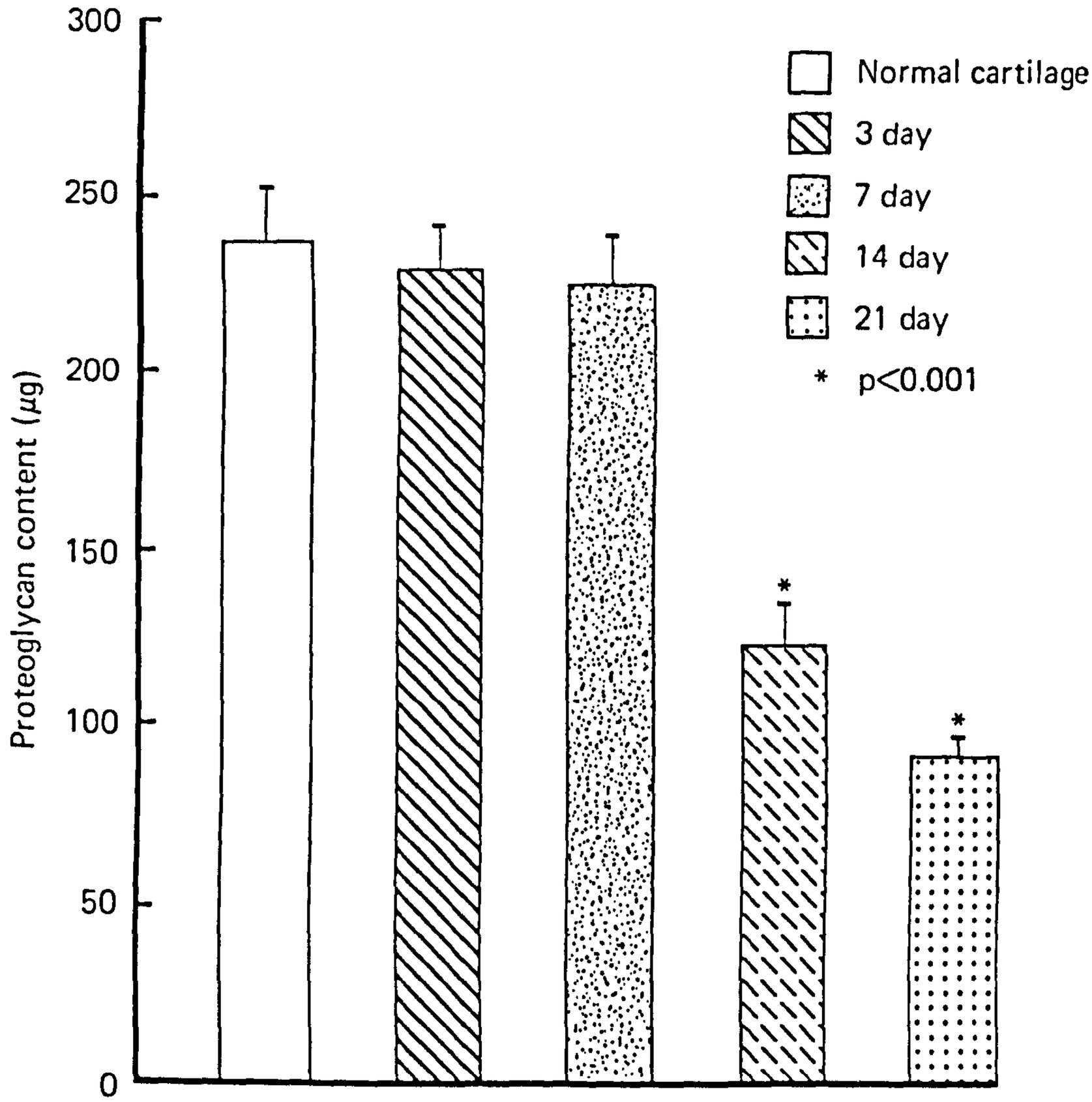

Fig. 35

Amount of proteoglycan (μg) in rat femoral head cartilage at 3, 7, 14 and 21 days following implantation into 6 day pouches of pertussis sensitised animals.

The histological changes in cartilage implanted into pertussis and carrageenan inflamed pouches were essentially the same. One difference, however, was that cartilage from pertussis inflamed animals was usually found in the exudate and was not adherent to the pouch wall.

Effect of drug treatment on proteoglycan content of articular cartilage implanted into pertussis inflamed pouches

Rat femoral head cartilage caps were implanted into six day pouches of pertussis sensitised animals. Cartilage caps were implanted one day after the animals were challenged with the pertussis vaccine. Animals were treated with different therapeutic agents. The treatment commenced on the day following cartilage implantation and the animals were given daily oral doses for fourteen days. The drugs used were indomethacin (1 mg and 3 mg/kg), D-penicillamine (25 mg and 50 mg/kg), dexamethasone (0.1 mg and 0.2 mg/kg) and levamisole (2.5 mg and 5.0 mg/kg). Implants obtained from non-treated groups (control) lost about 43% proteoglycan content.

Indomethacin

Indomethacin was found to protect the implanted cartilage significantly ($P < 0.001$) and this protective effect was observed with 1 mg and 3 mg/kg (table 31 , figure 36). This effect was observed to be dose-dependent. Cartilage recovered from 1 mg/kg treated animals lost approximately 16% proteoglycan content, compared with cartilage obtained from 3 mg/kg treated

Experiment	proteoglycan content (μg)	% change
Normal cartilage	234.0 ± 17.5	--
Bordetella pertussis	133.5 ± 6.5	-42.95
1.0 mg/kg indomethacin	196.0 ± 15.0**	-16.24
3.0 mg/kg indomethacin	202.0 ± 16.5**	-13.68

Table (31)

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into pertussis inflamed 6 day pouches of animals treated with a daily oral dose of either 1 or 3 mg/kg indomethacin throughout implantation period.

Mean values ± S.E.M.

6 rats per group

** $P < 0.01$

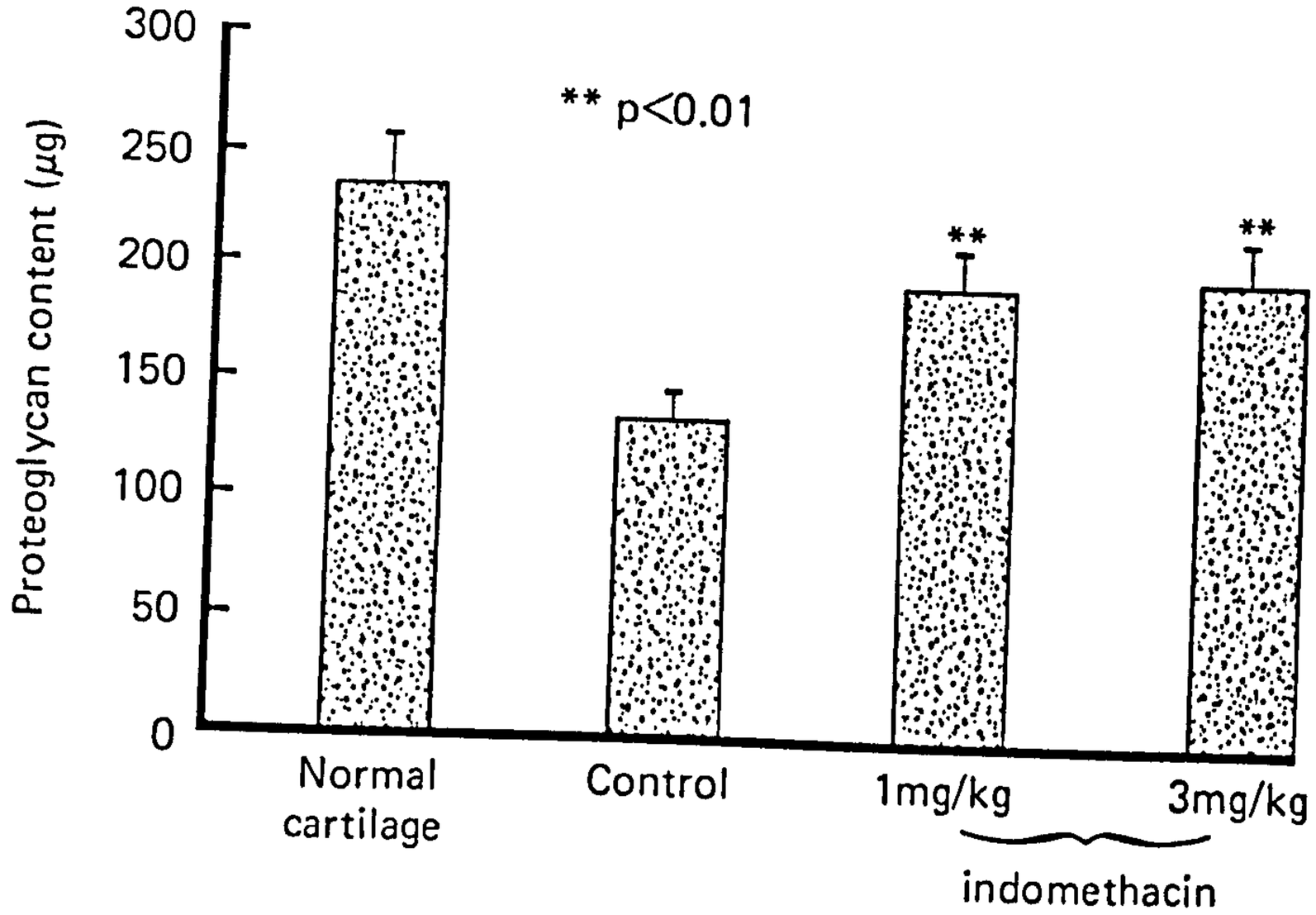

Fig. 36

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into pertussis inflamed 6 day pouches of animals treated daily with an oral dose of either 1 or 3mg/kg indomethacin throughout implantation period.

animals, which lost only 13% content. At the end of this experiment the animals were killed and their gastro-intestinal tracts were examined for ulceration. No ulcers were detected, either macroscopically or microscopically.

D-penicillamine

A significant ($P < 0.01$) protection to the implanted cartilage was also observed in samples obtained from 50 mg/kg D-penicillamine treated groups (table 32 , figure 37). Implants obtained from 50 mg/kg treated groups lost approximately 15% proteoglycan content, while the loss observed in 25 mg/kg treated groups was about 26%.

Dexamethasone

The same profile of protection was also observed with samples from dexamethasone treated animals. No protection was observed with implants obtained from 0.1 mg/kg treated groups and the loss was approximately 22%. On the other hand, there was a significant ($P < 0.01$) protection after fourteen days of treatment with 0.2 mg/kg dexamethasone where the loss was only 16%, compared with 43% observed in the control group (table 33 , figure 38).

Levamisole

Levamisole was the only tested drug which showed no protection to the implanted cartilage, and this effect was observed with both 2.5 mg and 5.0 mg/kg. Cartilage obtained from 2.5 mg and 5.0 mg/kg treated animals showed a loss in proteoglycan content of approximately 23% and 25%(table 34, figure 39).

Experiment	proteoglycan content (µg)	% change
Normal cartilage	234.0 ± 17.5	--
Bordetella pertussis	133.5 ± 6.5	-42.95
25.0 mg/kg D-penicillamine	171.5 ± 17.5	-26.71
50.0 mg/kg D-penicillamine	197.5 ± 19.0**	-15.6

Table (32)

Proteoglycan content (µg) of femoral head cartilage implanted for 2 weeks into pertussis inflamed 6 day pouches of animals treated with a daily oral dose of either 25 of 50 mg/kg D-penicillamine throughout implantation period.

Mean values ± S.E.M.

6 rats per group

** $P < 0.01$

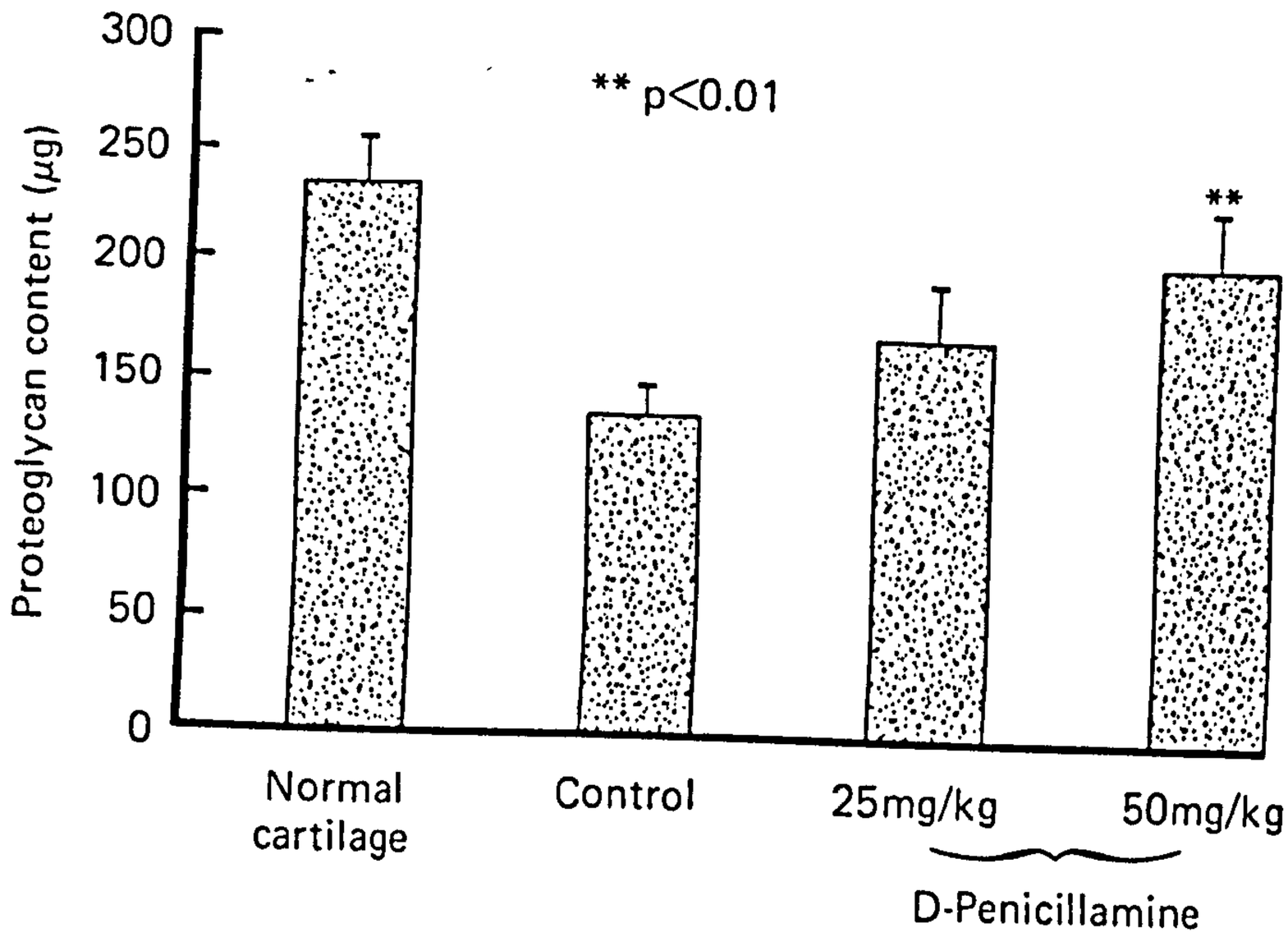

Fig. 37

Proteglycan content (μg) of femoral head cartilage implanted for 2 weeks into pertussis inflamed 6 day pouches of animals treated daily with an oral dose of either 25 or 50mg/kg D-Penicillamine throughout implantation period.

Experiment	proteoglycan content (µg)	% change
Normal cartilage	234.0 ± 17.5	--
Bordetalla pertussis	133.5 ± 6.5	-42.95
0.1 mg/kg dexamethasone	180.5 ± 17.0	-22.87
0.2 mg/kg dexamethasone	196.5 ± 15.0**	-16·03

Table (33)

Proteoglycan content (µg) of femoral head cartilage implanted for 2 weeks into pertussis inflamed 6 day pouches of animals treated daily orally with a dose of either 0.1 or 0.2 mg/kg dexamethasone throughout implantation period.

Mean values ± S.E.M.

6 rats per group

** $P < 0.01$

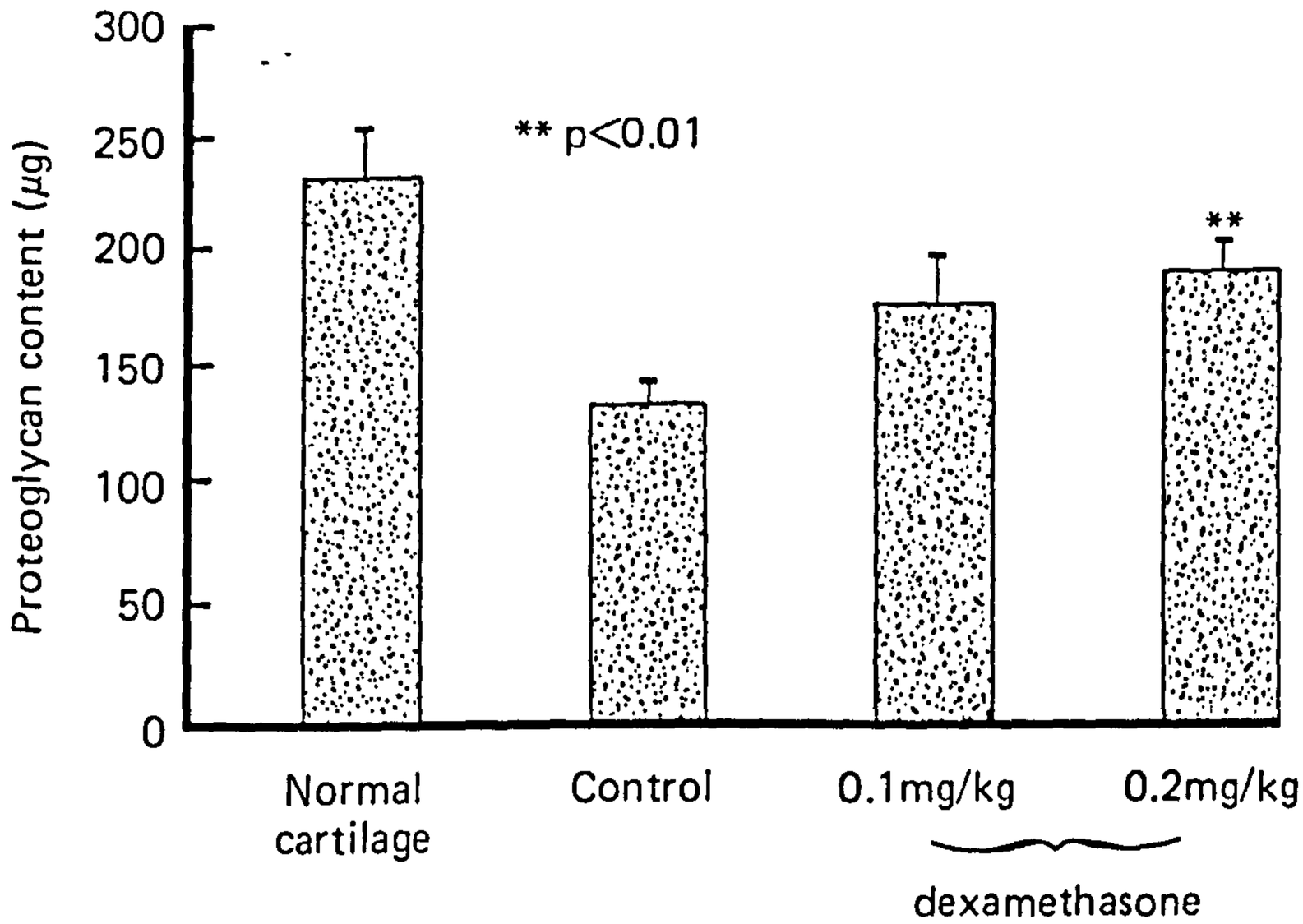

Fig. 38

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into pertussis imflamed 6 day pouches of animals treated daily orally with a dose of 0.1 or 0.2mg/kg dexamethasone throughout implantation period.

Experiment	proteoglycan content (μg)	% change
Normal cartilage	284.5 ± 17.5	--
Bordetella pertussis	170.5 ± 16.5	-40.08
2.5 mg/kg levamisole	219.5 ± 19.5	-22.85
5.0 mg/kg levamisole	214.0 ± 22.0	-24.79

Table (34)

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into pertussis inflamed pouches of animals treated daily orally with a dose of either 2.5 or 5.0 mg/kg levamisole throughout implantation period.
Mean values ± S.E.M.
6 rats per group

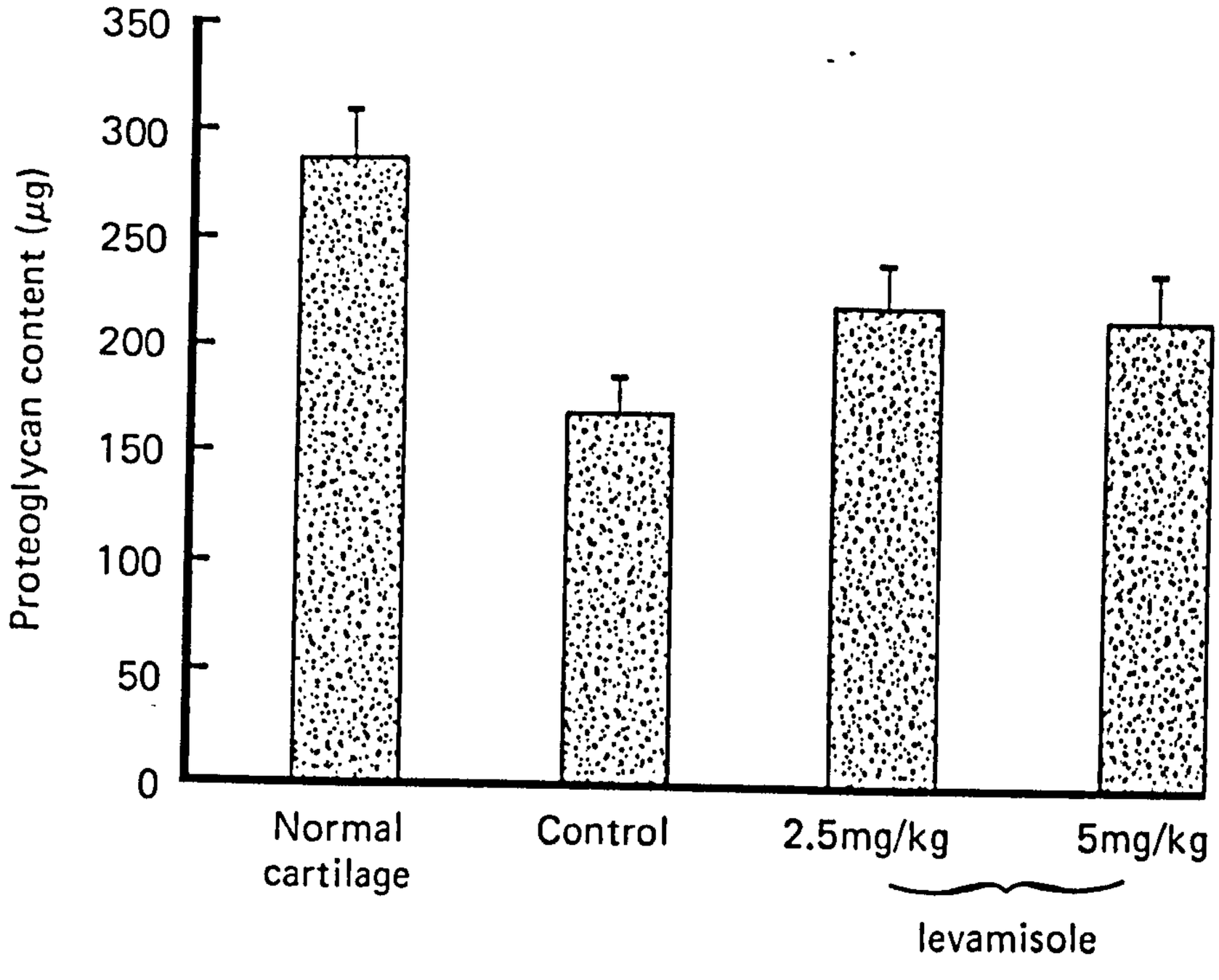

Fig. 39

Proteoglycan content (μg) of femoral head cartilage implanted for 2 weeks into pertussis inflamed 6 day pouches of animals treated daily orally with 2.5 or 5mg/kg levamisole throughout implantation period.

Histological observations following drug treatment

Histological examination of material obtained from drug treated animals showed no signifcant changes from control material with indomethacin, D-penicillamine and levamisole. There were gross changes observed following treatment with 0.1 mg and 0.2 mg/kg dexamethasone. Pouches from 0.1 mg and 0.2 mg/kg treatedanimals seven and fourteen days after challenge, were macroscopically thinner than those from control animals. This finding was confirmed microscopically, where it was observed that the pouch showed less fibrous connective tissue in the more superficial layer and considerably less infiltration by inflammatory cells.

Careful examination of lymph nodes from 0.1 mg and 0.2 mg/kg treated animals showed that they were macroscopically smaller than lymph nodes from control animals. There was an apparent reduction in granuloma content at three and seven days.

Determination of alpha-1-acid glycoprotein

Cooke et al in 1958, found that the levels of alpha-1-acid glycoprotein (α-1-GP) rise after trauma, in pregnancy, pneumonia, with some malignancies and in rheumatoid arthritis. In this study, the concentrations of α -1-GP were measured in sera and exudates from pertussis and carrageenan treated animals. The results summarised in table (35) figure (40) are for experiments where the concentrations of α -1-GP in sera and exudates from pertussis sensitised and carrageenan treated animals were measured at different times.

time after injection	Bordetella pertussis gp.		carrageenan gp.	
	serum	exudate	serum	exudate
1 day	3.9 ± 0.5	3.9 ± 0.45	3.1 ± 0.37	1.75 ± 0.2
2 days	3.8 ± 0.41	5.7 ± 0.5	3.85 ± 0.42	2.3 ± 0.2
3 days	5.9 ± 0.32	7.5 ± 0.6	4.4 ± 0.4	6.8 ± 0.8
7 days	4.6 ± 0.53	4.7 ± 0.61	1.2 ± 0.11	4.15 ± 0.51
14 days	4.5 ± 0.37	2.7 ± 0.3	4.4 ± 0.5	2.2 ± 0.15
21 days	0.9 ± 0.1	1.8 ± 0.11	1.55 ± 0.1	1.55 ± 0.11

Table (35) The concentrations (µg/ml) of alpha-1-acid glycoprotein (-1-GP) in sera and exudates of pertussis sensitised and carrageenan inflamed animals at different times.
Mean values ± S.E.M.
6 rats per group

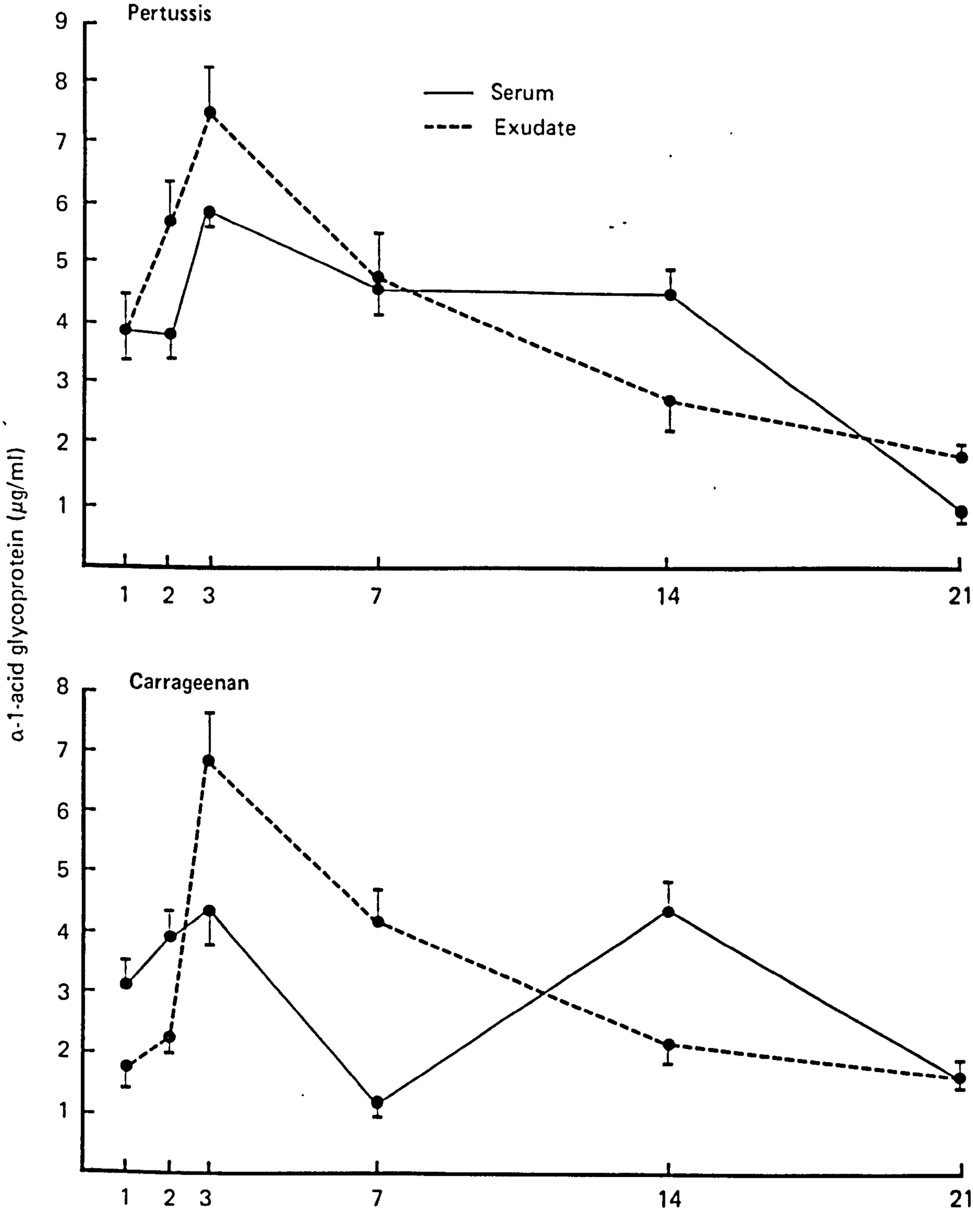

Fig. 40

The concentrations of α-1-acid glycoprotein in sera and exudates of pertussis sensitised and carrageenan inflamed animals at different times.

Pertussis groups

Determination of the levels of ∝ -1-GP in sera and exudates collected from pertussis inflamed animals showed that ∝ -1-GP peaked at three days in both serum and exudate, and was 5.9 and 7.5 µg/ml respectively. At seven and fourteen days, the serum levels were still high and they were 4.6 and 4.5 µg/ml. Twenty-one days after challenge with pertussis vaccine, the concentrations of ∝ -1-GP in serum and exudate dropped sharply to 0.9 µg/ml in serum and 1.8 µg/ml in exudate.

Carrageenan groups

Alpha-1-acid glycoprotein peaked three days after the injection of carrageenan, in both serum and exudate, and the concentrations were 4.4 and 6.8 µg/ml respectively. At seven days, the concentration dropped from 4.4 µg/ml to 1.2 µg/ml in serum and 6.8 µg/ml to 4.15 µg/ml in exudate. At the fourteenth day, the concentration of ∝ -1-GP in serum rose again to its maximum and was 4.4 µg/ml. Measurement of ∝ -1-GP in the inflammatory exudate at fourteen and twenty-one days, showed that the concentration was 2.2 µg/ml and 1.55 µg/ml. Twenty-one days after carrageenan injection, ∝ -1-GP dropped to its minimum, where it was 1.5 µg/ml.

Effect of drug treatment on the levels of ∝ -1-GP

Sera and inflammatory exudates were collected from animals of drug treated groups at different times. The tested drugs were indomethacin (1 mg and 3 mg/kg),

D-penicillamine (25 mg and 50 mg/kg), dexamethasone (0.1 mg and 0.2 mg/kg) and levamisole (2.5 mg and 5.0 mg/kg). Assays were performed on sera and exudates from animals receiving the drug treatment as described above. It was found that alpha-1-acid glycoprotein levels failed to show any correlation with the drug effects on the inflammatory process. Furthermore, there were considerable variations from experiment to experiment. It was decided not to pursue this line of research further.

D I S C U S S I O N

Arthritis is characterised by inflammation of the synovial membrane of joints. Ultimately, the problem for those who suffer from this disease, is destruction of the hard tissue, ie, cartilage and bone. Rheumatoid arthritis is a disease of joints where the hard tissue is eroded by a proliferative invasive tissue called pannus, which is derived from the synovial membrane and its microvasculature (Fasbender, 1975). The mechanisms involved in the destruction of joint tissue still remain unclear (Martel-Pelletier et al, 1985). Major advances have been made, however, and these are primarily the result of studies based on in vitro techniques. Animal models are still of considerable importance, both for the determining of basic cellular control mechanisms and for detecting and understanding the pharmacological control. Billingham (1983) in a recent review, emphasises the importance of in vivo approaches for the discovery of novel disease modifying agents. Paulus and Whitehouse in 1973, pointed out the difficulties in searching for novel disease modifying drugs. Certainly the main difficulty arises in that no single aetiological factor has been found against which to target drugs.

Many animal models of acute inflammation are available. Carrageenan-induced oedema is frequently used to study anti-inflammatory drugs followed by systems such as ultraviolet erythema in guinea pig skin (Winder et al, 1958). Further evaluation of a compound requires a chronic model such as adjuvant- induced arthritis (Billingham, 1983) or a proliferative model, such as cotton pellet granuloma (Meier et al, 1950). The main advantages of using an experimental

model of arthritis for examining pharmacological agents, is that the chronic inflammatory changes observed are likely to be useful in detecting novel compounds. Such agents may be undetected in acute models of inflammation.

The models of experimental arthritis currently available can be grouped under three categories (i) adjuvant-induced arthritis in the rat, (ii) chronic monoarticular arthritis induced by intra-articular injection of antigens, eg, fibrin (Dumonde and Glynn, 1962), and (iii) arthritides caused by infectious agents, eg, mycoplasma arthritides (Cole et al, 1971). Adjuvant-induced arthritis is restricted to the rat and it has been used for screening purposes. It is a useful model for the detection of certain kinds of drug activity but its real value as a model of rheumatoid arthritis remains to be justified. Although it detects compounds active against acute inflammation and immuno-suppressive agents, there are simpler models available for both purposes. It does not appear to detect the activity of disease modifying drugs like D-penicillamine, which seems to affect the human arthritic process. Antigen-induced chronic arthritis, as exemplifed by the model of Dumonde and Glynn (1962), closely resembles several aspects of rheumatoid arthritis, but its major drawback is that the only reliable method of assessment appears to be based on histological examination, which is accompanied by problems with quantification. Similarly, arthritis caused by an infectious agent may represent a closer approximation of the human disease than does adjuvant disease, but there is very little information available on the effect of drug

treatment, since the assessment of the disease activity is difficult.

The aim of the present study was to develop a novel animal model with relevance to human disease (arthritis), in order to study the mechanisms of cartilage degradation which could be quantified. Many workers have previously examined the effects of drugs on inflammation in soft tissue, either by using cavity models such as pleurisy (Willoughby, 1975) or polyester sponges (Higgs et al, 1980). Similarly, many workers have investigated the effects of soft tissue on cartilage degradation in vitro (Dingle, 1979). The present study has concentrated on bringing together these two approaches in one in vivo model. The model chosen to base these studies on was the air pouch. The air pouch granuloma technique was first introduced by Selye (1953) and modified by Robert and Nezamis (1957). The basic procedure consisted of making an air pouch in the back of the rat by injecting 25 ml air subcutaneously. Subsequently, dilute croton oil was introduced into the pouch to induce granulomatous tissue formation. The response was evaluated 4 - 14 days later, on the basis of the volume of the fluid collected from the pouch and the weight or thickness of the pouch wall. Besides croton oil, several other irritants have been used, especially carrageenan (Highton, 1963; Fukuhara and Tsurufuji, 1969).

Edwards, Sedgwick and Willoughby in 1981, first noticed that injecting air in the subcutaneous tissue of the back of mice and rats, resulted after six days in the formation of lining structure which closely resembled synovium.

Although the linings of the subcutaneous air cavities may not be identical with any particular piece of joint lining, they demonstrated all the essential features of a synovial lining. Moreover, these linings have similar cell sub-populations as judged by electron microscopy and light microscopy, using haematoxylin and eosin and Van Gieson stains, esterase activity and immunofluorescent staining for Ia antigen. The lining cells were of two main types; the commonest type (50 - 90%) were identified as fibroblasts and were indistinguishable from type B cells of the mouse knee synovial lining. The second major cell type (10 - 50%) took up the injected colloid carbon avidly and they could be described as macrophages or type A synovial lining cells.

Such a system differs from the traditional air pouch granuloma model, in that inflammation can be studied in a cavity similar to synovium. Edwards and his colleagues (1981) presented for the first time an <u>in vivo</u> model suitable for studying the inflammatory process, and the process of cartilage degradation (hard tissue) in contact with lining cells (soft tissue). Sedgwick et al in 1983 showed that the six day pouch responds to a greater degree than the one and the three day pouches to different inflammatory stimuli (carrageenan, calcium pyrophosphate dihydrate crystals and urate crystals). They attributed the increased inflammatory reactivity in the lining of the six day pouch of the rat to the following possibilities: (i) an increase in the vascularity of the lining, which results in more inflammatory cells recruited to the air pouch, (ii) the formation of an organised surface layer

of mononuclear phagocytes and fibroblastic cells, or (iii) the formation of an increasingly effective mechanical barrier that retains the irritants and the products of the inflammatory response.

Since the air pouch had features similar to synovium and could easily be inflamed, Sin et al (1984) introduced the idea that the introduction of cartilage into such a structure may provide information on hard tissue-soft tissue interaction. These workers used the mouse six day pouch to study cartilage degradation, where autologous living or dead intact or minced mouse xiphisterna were implanted into the air pouches of the original donors. They showed that only a modest loss of proteoglycan and collagen occurred in the living intact cartilage. Severe loss of proteoglycan, however, was detected in the minced living and dead intact cartilage. The air pouch cartilage implant system seemed to have potential for the examination of pharmacological agents. The present study, therefore, firstly verified the works of Sin et al (1984) in order to evaluate its use as a model for examining the mechanisms of actions of existing disease modifying agents and detecting novel agents.

The data described confirms the findings of Sin and his colleagues. Living and dead cartilage were used in order to verify the in vitro observations of Fell and Jubb (1977), that synovial tissue, when placed in contact with either living or dead articular cartilage, caused the loss of proteoglycan and collagen from the tissue matrix. Fell and Jubb proposed that the damage observed was due to either direct enzyme degradation or to the indirect action of

substances released from synovium stimulating the 'living' chondrocytes to degrade their own extracellular matrix. These indirect substances they have termed 'catabolins' and have been implicated as important mediators of cartilage degradation. Saklatvala et al (1984) recently confirmed that purified porcine mononuclear cell catabolin was in fact a form of interleukin-1. Minced samples were used in order to study the effect of mechanical trauma on cartilage degradation. Sin et al (1984) proposed two possible mechanisms by which mechanical trauma may trigger cartilage degradation. Firstly, trauma such as mincing may abolish the perichondrial protection and may cause the loss of stability of the matrix in the damaged area and make it vulnerable to attack by various types of mediators known to degrade cartilage. The second mechanism is that trauma may affect the activity of the chondrocytes, which have been known to play an anabolic role under certain circumstances (Mitrovic, 1982) and also produce substances which inhibit the degradative activity of proteolytic enzymes (Sorgente et al, 1975). The observation that minced cartilage fragments attract leucocytes would also suggest that the release of matrix components after injury amplifies the inflammatory response. It has been shown that mechanical deformation of cell membranes in articular cartilage can activate phospholipase and induce the generation of arachidonic acid (Mitrovic, 1982). The inflammatory response seen around fragments of cartilage may be due to the release of chemotactic prostanoids.

The present study improved the system of Sin et al to

examine the mechanism of action of non-steroidal anti-inflammatory drugs and disease modifying agents. Because of the small size of the cartilage fragments, retrieval of the cartilage was difficult. The present study improved the mouse xiphisternum model for the evaluation of pharmacological agents by introducing the millipore-half chamber technique, where the cartilage fragments were introduced into the chamber prior to implantation. This allowed 100% retrieval of the cartilage fragments. The introduction of this technique (millipore-half chamber) facilitated the screening of different pharmacological agents on the process of cartilage degradation. Initially, different groups of anti-inflammatory and anti-rheumatic compounds were examined on the process of inflammation and cartilage degradation. This study was designed to compare for the first time the reduction of soft tissue inflammation with the protection afforded on the cartilage.

Several non-steroidal anti-inflammatory agents were selected. Aspirin and indomethacin were used because they are both potent inhibitors of arachidonic acid metabolism affecting the cyclo-oxygenase metabolic pathway (Lombardino, 1983). In the present study, both these agents significantly reduced leucocyte accumulation into the mouse air pouch. These observations supportthose of others who showed that cyclo-oxygenase inhibitors could inhibit cell accumulation in experimental subcutaneous sponges (Higgs et al, 1980), carrageenan induced pleurisy (Mikami and Miyasaka, 1983), carrageenan induced paw oedema (Higgs and Mugridge, 1982) and the carrageenan granuloma pouch (Fukuhara and Tsurufuji, 1969).

It is possible that both of these compounds, by blocking the generation of prostaglandins, reduced the formation of exudate and,therefore, the concentrations of chemotactic factors. Di Rosa et al (1971) showed previously that indomethacin suppressed the total number of leucocytes emigrating into the rat paw after carrageenan injection. In contrast, Ackerman et al (1980), showed that indomethacin enhances the migration of mononuclear phagocytes into the pleural cavity three days after carrageenan injection. Ackerman and his colleagues explained their observations on the grounds that indomethacin, by blocking the cyclo-oxygenase pathway of arachidonic acid metabolism, increased the generation of lipoxygenase products. The present study showed that with these doses of cyclo-oxygenase blockers in the mouse, cell accumulation is reduced. The concept of substrate diversion and lipoxygenase products has recently been reviewed by Higgs and Vane (1983). Esterified arachidonic acid is cleaved from membrane phospholipids by the action of phospholipase-A2 in response to a variety of stimuli and may then be converted either by cyclo-oxygenase to prostaglandins and thromboxanes, or by lipoxygenase to leukotrienes. These metabolic pathways have been extensively reviewed (Bakhle, 1983 and Granstrom, 1984). Cyclo-oxygenase products have been detected in a variety of experimental inflammatory lesions (Tissot et al, 1984) and in human chronic joint inflammation (Brodie et al,1980). Leukotrienes have also been detected experimentally (Simmons et al, 1983) and in synovial fluid of rheumatoid arthritis (Davidson et al, 1982). Arachidonic acid metabolites are important mediators of

inflammation and have numerous and complex biological effects. Different arachidonic metabolites may be released depending on stimulus and cell type, and their effects may vary from site to site (Higgs et al, 1983a). Many of the agents with anti-inflammatory activity act via the blockade of the cyclo-oxygenase pathway of arachidonic acid (Higgs et al, 1983b). Benoxaprofen, however, is able to inhibit both cyclo-oxygenase and lipoxygenase pathways with preferential activity against lipoxygenase (Walker and Dawson, 1979).

In the present study, benoxaprofen also significantly reduced leucocyte accumulation in the mouse air pouch. This supports previous observations of Meacock and Kitchen (1979). Piroxicam was chosen as an example of a reversible cyclo-oxygenase blocker (Goodwin, 1984). This agent similarly inhibited cell accumulation. Although these non-steroidals blocked cell accumulation, thus inhibiting the secretion of cellular products, none of these agents has any effect on the implanted cartilage. These observations contrast to those of others using the adjuvant arthritis model. Non-steroidal anti-inflammatory agents have been shown to effectively suppress adjuvant arthritis. The effectiveness of NSAID on adjuvant arthritis appears to vary from laboratory to laboratory. Glenn (1966) obtained an ED50 for indomethacin of 0.2 mg/kg using an arthritic score to measure effectiveness, whereas Di Pasquale et al (1975) found and ED50 of 2.0 mg/kg. It would appear from the present data that prostaglandins/eicosanoids are not important as mediators of cartilage degradation.

In contrast to the findings using NSAID , D-penicillamine

had no signficant effect on soft tissue cell accumulation, but significantly reduced proteoglycan loss from cartilage implants. The view that D-penicillamine is not an anti-inflammatory drug is supported by its lack of effect on other conventional models. Maddox (1973) showed that D-penicillamine failed to suppress carrageenan-induced paw oedema. Arrigoni-Martelli and Bramm (1975) found no effect on the primary lesions of adjuvant arthritis, but an enhancement of the secondary lesions. Secondary lesions of adjuvant arthritis are usually regarded as a manifestation of cell-mediated immunity. This is supported by its effects in models of delayed hypersensitivity using pertussis vaccine as antigen (Dieppe et al, 1976). In this model, the inflammatory reaction to pertussis vaccine in sensitised animals is enhanced by D-penicillamine. Also in support are enhancement of skin responsiveness to tuberculin in patients with rheumatoid arthritis after treatment with D-penicillamine (Berry and Huskisson, 1976). These observations prompted various authors to speculate on the mode of action of D-penicillamine via the modulation of T-cell function.

These findings suggest that agents such as D-penicillamine can fundamentally reduce the degradation of cartilage. The final group of agents tested in this system were the corticosteroids. Previous in vitro studies have shown that D-penicillamine and corticosteroids can reduce cartilage degradation (Sheppeard et al, 1982). Apart from the work of Hunneyball et al (1977) using the rabbit monoarticular arthritis, the mouse xiphisternal system is the first model reported to detect D-penicillamine. Similarly, hydrocortisone

reduced proteoglycan loss in this system. A detailed discussion on the mechanism of action of these drugs follows. A possible criticism of the model described was firstly that xiphisternum is structurally different from other cartilage surfaces, as it has a perichondrium. Mechanical damage is required to induce proteoglycan loss, and furthermore it is not articular. Since articular cartilage is the important tissue in arthritis, it was decided to try and further develop the model to overcome these problems. Furthermore, the cell numbers in the mouse model were found to vary considerably from animal to animal. It was decided to move to the rat.

In order to develop an improved system, the cartilage cap of the mouse femoral upper end was used. This, however, was found to be technically difficult. It was, however, found that the same cartilage obtained from rats proved to be a good source to study cartilage degradation. Initially, experiments were carried out to see whether femoral head articular cartilage, if implanted into inflamed or non-inflamed soft tissue, degraded. One of the problems of the rat femoral head system is that the cartilage transplantation cannot be autologous. Firstly, therefore, it was necessary to examine whether cartilage was degraded by the soft tissue inflammation or stimulation of the immune system.

Cartilage has been considered to be immunologically privileged since it survives longer after transplantation than any other tissue, except the cornea (Brown and Cruess, 1982). In the present study, cartilage from donor rats had been implanted into pouches of recipient animals

(allografts) on the basis that cartilage is so weakly antigenic (Gertzbein and Lance, 1976). The findings that outbred and inbred strains of rat degraded cartilage to the same degree, leads to the conclusion that no auto-immune rejection process is involved. This was further supported by the histological findings of this study, that there was no regional lymphoid tissue involvement. Malseed and Heyner (1976) successfully elicited an immune response with isolated chondrocytes. These authors suggested that chondrocytes were not weakly antigenic but could induce a significant serological response. Yoshiyoka et al (1982) also found that they could detect delayed hypersensitivity in animals immunised with articular cartilage antigens. Surprisingly, animals immunised against articular cartilage degraded implants to the same degree as non-immunised animals, and this further supports the conclusion that the air pouch cartilage-implant model is not measuring tissue rejection.

Carrageenan was initially chosen as irritant in this study, in order to examine the effect of soft tissue inflammation on cartilage degradation. Carrageenan is able to induce both acute and chronic inflammation (Di Rosa, 1972). Sedgwick et al (1983) showed that if the air pouch is formed for six days, it behaves differently in response to carrageenan. The present study was able to to examine the effects of prolonged inflammatory lesions on cartilage degradation, using the six day pouch system. It has been shown that carrageenan is rapidly taken up by mononuclear phagocytes with the resultant release of hydrolytic enzymes (Allison et al, 1966, Cantazaro et al, 1971). Thus, carrageenan can induce an

environment of tissues and cells which have been proposed to induce cartilage degradation (Decker et al, 1982, Starky et al, 1977)

The results described in this study suggest that the presence of an inflammatory reaction, of the type induced by carrageenan, is unable to accelerate proteoglycan loss from cartilage. Since loss of matrix is a primary factor in rheumatoid arthritis, it would appear that the type of inflammation used in this study is not representative of the rheumatoid disease process. The findings that proteoglycan loss occurs in 'normal' , ie, non-inflamed tissue , is not understood. However, it could be due to the direct contact of cartilage with the soft connective tissue cells. The six day pouch tissue, which has previously been shown to have an organised layer of lining cells (macrophages and fibroblastic cells), was not found to degrade cartilage to a greater extent than subcutaneous tissue. It is of great importance in such experiments to provide 'normal' tissue controls. Subcutaneous implantation following blunt dissection may itself induce tissue damage and the generation of inflammatory mediators. Fell and Jubb (1977) showed that catabolin acts as a messenger which is capable of stimulating chondrocytes in living cartilage to resorb their own extracellular matrix. If catabolin was generated during carrageenan inflammation, dead cartilage would be expected to degrade at a slower rate than living. The results described in this study, showed that dead cartilage lost proteoglycan substantially after only one week. Furthermore, no difference was observed between implantation into 'normal' or 'inflamed' tissue. This suggests that degradation of cartilage is more

likely due extrinsic (damaged tissue) cells rather than by intrinsic (viable chondrocytes) enzymes.

Sin and his colleagues (1984) proposed that cartilage is not normally broken down because it has self-protective mechanisms. This protection could be reduced by trauma induced mechanically. In the articular cartilage system, mincing of cartilage induced greater loss of proteoglycan but the effects were not as dramatic as with xiphisternal cartilage. These results may have been observed because articular cartilage has no perichondrium. The perichondrium of xiphisternal cartilage may afford some protection. It is possible that the loss of cartilage matrix seen in this system is more closely related to soft tissue adhesion than to inflammation. The fact that implanted cartilage is present in an immovable state, could be an important factor in the process of cartilage degradation. In this respect, the work of Salter et al (1980) is of interest. These authors showed that movement has a major protective role for cartilage in joints injured by infection or trauma.

It was decided to assess the value of the air pouch as a site for immune inflammation. Immune complexes have been proposed to localise in joint cavities or across blood vessel walls, inducing inflammation (Ziff, 1980). In this study, an active Arthus reaction was induced first, which persisted for about 72 hours, giving a predominantly cellular response peaking at four hours, with the dominance of polymorpho-nuclear leucocytes. It was decided also to attempt a reverse passive arthus reaction. This, however, proved difficult to produce in the air pouch.

In order to determine the pathological mechanisms involved in rheumatoid arthritis, it was necessary to develop a chronic immune model in animals which simulate the disease. Bordetella pertussis vaccine has for a long time been used as an antigen for producing hypersensitivity in the rat. Rowley et al (1959) showed that pertussis vaccine could produce a severe active cutaneous hypersensitivity in the rat. These authors showed that this hypersensitivity could be transferred by lymph node cells but not with serum. Willoughby in 1966, confirmed these findings by showing that inflammation could be induced in the paw of animals previously sensitised with pertussis. Willoughby found that the paw oedema reached a maximum after 24 hours and that on histological examination the lesion was characterised by massive mononuclear cell invasion. Because of the problems in quantification of paw oedema, Dieppe et al (1976)induced pertussis hypersensitivity in the pleural cavity of rats. These authors attempted to design a system which would detect drug activity useful for the treatment of rheumatoid arthritis. The rationale for these studies was that rheumatoid arthritis was thought to have a delayed hypersensitivity component (Yu and Peter, 1974).

Ideally, an experimental model of inflammation with relevance to rheumatoid arthritis should be prolonged and have an immune component. The present study has attempted to use the previous knowledge of the structure of the six day pouch and bordetella pertussis vaccine to produce such a model. The present findings have shown that it is necessary to use the six day old pouch to produce a

prolonged inflammatory response to pertussis vaccine. Using this aged pouch, a single challenge of pertussis vaccine into previously sensitised rats can produce an inflammatory reaction which persists over a 21 day period. Cell transfer experiments were not attempted but histological studies of the lymph nodes revealed activation of paracortical areas with large numbers of small lymphocytes and also activation of medullary areas with large tracts of immunoblasts and plasma cells being present.

Turk (1980) had previously classified the changes occurring in the lymph nodes during cell mediated immunity and immediate hypersensitivity. Turk showed that in pure cell mediated reactions, only changes in the paracortical areas were observed. Whereas immediate hypersensitivity changes in the medullary area were seen leading to the formation of plasma cells. Turk also showed the 'mixed reaction ' where both types of histological dye were seen in the lymph nodes. It seems probable that with the histological changes that were noted during these experiments, bordetella pertussis was leading to a mixed hypersensitivity reaction, ie, both immediate and delayed. This may be important since, in rheumatoid arthritis, both types of hypersensitivity reaction are seen often together (Ziff, 1973).

In this study, the level of one of the acute phase reactants (α-1-glycoprotein) has been measured in the exudate and sera of animals with carrageenan and pertussis-induced inflammation. Billingham (1983) suggested that biochemical changes can provide a considerable amount of information about the extent of an ongoing chronic inflammation.

With the implication that interleukin-1 released from active mononuclear phagocytes can elevate acute phase reactants, it would seem that the level of these proteins is a good measure of cell activity (Bornestein, 1982). In the present study, the peak of both serum and exudate α-1-glycoprotein was seen when the lesion was dominated by polymorphonuclear leucocytes. This was to some extent surprising, since it would be expected that α-1-glycoprotein concentration would be at its highest when mononuclear cells dominate the lesion. These results may indicate that mononuclear cells present in large numbers are relatively non-active. This does not agree, however, with the work of Spector (1969), who showed granulomas produced with pertussis were high in cell turnover, implying that these cells were highly active.

The advantage of the present system over other immune air pouch systems, is the prolonged life of the lesion. Tsurufuji et al (1982) induced an allergic air pouch inflammation using azobenzenearsonate conjugated acetyl bovine serum. Tsurufuji et al (1982) produced with this antigen a cellular reaction which peaked 1 - 2 days after challenge, rapidly waning thereafter. Further more, the antigen itself in non-sensitised animals was able to induce an oedematous reaction and cell accumulation. In this study, the reaction is highly specific, no inflammation being produced in response to challenge in non-sensitised animals. Recently, Yoshino et al (1984) have proposed a persistent antigen induces chronic inflammation in the rat air pouch. This model,

although being interesting, has the disadvantage of requiring continuous acute inflammatory insults. Their system had the added complication of using carboxy methyl cellulose as the irritant vehicle.

Because inflammation caused by different irritants can induce very different chemical mediator release (Turk and Willoughby, 1978), we have compared the loss of proteoglycan by cartilage implanted into non-immune and immune lesions. Articular cartilage was implanted into pertussis-inflamed six day pouches for different times. In this type of prolonged inflammation, the loss of proteoglycan from the implanted cartilage was not significantly different from the loss seen by tissue in normal pouches. It seems that, at least at the level of inflammation and cartilage breakdown mediators present in the carrageenan and pertussis-inflamed pouches, cartilage degradation is not accelerated by direct effect of these mediators.

The effects of indomethacin, dexamethasone and the DMARD's (D-penicillamine and levamisole), on inflammation and proteoglycan loss of implanted articular cartilage were investigated using the two inflammatory models in the rat (carrageenan and pertussis-inflamed six day pouches). Indomethacin was found to suppress carrageeenan and pertussis-induced inflammation in both the tested doses (1 mg and 3 mg/kg). Protection of the implanted cartilage was observed after fourteen days of a daily treatment with 3 mg/kg in carrageenan system and with both the tested doses in pertussis system. Dexamethasone was found to be a potent anti-inflammatory agent, both types of inflammation

being strongly suppressed and the pouch linings were thinner than those of control groups. There was a significant protection observed in implants recovered from both carrageenan and pertussis-inflamed pouches.

On the other hand, the DMARD (D-penicillamine and levamisole) were found to have no anti-inflammatory activity. Furthermore, there was a trend towards potentiation for the inflammatory process. However, this did not reach significance. Surprisingly, these two drugs showed a significant protection to the implants recovered from carrageenan-inflamed pouches. There was also a protection to the implanted cartilage recovered from pertussis-inflamed pouches after treatment with D-penicillamine and levamisole, however, the significant protection was only observed with 50 mg/kg D-penicillamine.

The effects of indomethacin, dexamethasone, D-penicillamine and levamisole on the loss of proteoglycan from articular cartilage implanted into normal subcutaneous tissue were also investigated throughout this study. It was only dexamethasone which showed a significant protection to the implanted cartilage after fourteen days of treatment, while the rest of the tested drugs failed.

The anti-inflammatory action of the NSAID tested on the different models of this study is in agreement with published data in other inflammatory models, like sponge implantation (Doyle et al, 1983), carrageenan-induced pleurisy (Mikami and Miyasaka, 1983), carrageenan-induced paw oedema (Higgs et al, 1980), pertussis-induced pleurisy (Dieppe et al, 1976), pertussis-induced paw oedema

(Arrigoni-Martelli et al, 1976) and carrageenan granuloma pouch (Fukuhara and Tsurufuji, 1969). Non-steroidal anti-inflammatory drugs were also found to inhibit adjuvant-induced arthritis (Billingham, 1983) and collagen type-II induced arthritis (Phadke and Nanda, 1982). As described earlier, NSAID act via the blockade of the cyclo-oxygenase pathway of arachidonic acid (Higgs et al, 1983b). Whether this effect represents their exclusive site of action is uncertain (Weissman, 1983). Arachidonic acid metabolites are important mediators of inflammation and have numerous and complex biological effects. Different arachidonic metabolites may be released, depending on stimulus and cell type, and their effects may vary from site to site (Higgs et al, 1983a). Prostanoids have been implicated in the cartilage damage, since these substances have been shown to induce and potentiate synovial inflammation (Robinson et al, 1975). In vitro, the NSAID have been reported to inhibit polymorphonuclear leucocytes' adherence (Buchanan et al, 1983), chemotaxis (Rivkin et al, 1976), aggregation (Kaplan et al, 1984), lysosomal enzyme release (Smith and Iden, 1980) and superoxide anion formation (Lombardino, 1983). Some of these effects are drug specific and suggest that the NSAID have effects unrelated to cyclo-oxygenase inhibition (Kaplan et al, 1984). Indeed, phospholipase-A2 inhibition has been reported for indomethacin (Kaplan et al, 1978).

Although a reduction in both fluid exudation and total cell number was observed in animals treated with NSAID ,

only the steroidal anti-inflammatory drugs tested throughout this study totally inhibited the formation of the granuloma. There was also a marked histological change in the pouch linings following treatment with steroidal anti-inflammatories. Originally, Selye (1953) used the granuloma pouch technique for the study of antiphlogistic corticoids. He showed that the introduction of small amounts of hydrocortisone directly into the wall of the pouch completely prevents granuloma production. Furthermore, histologically, the animal's pouch wall seemed extremely thin. Steroids have been shown to inhibit cotton pellet granuloma (Lerner et al, 1964), carrageenan-induced pleurisy (Mikami and Miyasaka, 1983), carrageenan-induced oedema (Higgs et al, 1980). Steroidal anti-inflammatory compounds have been shown to inhibit leucocyte migration in vitro and in vivo (Koh et al, 1979). The steroidal anti-inflammatory dexamethasone was found to inhibit leucocyte migration to allergic inflamed air pouch (Ohuchi et al, 1982). Steroidal anti-inflammatories have been shown to stabilise cell membrane (Pollock and Brown, 1971). It is therefore possible that the stabilisation of these drugs on blood vessel walls and leucocytes may prevent leakage of exudate and migration of cells into the air pouch.

The potent anti-inflammatory action of the steroids is due to inhibition of the release of arachidonate substrate. This action has been explained by the discovery that steroids induce intracellular synthesis of an endogenous phospholipase inhibitor called macrocortin (Blackwell et al, 1980) or lipomodulin (Hirata et al, 1980). Partially purified macrocortin inhibits the formation of arachidonate metabolites in vitro and in vivo (Parente et al, 1984).

Steroids have been shown to inhibit all phases of adjuvant-induced arthritis in the rat (Billingham, 1983) and monoarticular arthritis (antigen-induced arthritis) in the rabbit (Pitt and Lewis, 1984). According to Hunneyball (1981) steroids may exert their anti-arthritic effect by suppressing cell-mediated immune responses within the synovium. Tissue culture studies have provided information on the possible molecular events that occur in arthritic joints. Interactions between T-lymphocytes and monocytes produce a molecular cell factor, which stimulates the biosynthesis and release of collagenase and prostaglandins from macrophage-type synovial cells (Dayer et al, 1979). Corticosteroids indirectly lower both prostaglandins and collagenase levels by inhibitng the production and release of mononuclear cell factor (McGuire et al, 1981). Collagenase and other metallo-proteins are inhibited by the anti-proteinase TIMP (tissue inhibitor of metallo-proteinases). Corticosteroids stimulate TIMP synthesis in synovial cells (McGuire, Murphy, Reynolds and Russell, 1981). Dexamethasone was found to inhibit the plasminogen activator activities of both unstimulated and stimulated (mitogen) peripheral

blood mononuclear cell stimulated fibroblasts (Hamilton et al, 1981). Plasmin is generated after interaction of another neutral protease, plasminogen activator, with the substrate plasminogen. Plasmin can degrade cartilage (Lack and Rogers, 1958), and activate latent collagenase (Werb et al, 1977). Martel-Pelletier et al (1985) showed that steroids induced suppression of the active neutral metallo-proteoglycan degrading enzyme activity in the cartilage of rheumatic patients.

In this study, D-penicillamine showed a protection to the implanted cartilage and was found to have no anti-inflammatory effect. Maddox (1973) showed that penicillamine failed to suppress carrageenan-induced paw oedema. The same effect of penicillamine on inflammation was also observed in pertussis-induced paw oedema (Arrigoni-Martelli et al, 1976), pertussis-induced pleurisy (Dieppe et al, 1976). D-penicillamine also tends to improve the diminished neutrophil chemotaxis observed in rheumatoid arthritis (Lyle, 1983). D-penicillamine has been shown to diminish antigen-induced arthritis in the rabbit, as assessed by both measurement of joint circumference and histological examination (Hunneyball et al, 1977). Liyanage and Currey (1972) were unable to inhibit adjuvant-induced arthritis with a dose of D-penicillamine up to 200 mg/kg. Moreover, D-penicillamine has been shown to markedly inhibit the radiographic destruction of the joint and bone with concomitant lowering of acute phase protein levels in serum (Sloboda et al, 1981).

A number of observations in treated patients have suggested that D-penicillamine might exert an immuno-suppressive action and, thus, act by suppressing the on-going immune response that underlies the chronic inflammation (Lipsky, 1984). Direct confirmation of this conclusion will require assessment of T-lymphocyte function in treated patients. Therapy with D-penicillamine often results in decreased rheumatoid factor titers, decreased levels of circulating immune complexes (Jaffe, 1965) and diminished levels of serum immunoglobulins (Bluestone and Goldberg, 1973). Moreover, Hunneyball and his colleagues (1978) found that prolonged administration of D-penicillamine to rabbits could suppress both humoral immune response and delayed-type hypersensitivity.

In this study, levamisole was also shown to have nearly the same effects on inflammation and proteoglycan loss as those observed with D-penicillamine treatment. The activity of D-penicillamine in rheumatoid arthritis is similar to that effected by a number of other agents, including levamisole (Multicentre Study Group, 1978). However, while D-penicillamine showed some immunosuppressive activity, levamisole was found to be immunostimulant (Stecher and Carlson, 1983). However, a number of indirect clinical observations support this point of view. Huskisson in 1979, showed that levamisole was able to restore depressed cell-mediated immune parameters both <u>in vitro</u> and <u>in vivo</u> through effects on macrophage and lymphocyte populations. Levamisole

also stimulates precursor T-lymphocytes to differentiate into mature T-cells and restores to normal the depressed effector functions of peripheral T-lymphocytes and phagocytes (Symoens and Rosenthal, 1977). Levamisole does not directly stimulate B-cells, it does not increase their proliferative response to mitogens and it has no direct effect on antibody production (Symoens and Schuemmans, 1983).

From the experiments described here, it seems reasonable to attempt to form a working hypothesis as to their various modes of action. One thing is evident inflammation per se in the model used in this thesis appears not to induce proteoglycan loss from cartilage implants. It is conceivable that cartilage floats free in the exudate and does not sit in juxtaposition to macrophage and fibroblasts of the lining cells. This is possible since De Brito (umpublished results) has found that increasing granulation tissue around implanted cartilage enhances breakdown. This lends support to the key role of macrophage and fibroblast interaction.

Indomethacin is known to inhibit cyclo-oxygenase and block formation of prostaglandins. It reduces cell infiltration and volume of exudate. In this series of experiments it has been shown to protect femoral head cartilage from proteoglycan loss. This is probably independent of its anti-inflammatory activity, as reduction of inflammation could be supposed to enhance proteoglycan loss. It has previously been reported that indomethacin in granulomatous type inflammation will impair the function of normal macrophages. Di Rosa et al (1971) observed that macrophages

in vivo in animals treated with indomethacin, engaged in autophagocytosis and lysosomes could be seen to be fusing with mitochondria within the same macrophages. Possibly, the protection afforded by indomethacin may be attributed to impaired macrophage function. Thus, the macrophage could fail to secrete interleukin-1 to act on the fibroblasts and thus attack the cartilage.

When we turn to dexamethasone, this has a general inhibitory effect on cells. It inhibits movement and secretion of cellular products. It is interesting that this drug reduces the thickness of the pouch wall, suggesting that the integrity of the pouch wall is maintained by a proliferative process and not merely by migration of cells, as this effect was not observed with indomethacin, which would reduce cell migration. In the implant experiments described here, dexamethasone protected cartilage from proteoglycan loss. It seems likely that this phenomenon was brought about by a different mechanism to that postulated for indomethacin. Thus, dexamethasone, by reducing the thickness of the pouch wall, is reverting the pouch to an immature pouch, which has less reactivity and fewer macrophages and fibroblasts to interact. In addition, those cells that are present would be secreting fewer products. Once again, it is proposed that this is not related to the anti-inflammatory effect of the steroid on the exudate.

D-penicillamine would appear immediately to have separate mechanisms. It is evident that in the present experiments it potentiates the inflammatory response. We do know that in man, D-penicillamine lower acute phase proteins

(McConkey, 1976). In addition, Billingham (1983) has shown that in the rat, acute phase proteins move in direct correlation with interleukin-1, ie, acute phase proteins increase equally with a rise in interleukin-1, and conversely fall with an equal decrease.

It seems possible that this represents the key to the protective effect of D-penicillamine, namely a drop in interleukin-1 production by macrophages will fail to activate fibroblasts. This drug also supports the concept of the relative non-importance of inflammation in bringing about cartilage degradation.

Levamisole, which has been described as a disease modifying drug (Stecher, 1983), fails to exert a demonstrable effect on the inflammatory response, as shown in the air pouch models. Yet this drug protects cartilage against proteoglycan loss. Levamisole, like D-penicillamine, also causes a drop in acute phase proteins in man (McConkey, 1976). Thus, we could speculate a similar mode of action to that described for D-penicillamine. However, in addition to their properties, it has been suggested that levamisole can, under certain conditions, increase T-suppressor cell activity. It is interesting to recall that Rothwell and Spector (1972) showed that granulation requires T-cell participation, and neonatal thymectomy can block the formation of cotton pellet granulomas. This type of activity should be inhibitory towards granuloma formation, ie, interation of macrophages and fibroblasts.

The recurrent theme in this speculation is the interaction of macrophages and fibroblasts. Indeed, Leibovich and Ross (1975) have shown previously that wound healing

cannot proceed in the absence of macrophages and they suggest that macrophages occupy the key role in directing the fibroblasts to their appropriate function. Another support for this speculation comes from the work of Vaes (1980), who for many years has proposed the interaction of macrophages and fibroblasts to be all important in chronic inflammation. If this hypothesis is correct regarding the mode of action of these therapeutic agents, it could have some importance in the search for new compounds. Ideally, one would seek a compound that may have clear activity in acute phase proteins. Unfortunately, in the present work, drug action on acute phase protein was not pursued to fruition. The results obtained were variable and it was not deemed reasonable to repeat all these experiments. It now seems important to return to this aspect as soon as possible, and also to examine the effect of drugs on macrophage / fibroblast interaction. It is interesting that this hypothesis embraces interleukin-1. Dingle (1979) has suggested catabolin as the important factor in causing chondrocytes to destroy cartilage, as has been discussed earlier. Should interleukin-1 and catabolin be found to be identical, possibly in the experiments described in this thesis, catabolin may be important but not from the point of view held by the Cambridge group, ie, on chondrocytes but instead by its action on fibroblasts.

To conclude, this thesis describes the development of a novel <u>in vivo</u> system for examining soft tissue-hard tissue interaction. This system may be suitable for elucidating the

underlying mechanisms of arthritic bone and cartilage erosion and for detecting and evaluating new therapeutic agents.

R E F E R E N C E S

Ackerman, G.A. (1968), Lab. Invest. 19, 290.

Ackerman, N., Tomolonis, A., Miram, L., Kheifets, J.B., Martinez, S. and Carter, A. (1980), J. Pharmacol. Exp. Ther. 215, 588.

Addison, W. (1843) cited Jarcho, S. (1971), Am. J. Cardiol, 28, 223.

Adolph, M., Fontagne, J., Pelletier, M. and Giroud, J.P. (1975), Nature, 253, 637.

Allison, A.C., Harrington, J.S. and Birbeck, M. (1966), J. Exp. Med., 124, 141.

Ammendola, G., Di Rosa, M. and Sorrentino, L. (1975), Agents Actions, 5, 250.

Anderson, R., Glover, A. and Rabson, A.R. (1977), J. Immunol., 118, 1690.

Ansell, B.M. (1978), J. Rheum. 5, Suppl. 4.

Arnold, J., (1873) cited M. Rocha E. Silva (1978), In inflammation Ed. Vane, J.R., Ferreira, S.H., Springer-Verlag, Berlin.

Arrigoni-Martelli, E. (1982), Drugs of Today, 18, 461.

Arrigoni-Martelli, E.,Bramm, E., Huskisson, E.C., Willoughby, D.A. and Dieppe, P.A. (1976), Agents Actions, 6, 613.

Bainton, D.F. and Farquhar, M.G. (1968), J. Cell Biol. 39, pages 286 & 299.

Bakhle, Y.S. (1983), Br. Med. Bull., 39 (3), 214.

Ballonius, G. (Guillaume de Baillou), 1642, Libre de rheumatismo et Pleuritide dorsale, Paris, Quesnel.

Barada, F.A., O'Brien, W. and Horwitz, D.A. (1982), Arth. Rheum. 25, 10.

Barger, G. and Dale, H.H. (1910-1911) J. Physiol., 41, 499.

Barland, P., Novikoff, A.B. and Hamerman, D. (1962), J. Cell Biol., 14, 207.

Barnett, A.J. (1968), Cartilage In : Comprehensive Biochemistry, Vol. 26B, M. Florkin and E.H. Stotz (eds.) Elsevier, Amsterdam.

Barnett, C.H. (1956), J. Bone Joint Surg., 38(B), 567.

Barrett, A. (1981), In : Cellular interactions, J.T. Dingle and J.I. Gordon (eds.), pp.185, Elsevier/ North-Holland Biomedical press, Amsterdam.

Barrett, A. and Starkey, P.M. (1977), In : Rheumatoid arthritis, J.I. Gordon and B.I. Hazelman (eds.), pp.211, Elsevier/North-Holland Biomedical press, Amsterdam.

Baum, S.J., McVittie, T.J., Brandenburg, R.T., and Levin, S.G. (1978), Exp. Haematol., 6, 405.

Baur, E.A., Stricklin, G.P., Jeffrey, J.J. and Eisen, A.Z. (1975), Biochem. Biophys. Res. Commun., 64, 232.

Benitz, K.F. and Hall, L.M. (1963), Arch. Int. pharmacodyn., 144, No. 1-2.

Bergstrom, S., Duner, H., Von Euler, U.S., Pernow, B., and Sjovall, J. (1959), Acta Physiol. Scand., 45, 145.

Berry, H. (1983), In : Rheumatology and Rehabilitation, H. Berry, E. Hamilton and J. Goodwill (eds.), Croom Helm Publications, London, Canberra.

Berry, H. and Huskisson, E.C. (1976), In : Anti-inflammatory drugs, J.R. Vane and S.H. Ferreira (eds.) pp.404, Springer-Verlag, Berlin.

Bierman, H.R. (1964), Ann. N.Y. Acad, Sci. 113, 753.

Billingham, M.E.J. (1983), Pharmac. Ther., 22, 389.

Blackwell, G.J., Carnuccio, R., Di Rosa, M., Flower, R.J., Parente, L. and Persico, P. (1980), Nature, 287, 147.

Bloom, W. (1921),Bull. Johns Hopk. Hosp., 33, 185.

Bluestone, R. and Goldberg, L.S. (1973), Ann. Rheum. Dis., 32, 50.

Bornestein, D.L. (1982), Ann. N.Y. Acad. Sci. 389, 322.

Bouillaud, J.B. (1840), Traite clinique du rheumatisme articulaire et de la loi de coincidence des inflammations du coeur avec cette malaide. Paris : Dodsley.

Boyden, S.V. (1962), J. Exp. Med., 115, 453.

Brain, S.D., Williams, T.J., Tippins, H.R., Morris, H.R. and MacIntyre, I. (1985), Nature 313, 54.

Brown, K.L. and Cruess, R.L. (1982), J. Bone Joint Surgery, 64 (A), 70.

Buchanan, M.R., Vasquez, M.J. and Gimbrone, M.A. (1983), Blood, 62, 889.

Cantazaro, P.J., Schwartz, H.J., Graham, R.C. (1971), Am. J. Pathol., 64, 387.

Capasso, F., Dunn, C.J., Yamamoto, S. and Willoughby, D.A. (1975), J. Path., 116, 117.

Carnuccio, R., Di Rosa, M. and Persico, P. (1980), Br. J. Pharmac., 76, 186.

Castor, C.W. and Whitney, S.L. (1978), J. Lab. Clin. Med. 91, 811.

Cervini, C. (1977), Clin. Ter., 8, 495.

Chambers, R. and Zweifach, B.W. (1947), Physiol. Rev., 27, 436.

Clark, E.R., Clark, E.L. and Rex, R.O. (1936), Am. J. Anat., 59, 123.

Cochrane, C.G. and Muller-Eberhard, H.J. (1968), J. Exp. Med., 127, 371.

Cohnheim, J. (1867), Arch. Path. Anat., 40, 1.

Cole, B.C., Ward, J.R., Jones, R.S. and Cahill, J.F. (1971), Infect. Immunol., 4, 344.

Collier, H.O.J. (1958), In : 5-hydroxytryptamine, G.P. Lewis (ed.) pp.5., Pergamon Press, London.

Collier, H.O.J. (1969), Nature, 223, 35.

Commandon, J. (1919), Comp. Rend. Soc. Biol., 82, 1305.

Conner, N.S., Ford-Hutchinson, A.W. and Smith, M.J.H. (1980), In : Inflammation, mechanisms and treatment. D.A. Willoughby, and J.P. Giroud (eds.) MTP press.

Cooke, W.T., Fowler, D.I., Cox, E.V., Gaddie, R. and Maynell, M.J. (1958), Gastroen., 34, 910.

Cornely, H.P. (1966), Proc. Soc. Exp. Biol. Med. 122, 831.

Cotran, R.S. and Majno,G. (1964), Ann. N.Y. Acad. Sci., 116, 750.

Crunkhorn, P. and Willis, A.L. (1971), Brit. J. Pharmacol., 41, 49.

Dale, H.H. and Laidlaw, P.P. (1910), J. Physiol., 41, 318.

Dale, H.H. and Laidlaw, P.P. (1911), J. Physiol., 43, 182.

Dale, H.H. and Richards, A.N. (1918), J. Physiol., 52, 110.

Dale, H.H. and Laidlaw, P.P. (1918), J. Physiol., 52, 355.

Danon, A. and Assouline, G. (1978), Nature, 273, 552.

Davidson, E.M., Ford Hutchinson, A.W., Smith, M.J.H. and Walker, J.R. (1978), Brit. J. Pharmacol., 64 (3), 437P.

Dayer, J.M., Breard, J., Chess, L. and Krane, S.M. (1979), J. Clin. Invest., 64, 1386.

Dayer,J.M., Krane, S.M., Russell, R.C.G. and Robinson, D.R. (1976), Proc. Natl. Acad. Sci. USA., 73, 945.

Dayer, J.M., Robinson, D.R. and Krane, S.M. (1977), J. Exp. Med., 145, 1399.

Dayer, J.M., Russell, R.G.G. and Krane, S.M. (1977), Science, 195, 181.

Decker, R.S. and Dingle, J.T. (1982), Science, 215, 987.

Deshmukh-Phadke, K., Nanda, S. and Lee, K. (1980), Eur. J. Biochem., 104, 175.

De Vries, C.J.P., Haasnoot, J.P., Van Der Weij and Cats, A. (1982), Clin. Exp. Immunol., 47, 474.

Dieppe, P.A., Willoughby, D.A., Huskisson, E.C. and Arrigoni-Martelli, E. (1976), Agents and Actions, 6 (5), 618.

DiMartino, M.J. and Walz, D.T. (1977), Inflammation, 2, 131.

Dingle, J.T. (1962), Proc. Roy. Soc. Med., 55, 109.

Dingle, J.T. (1979), Ann. Rheum. Dis., 38, 201.

Dingle, J.T., Saklatvala, J., Humbry, R., Tyler, J., Fell, H.B. and Jubb, R. (1979), Biochem. J., 184, 177.

Di Pasquale, G., Rassaert, C., Richter, R., Welas, P., Gingold, J. and Singer, R. (1975), Agents Actions, 5, 256.

Di Rosa, M. (1972), J. Pharm. Pharmacol. 24, 89.

Di Rosa, M., Giroud, J.P. and Willoughby, D.A. (1971), J. Path., 104, 15.

Di Rosa, M., Papadimitriou, J.M. and Willoughby, D.A. (1971), J. Path., 105, 239.

Doyle, A.B., Hambleton, P., Miller, P. and Parker, F. (1983), Br. J. Pharmacol., 79, 350P.

Dumonde, D.C. and Glynn, L.E. (1962), Br. J. Exp. Path., 43, 373.

Dumonde, D.C., Wolstencroft, R.A., Panayi, G.A., Mathew, M., Morley, J. and Howson, W. (1969), Nature, 224, 38.

Duthie, E.S. and Chain, E. (1939), Brit. J. Exp. Path., 20, 417.

Ebert, R.H. (1965), In : The inflammatory process. B.W. Zweifach, L. Grant and R.T. McCluskey(eds.), Academic Press Inc., New York.

Ebert, R.H. and Florey, H.W. (1939), Brit. J. Exp. Path., 20, 342.

Edwards, J.C.W., Sedgwick, A.D. and Willoughby, D.A. (1981), J. Path., 134, 147.

Eichwald, (1864), Wiezb. Med. Zeitschr. 335 (As quoted by Hofmeister, 1880).

Elford, P.R., Richardson, H.J., Sharrard, R.M., Meats, J.E., Skjodt, R.A.D., Bunning, A., Crawford, A. and Russell, R.G.G. (1985), Br. J. Rheum., 24, (suppl. 1), 55.

El-Ghobarey, A.F., Mavrikakis, M.E., Macleod, M. (1978), Q. J. Med., 47, 385.

Elliott, D.F., Horton, E.W. and Lewis, G.P. (1960a), J. Physiol., 150, 6P.

Elliott, D.F., Horton, E.W. and Lewis, G.P. (1960b), Biochem. J., 74, 15P.

Empire Rheumatism Council, (1961), Ann. Rheum. Dis., 20, 315.

Erspamer, V. (1954), Pharmacol. Rev., 6, 425.

Everett, N.B. and Tylor, R.W. (1968), Biochem. Pharmacol., 17, 185.

Faltynek, C.R. and Silbert, J.E. (1978), J. Biol. Chem. 253, 6746.

Fantozzi, R., Brunelleschi, S., Campi, P., Blandina, E., Masini, E. and Mannaioni, P.F. (1984), Agents and Actions, 14, 441.

Farndale, R.W., Sayers, C.A. and Barrett, A.J. (1982), Conn. Tiss. Res., 9, 247.

Fassbender, H.G. (1975), In : Pathology of rheumatic diseases (Springer-Verlag, Berlin), pp.353.

Fell, H.B. and Jubb, R.W. (1977), Arthr. Rheum., 20, 1359.

Ferreira, S.H., Moncada, S. and Vane, J.R. (1971), Nature, (New Biol.), 231, 237.

Finkelstein, A.B., Roisman, F.R. and Walz, D.T. (1977), Inflammation, 2, 143.

Florey, L. (1970) : General Pathology, 4th edition, Publ. W.B. Saunders Company, Philadelphia & London.

Florey, H.W. (1925), Brain, 48, 43.

Flower, R.J. and Blackwell, G.J. (1979), Nature, 278, 456.

Flower, R.J. and Vane, J.R. (1974a), Biochem. Pharmacol. 23, 1439.

Flower, R.J. and Vane, J.R. (1974b), In :Prostaglandin biosynthesis inhibitors, pp.1, New York: Raven Press.

Foot, N.C. (1925), Anat. Rec., 30, 15.

Ford-Hutchinson, A.W., Bray, M.A. Doig, M.V., Shipley, M.E., and Smith, M.J.H. (1980), Nature (London), 286, 264.

Freedman, A. (1956), Ann. Rheum. Dis., 15, 251.

Fukuhara, M. and Tsurufuji, S. (1969), Biochem. Pharmacol., 18, 475.

Galen, C. (1821 - 33), Opera Omnia, Leipzg.

Gertzbein, S.D. and Lance, E.M. (1976), Clin. Exp. Immunol., 24, 102.

Gery, I., Gershon, R.K. and Waksman, B.H. (1971), J. Immunol., 107, 1778.

Gery, I., Gershon, R.K. and Waksman, B.H. (1972), J. Exp. Med., 136, 128.

Giroud, J.P., Fontagne, J., Blondelon, D., Adolphe, M., Dunn, C.J. and Willoughby, D.A. (1977), Biomedicine, 27, 19.

Giroud, J.P., Pelletier, M. and Girre, G. (1977), C.R. Acad. Sc. Paris. t 285, 1143.

Glenn, E.M. (1966), Am. J. Vet. Res., 27, 339.

Goetzl, E.J., Derian, C.K., Tauber, A.I. and Valone, F.H. (1980), Biochem. Biophys. Res. Comm., 94, 881.

Goetzl, E.J. and Sunn, F.F. (1979), J. Exp. Med., 150, 406.

Goetzel,E.J., Woods, J.M. and Gorman, R. (1977), J. Clin. Invest., 59, 179.

Goldstein, I.M., Rous, D. and Kaplan, H.B. (1975), J. Clin. Invest., 56, 1115.

Goldstein, J. (1978), J. Rheumatol., 5, suppl. 4, 5.

Goodwin, J.S. (1984), Am. J. Med., 77 (1A), 57.

Goud, T., Schotte, C. and Van Furth, R. (1975), J. Exp. Med., 142, 1180.

Gowen, M., Wood, D.D., Ihrie, E.J., McGuire, M.K.B. and Russell, R.G.G. (1983), Nature, 306, 378.

Cowen, M., Wood, D.D., Ihrie, E.J., Meats, J.E. and Russell, R.G.G. (1984), Biochem. Biophys. Acta, 797, 186.

Granstrom, E. (1984), Inflammation, 8, suppl., S15

Greenwald, R.A., Moy, W.W. and Lazarus, D. (1976), Arth. Rheum., 19, 799.

Gregory, J.D. (1973), Biochem. J., 133, 383.

Hadden, J.W., Sadlik, J.R. and Hadden, E.M. (1975), Nature, 257, 483.

Haeckel, E.H. (1862) cited in Vaughan, R.B. (1965), Medical Hist. 9, 201.

Hamberg, M. and Samuelsson, S. (1974), Proc. Nat. Acad. Sci. (Wash), 70, 899.

Hammerman, D. et al (1967), J. Exp. Med., 1126, 1005.

Harris, E.D., Jr. (1981) In : Textbook of rheumatology. W.N. Kelley;E.D., Jr. Harris; S. Ruddy and C.B. Sledge (eds.) pp.896. W.B. Saunders Co., Philadelphia.

Harth, M., Stiller, C.R., St. C. Sinclair, Evans, N.R., McGirr, D. and Zuberi, R. (1977), Clin. Exp. Immunol., 27, 357.

Hascall, V.C. (1981),In : Biology of carbohydrates. V. Ginsburg and P. Robbins (eds.) pp.1. Wiley interscience, New York.

Hauser, P. and Vaes, G. (1978), Biochem. J., 172, 275.

Heberden, W. (1802),Commentaries on the History and Cure of Disease. London : Payne.

Hemler, M.E. and Lands, E.M. (1980), J. Biol. Chem., 225, 6253.

Higgs, G.A., Eakins, K.E., Mugridge, K.G., Moncada, S. and Vane, J.R. (1980), Europ. J. Pharmacol. 66, 81.

Higgs, G.A., Flower, R.J. and Vane, J.R. (1979), Biochem. Pharm. 28, 1959.

Higgs, G.A., Moncada, S., Salmon, J.A. and Seager, K. (1983a), Br. J. Pharmacol., 79, 863.

Higgs, G.A., Moncada, S. and Vane, J.R. (1983b), In : Anti-rheumatic drugs, E. Huskisson (ed.), pp.11. Prager Scientific.

Higgs, G.A. and Mugridge, K.G. (1982), Br. J. Pharmacol., 76, 284P.

Higgs, G.A. and Vane, J.R. (1983), Br. Med. Bul.,39(3), 265.

Highton, T.C. (1963), Br. J. Exp. Path., 44, 137.

Hippocrates. The Genuine Works of Hippocrates. Translated from the Greek by Francis Adams (1849), London : Sydenham Society.

Hirata, F., Schiffman, E.,Venkatasubramanian, K., Salmon, D. and Axelrod, J. (1980), Proc. nat. Acad. Sci, U.S.A, 77, 2533.

Horton, E.W. (1963), Nature (London), 200 892.

Humphrey, J.H. and Jaques, R. (1954), J. Physiol,,124, 305.

Humphrey, D.M., McManus, L.M., Satouchi, K., Hanahan, D.J. and Pinckard, R.N. (1982), Lab. Invest., 46, 422.

Hunneyball, L.M. (1981), Agents Actions, 11(5), 490.

Hunneyball,I.M., Stewart, G.A. and Stanworth, D.R. (1977), Ann. Rheum. Dis., 36, 378.

Hunneyball, I.M., Stewart, G.A. and Stansworth, D.R. (1978), 35, 159.

Hunter, J. (1794), In "Treatise on the blood, inflammation and gun-shot wounds", London, Richardson.

Hurley, J.V., Ryan, G.B. and Friedman, A. (1966), J. Path. Bact., 91, 575.

Hurley, J.V. and Spector, W.G. (1961), J. Path. Bact., 82, 403.

Huskisson, E.C. (1979), Clin. Rheum. Dis., 5, 351.

Huskisson, E.C., Dieppe, P.A., Scott, J., Trapnell, J., Balme, H.W. and Willoughby, D.A. (1976), Lancet, 1, 393.

Huybrechts-Godin, G. and Vaes, G. (1978), FEBS Lett., 91, 242.

Ishikawa, H. and Ziff, M. (1976), Arth. Rheum., 19, 1.

Jaffe, I.A. (1963), Ann. Rheum. Dis., 22, 71.

Jaffe, I.A. (1965), Arthritis Rheum.,8, 1064.

Janoff, A. and Blondin, J. (1970), Proc. Soc. Exp. Biol. Med., 135, 302.

Johnston, J.B., Lehmeyer, J.E. and Guthrie, L.A. (1976), J. Exp. Med., 143, 1551.

Kaley, G. and Weiner, R. (1971), N.Y. Acad. Sci., 180, 338.

Kampschmidt, R.F. and Upchurch, H.F. (1977), Proc. Soc. Exp. Biol. Med., 155, 89.

Kaplan, H.B., Edelson, H.J., Korchak, H.M., Given, W.B., Abramson, S. and Weissmann, G. (1984), Biochem. Pharmacol., 33, 371.

Kaplan, A.P., Mayer, H.L. and Austen, K.F. (1972), J. Exp. Med., 135, 81.

Kaplan, L., Weiss, J. and Elsbach, P. (1978), Proc. Nat. Acad. Sci. U.S.A., 75, 2955.

Kelly, W.N., Harris, E.D., Ruddy, S. and Sledge, C.B. (1981), In : Textbook of Rheumatology, Philadelphia : J.W.B. Saunders, pp. 2504.

Kempson, G.E. et al (1976), Biochem. Biophys. Acta, 428, 741.

Kempson, G.E., Muir, H., Pollard, C. and Tuke, M. (1973), Biochem. Biophys. Acta, 297, 456.

Kempson, G.E., Muir, H., Swanson, S.A.V. and Freeman, M.A.R. (1970), Biochem. Biophys. Acta, 215, 70.

Ketchel, M.M. and Favour, C.B. (1955), J. Exp. Med., 135, 81.

Kimura, J.H., Hardingham, T.E. and Hascall, V.C. (1980), J. Biol. Chem., 225, 7134.

Kobayashi, I. and Ziff, M. (1975), Arth. Rheum., 18, 475.

Koh, M.S., Parente, L. and Willoughby, D.A. (1979), Eur. J. Rheum. Inflamm.,2(2), 87.

Krogh, A. (1920), J. Physiol.,53, 399.

Lack, C.H. and Rogers, H.J. (1958), Nature, 182, 948.

Lawrence, H.S. and Landy, M. (1960), Mediators of Cellular immunity, New York : Academic press.

Learner, L.J., Bianchi, A. and Turkheimer, A.R. (1964), Ann. N.Y. Acad. Sci., 116, 1071.

Leeson, C.R. and Leeson, T.S. (1976), Histology (3rd Edn.) pp.158, W.B. Saunders Co. London.

Lehmeyer, J.E. and Johnston, R.B. (1978), Clin. Immunol. Immunopath. 9, 482.

Leibovich, S.G. and Ross, R. (1975), In : Mononuclear phagocytes in immunity, infection and pathology, R. Van Furth (ed.), Blackwell Scientific publications, London, pp.347.

Lewis, G.P. (1961), Nature (London), 192, 596.

Lewis, T. (1927), "Blood vessels of the human skin and their responses". (Shaw and Sons, London).

Lewis, W.H. (1925-1926), Harvey Lectures, 21, 77.

Lippiello, L., Yamamoto, K., Robinson, D. and Mankin, H.J. (1978), Arth. Rheum., 21, 909.

Lipsky, P.E. (1984), Advances in inflammation research, 7, 175.

Liyanage, S.P. and Currey, H.L.F. (1972), Ann. Rheum., Dis., 31, 521.

Lombardino, J.G. (1983), Eur. J. Rheum., Inflam. 6, 24.

Lorber, A., Gifford, D.B., Chandor, S.B. and Carrol, P.L. Jr. (1974), Clin. Pharmacol. Ther. 15, (Abstr.): 211.

Luger, T.A., Charon, J.A., Colot, M., Micksche, M. and Oppenheim, J.J. (1983), J. Immunol., 131, 816.

Lyle, W.H. (1983), In : Anti-rheumatic drugs, E.C. Huskisson (ed.) pp.528, Prager Scientific.

MacGregor, R.R. (1976), Am. J. Med., 61, 597.

Maclagan, T.J. (1881), Rheumatism its Nature, its Pathology, and its successful Treatment. London : Pickering.

Maclagan, T.J. (1874), cited "Aspirin" Collier, H.O.J. (1963). Scientific American.

Maddox, I.S. (1973), Biochem. Biophys. Acta, 306, 74.

Majno, G. and Leventhal, M. (1967), Lancet, 2, 99.

Majno, G., Palade, G.E. and Schoeffl, G.I. (1961), J. Biophys. Biochem. Cytol., 11, 607.

Malemud, C.J. and Janoff, A. (1975), Arth. Rheum., 18, 361.

Mallory, F.B. (1898), J. Exp. Med., 3, 611.

Malseed, Z. and Heyner, S. (1976), Arth. Rheum., 19(2), 223.

Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965), Int. J. Immunochem., 2, 235.

Mariano, M. and Spector, W.G. (1974), J. Path., 113, 1.

Martel-Pelletier, J., Cloutier, J-M., Howell, D.S. and Pelletier, J-P. (1985), Arth. Rheum., 28(4), 405.

Massart, J. and Bordet, C. (1890), J. de Med. Chir. Pharmacol., 40, 169.

Maximow, A. (1927), Arch. Path., 4, 557.

McCall, E. and Yolton, L.J.F. (1974), Brit. J. Pharm., 52, 452.

McConkey, B. (1976), Agents Actions, 6, 593.

McGuire, M.K.B., Meats, J.E. and Ebsworth, N.M. (1982), In : Articular synovium. Int. symp. Bruges, pp.75, Krager, Basel.

McGuire, M.K.B., Meats, J.E., Ebsworth, N.M., Russell, R.G.G., Murphy, G. and Reynolds, J.J. (1981), In : Seminar in arthritis and Rheumatism, Vol. XI, Suppl. (1), D.S. Howell and J.H. Talbot (eds.) pp.138, Grune and Stratton Inc., New York.

McGuire, M.K., Murphy, G., Reynolds, J.J. and Russell, R.G.G. (1981), Clin. Sci., 61, 703.

McManus, L.M., Pinckard, R.N. Fitzpatrick, F.A., O'Rourke, R.A., Crawford, M.D., and Hanahan, D.J. (1981), Lab. Invest., 45, 303.

Meacock, S.C.R. and Kitchen, E.A. (1979), J. Pharm. Pharmacol., 31, 366.

Meats, J.E., McGuire, M.B. and Russell, R.G.G. (1980), Nature, 286, 891.

Meier, R., Schuler, W. and desaulles, P. (1950), Experientia (Basel), 16, 204.

Mellbye, O.J. and Munthe, E. (1977), Ann. Rheum. Dis. 36, 453.

Menkin, V. (1936), J. Exp. Med., 64, 485.

Menkin, V. (1938), J. Exp. Med., 67, 129.

Merryman, P. and Jaffe, I.A. (1978), Proc. Soc. Exp. Biol. Med., 157, 155.

Metchnikoff, E. (1882), cited in Pelner L. (1969), "Elie Metchnikoff PHD" N.Y. State J. of Med. 69, 2371.

Metchnikoff, E. (1883), cited in Vaughan, R.B. (1965), Medical Hist., 9, 201.

Metchnikoff, E. (1888), Arch. Path., 113, 63.

Mikami, T. and Miyasaka, K. (1983), Europ. J. Pharmacol., 95, 1.

Miles, A.A. and Wilhelm, D.L. (1955), Brit. J. Exp. Path., 36, 71.

Mitrovic, D. (1982), Europ. J. Rheuma.Inflam., 5, 3.

Mizel, S.B., Dayer, J.M., Krane, S.M. and Mergenhagen, S.E. (1981), Proc. Natl. Acad. Sci. USA., 78, 2474.

Mizel, S.B. and Farrar, J.J. (1979), Cell Immunol., 48, 433.

Multicentre Study Group (1978), Lancet ii, 1007.

Murphy, P.A., Simon, P.L. and Willoughby, W.F. (1980), J. Immunol., 124, 2498.

Nasjletti, A. and Malik, K.U. (1979), Life sci., 25, 99.

Nijkamp, F.P., Flower, R.J., Moncada, S. and Vane, J.R. (1976), Nature, 263, 479.

Ohuchi, K. Yoshino, S., Kanaoka, K., Tsurufuji, S. and Levine, L. (1982), Int. Arch. Allergy Appl. Immunol., 68, 326.

Opie, E.L. (1906), J. Exp. Med., 8, 410.

Opie, E.L. (1910), Arch. Intern. Med., 5, 541.

Opie, E.L. (1922), Physiol. Revs., 2, 552.

Oppenheim, J.J., Togawa, A. and Wahl, S. (1979), In : The cell biology and Immunology of leucocytes, M. Quastel (ed.), pp.499, New York, Pub Acad. Press, N.Y.

Orning, L., Hammarstrom, S. and Samuelsson, B. (1980), Proc. Nat. Acad. Sci., U.S.A., 77, 2014.

Page, A.R. and Good, R.A. (1958), Am. J. Pathol., 34, 645.

Panayi, G.S., Neill, W.A., Duthie, J.J.R. and McCormick, J.N. (1973), Ann. Rheum. Dis., 32, 316.

Pappenheimer, J.R. (1953), Physiol. Rev., 33, 387.

Parente, L., DiRosa, M., Flower, R.J., Ghiara, P., Meli, R., Persico, P., Salmon, J.A. and Wood, J.N. (1984), Eur. J. Pharmacol., 99, 233.

Parratt, J.R. and West, G.B. (1957a), J. Physiol., 137, 169.

Parratt, J.R. and West, G.B. (1957b), J. Physiol., 137, 179.

Paulus, H.E. and Whitehouse, M.W. (1973), Ann. Rev. Pharmacol., 13, 107.

Paz, R.A. and Spector, W.G. (1962), J. Path. Bact., 84, 85.

Pearson, C.M. (1956), Proc. Soc. Exp. Biol. (N.Y.), 91, 95.

Peeters-Joris, Ch., Emonds-Alt, X. and Vaes, G. (1981), Biochem. J., 196, 95.

Permar, H.H. (1924), Am. Rev. Tub., 60,(6).

Pfeffer, W. (1884), Untersuchungen aus dem Botanis-chen Institut Tubingen, 1, 363.

Phadke, K. and Nanda, S. (1982), Eur. J. Rheum. Inflam., 5, 170.

Pick, E., Krejci, J., Cech, K. and Turk, J.L. (1969), Immunology, 17, 741.

Polgar, P. and Taylor, L. (1980), Prostaglandins, 19, 693.

Pollock, S.H. and Brown, J.H. (1971), J. Pharm. Exp. Therapeutics, 178, 609.

Poole, A.R., Hembry, R.M., Dingle, J.T., Pinder, I., Ring, E.F.J. and Cosh, J. (1976), Arth. Rheum., 19, 1295.

Rainsford, K.D., Peskar, B.M. and Brune, K. (1980), J. Pharm. Pharmacol., 33, 127.

Ratnoff, O.D. and Lepow, I.H. (1963), J. Exp. Med., 118, 681.

Renkin, E.M., Carter, R.D. and Joyner, W.L. (1974), Microvasc. Res., 7, 49.

Ridge, S.C., Oronsky, A.L. and Kerwar, S.S. (1980), Arth. Rheum., 23, 448.

Rivkin, I., Foschi, G.V. and Rosen, C.H. (1976), Proc. Soc. Exp. Biol. Med., 153, 236.

Robert, A. and Nezamis, J.E. (1957), Acta. Endocrinology, 25, 105.

Robertson,W., Van, B. and Schwartz, B. (1953), J. Biol. Chem., 201, 689.

Robinson, B.V. (1982), J. Pharm. Pharmac., 34, 458.

Robinson, D.R., McGuire, M.B. and Levine, L. (1975), Ann. N.Y. Acad. Sci., 265, 318.

Rocha E. Silva, M., Beraldo, W.T. and Rosenfeld, G. (1949), Am. J. Physiol., 156, 261.

Rosenwasser, L.J., Dinarello, C.A., Rosenthal, A.S. (179), J. Exp. Med., 150, 709.

Roth, S.H. (1982), J. Rheumatol., 9, suppl. 8, 120.

Rous, P., Gilding, H.P. and Smith, F. (1930), J. Exp. Med., 51, 807.

Rowley, D.A. and Benditt, E.P. (1956), J. Exp. Med., 103, 399.

Rowley, D.A., Chutkow, J. and Attig, C. (1959), J. Exp. Med., 110, 751.

Rothwell, T.I. and Spector, W.G. (1972), J. Path., 108, 15.

Ryan, G.B. and Majno, G. (1977), Acute inflammation; A review. Am. J. Path., 86, 184.

Ryan, G,B. and Spector, W.G. (1970), Proc. Roy. Soc. Lond. B., 175, 269.

Sabin, F.R., Doan, C.A. and Cunningham, R.S. (1925), In : Contributions to Embryology, 16,(361), 125, Carnegie Inst., Wash., Pub.

Saklatvala, J., Curry, V.A. and Sarsfield, S. (1983), Biochem. J., 215, 385.

Saklatvala, J., Pilsworth, L.M.C., Sarsfield, S., Gavrilovic, J. and Heath, J.K. (1984), Biochem. J., 224, 461.

Salter, R.B., Simmonds, D.F., Malcolm, B.W., Rumble, E.J., MacMichael, D. and Clements, N. (1980), J. Bone Joint Surg., 62(A), 1232.

Sato, H., Hashimoto, M., Sugio, K., Ohuchi, K. and Tsurufuji, S. (1980), J. Pharm. Dyn., 3, 345.

Schmidt, J.A., Mizel, S.B. and Cchen, D. (1982), J. Immunol., 128, 2177.

Scott, R.B. and Still, W.J.S. (1968), J. Clin. Invest., 47, 353.

Sedgwick, A.D., Sin, Y.M., Edwards, J.C.W. and Willoughby, D.A. (1983), 141, 483.

Sedgwick, A.D., Sin, Y.M., Mackay, A.R., Al-Duaij, A.D. and Willoughby, D.A. (1984), J. Pharm. Pharmacol., 36, 171.

Selye, H. (1953), J. Amer. Med. Ass., 152, 1207.

Sheppeard, H., Pilsworth, L.M.C., Hazleman, B. and Dingle, J.T. (1982), Ann. Rheum. Dis., 41, 463.

Simoens, J. and Rosenthal, M. (1977), J. Reticuloendothel. Soc., 21, 175.

Simoens, J. and Schuermans, Y. (1983), In : Anti-rheumatic drugs, Huckisson, E.C. (ed.) Praeger Scientific (Publisher) pp.573.

Simon, T.M., Wilcox, S.A. and Lorber, A. (1977), In Vitro J. Tissue culture Assoc., 13, 159.

Sin, Y.M., Sedgwick, A.D. and Willoughby, D.A. (1984), J. Path., 142, 23.

Sloboda, A.E., Birnbaum, J.E., Oronsky, A.L. and Kerwer, S.S. (1981), Arth. Rheum., 24, 616.

Smith, K.A., Lachman, L.B., Oppenheim, J.J. (1980), J. Exp. Med., 151, 1551.

Smith, R.J. and Iden, S.S. (1980), Biochem. Pharmacol., 29, 2389.

Smith, J.B. and Willis, A.L. (1971), Nature (New Biol), 231, 235.

Snyderman, R., Shin, H.S., Phillips, J.K., Gerwurtz, H. and Mergenhagen, S.E. (1969), J. Immunol., 103, 413.

Sokoloff, L. (1984), In : Internaticnal review of experimental pathology, vol. 26, G.W. Richter and M.A. Epstein (eds.) Academic press publications.

Sollman, T. (1916), J. Pharmacol. Exp. Therp., 9, 391.

Sorgente, N., Kuettner, K.E., Soble, L.W. and Eisenstein, R. (1975), Lab. Invest., 32, 217.

Spector, W.G. (1951), J. Path. Bact., 63, 93.

Spector, W.G. (1956), J. Path. Bact., 72, 367.

Spector, W.G. (1969), Int. Rev. Exp. Path., 5, 79.

Spector, W.G. (1977), An introduction to general pathology. Churchill, Livingstone, New York.

Spector, W.G., Walters, M.N.I., and Willoughby, D.A. (1965), J. Path. Bact., 90, 181.

Spector, W.G., Walters, M.N.I. and Willoughby, D.A. (1965), J. Path. Bact., 90, 635.

Spector, W.G. and Willoughby, D.A. (1957), J. Path. Bact., 74, 57.

Spector, W.G. and Willoughby, D.A. (1968), J. Path. Bact., 96, 389.

Spector, W.G. and Willoughby, D.A. (1974), In : White cells in inflammation, C.G.V. Norman (ed.). MTP press.

Spector, W.G. and Wynne, K. (1975), In : Future trends in inflammation, II, D.A. Willoughby, J.P. Giroud and G.P. Velo (eds.) MTP press.

Starky, P.M., Barrett, A.J., Burleigh, M.C. (1977), Biochem. Biophys. Acta, 483, 386.

Starling, E.H. (1896), J. Physiol. (London) 19, 312.

Stecher, V.J. and Carlson, J.A. (1983), Ann. Rep. Med. chem., 18, 171.

Stecher, V.J., Liauw, H.L. and Chinea, G.C. (1981), Current Ther. Res., 30 suppl., 34.

Stone, E. (1763), In : Aspirin, H.O.J. Collier (ed.), 1963, Scientific American.

Stone, K.J., Mather, S.J., and Gibson, P.P. (1975), Prostaglandins, 10, 241.

Sultzer, B.M. (1968), Nature, 219, 1253.

Sutton, J.S. and Weiss, L. (1966), J. Cell Biol., 28, 303.

Sydenham, T. (1848) Works of Thomas Sydenham, M.D. Translated from the Latin edition of Dr. Greenhill, London : Sydenham society.

Sztein, M.B., Vogel, S.N. and Sipe, J.D. (1981), Cell Immunol., 63, 164.

Tam, S., Hong, S.-C.L. and Levine, L. (1977), J. Pharmac. Exp. Ther., 203, 162.

Teitz, C.C. and Chrisman, D.D. (1975), Clin Orthop., 108, 264.

Thomas, L. (1956), J. Exp. Med., 104, 245.

Thompson, J. and Van Furth, R. (1970), J. Exp. Med., 131, 429.

Tissot, M., Bonne, C., Martin, B., Solier, M. and Giroud, J.P. (1984), Agents Actions, 14, 76.

Trang, L.E. (1980), Semin. Arthr. Rheum., 9, 153.

Trentham, D.E., Dynesius, M.S., Rocklin, R.E. and David, J.R. (1978), N. Engl. J. Med., 299, 327.

Trentham, D.E. Townes, A.S., and Kang, A.H. (1977), J. Exp. Med., 146, 857.

Tsurufuji, S. (1979), Exp. Clin. Res., 5, 79.

Tsurufuji, S., Sato, H., Rak, Min, K. and Ohuci, K., (1978), J. Pharm. Dyn., 1, 8.

Tsurufuji, S., Yoshino, S. and Ohuchi, K. (1982), Int. Arch. Allerg. Appl. Immunol., 69, 189.

Turk, J.L. (1980), Delayed hyper sensitivity, 3rd edition. Research monographs in immunology, 1. Elsevier/North Holland, Amsterdam.

Turk, J.L. and Willoughby, D.A. (1978), In : Handbook of experimental pharmacology, C.V.R. Born; A. Farah; H. Herken and A.D. Welch (eds.), pp.231, Springer-Verlag, Berlin.

Turner, S.R., Campbell, J.A. and Lynn, W.S. (1975), J. Exp. Med., 141, 1437.

Van Furth, R., Diesselhoff-Den Dulk, M.M.C., Mattie, H. (1973), J. Exp. Med., 138, 1314.

Vaes, G. (1980). Agents Actions, 10 (6),474.

Vane, J.R. (1971), Nature, 231, 233.

Vane, J.R. (1974), In : Prostaglandin synthetase inhibitors, pp.155, New York : Raven Press.

Velo, G.P., Dunn, G.J., Timsit, J. and Willoughby, D.A. (1973), J. Path., 111, 149.

Vernon-Roberts, B. (1980), In : Mason and Curry's Clinical Rheumatology, Eds. Curry, H.L.F., Pitman Medical publications.

Virchow, R. (1871), Cellular pathologie, 4th Ed. Berlin, A. Hirschwald.

Virolainen, M. and Defendi, V. (1967), Wistar. Inst. symp. Monogr. 7, 67.

Volkman, A. and Gowans, J.L. (1965a), Brit. J. Exp. Path., 46, 50.

Volkman, A. and Gowans, J.L. (1965b), Brit. J. Exp. Path. 46, 62.

Wahl, L.M., Wahl, S.M., Mergenhagen, S.E. and Martin, G.R. (1974), Proc. Natl. Acad. Sci. USA, 71, 3598.

Walker, J.R. and Dawson, W. (1979), J. Pharm. Pharmacol., 31, 778.

Wallach, D. (1978), J. Biol. Chem., 253, 24.

Walz, D.T., DiMartino, M.J. and Misher, A. (1971), Ann. Rheum. Dis., 30, 303.

Wantnick, A.S., Gilchrist, H., Kearney, S. and Sabin, C. (1974), In : Future trends in inflammation, G.P. Velo; D.A. Willoughby, and J.P. Giroud (eds.), Padua Piccin Medical Books.

Ward, P.A., Cochrane, C.G., and Muller Eberhard, H.J. (1965), J. Exp. Med., 122, 327.

Watson, J.C. and Tielemans, L. (1971), J. Cell Biol., 49, 817.

Weissman, G. (1983), Semin. Arthr. Rheum., 13, (1 Suppl. 1), 123.

Werb, Z. and Burleigh, M.C. (1974), Biochem. J. 137, 373.

Werb, Z., Mainardi, M.D., Vater, C.A. and Harris, E.D. Jr. (1977), N. Eng. J. Med., 296, 1017.

Wetzel, B.K., Spicer, S.S. and Horn, R.G. (1967), J. Histochem. cytochem. 15, 311.

Wilhelm, D.L. and Mason, B. (1958), Br. J. Exp. Path., 41, 487.

Wilhelm, D.L., Miles, A.A. and Mackay, M.E. (1955), Br. J. Exp. Path., 36, 82.

Willis, A.L. (1969), J. Pharm. Pharmacol., 21, 126.

Williams, T.J., Jose, P.J., Forrest, M.J., Wedmore, C.V. and Clough, G.F. (1984), KROC Found Ser., 16, 195.

Williams, T.J. and Morley, J. (1973), Nature, 246, 215.

Williams, T.J. and Morley, J. (1974), Brit. J. Exp. Path., 55, 1.

Willoughby, D.A. (1966), J. Path., 92, 139.

Willoughby, D.A. (1968), J. Path. Bact., 96, 381.

Willoughby, D.A., Polak, L. and Turk, J.L. (1968), Nature, (London), 219, 192.

Willoughby, D.A. and Spector, W.G. (1964), Ann. N.Y. Acad. Sci., 116, 874.

Winder, C.V., Wax, J., Burr, V., Been, M. and Rosiere, C.E. (1958), Arch. Int. Pharmacodyn., 116, 261.

Winter, C.A., Risley, E.A. and Nuss, G.W. (1962), Proc. soc. exp. Biol. (N.Y.), 111, 544.

Wood, D.D. (1983), In : The reticuloendothelial system : A comprehensive treatise, J.A. Bellanti and H.B. Herscowitz (eds.), Plenum Publications.

Woolley,D.E., Crossley, M.J. and Evanson, J.M. (1977), Arth. Rheum., 20, 1231.

Wynne, K.M., Dieppe, P.A., Scott, J. and Huskisson, E.C. (1981), Ann. Rheum. Dis., 40, 382.

Yamamoto, Y. and Onoue, K. (1979), J. Immunol., 122, 924.

Yoshino, S., Bacon, P.A., Blake, D.R., Scott, D.L., Wainwright, A.C. and Walton, K.W. (1984), Br. J. Exp. path., 65, 201.

Yoshiyoka, Y., Tsuru, S., Yokoyama, M. and Nomoto, K. (1982), Int. Archs. Allergy Appl. Immun., 68, 152.

Yu, D.T.Y. and Peter, J.B. (1974), Arth. Rheum., 4, 25.

Ziff, M. (1973), Fed. Proc., 32, 131.

Ziff, M. (1980), In : Inflammation, mechanisms and treatment, D.A. Willoughby and J.P. Giroud (eds.) pp.47, MTP Press.

Zurier, R.B. and Ballas, M. (1973), Arth. Rheum., 16, 251.

Zvaifler, N.J. (1971), Modern Treatment, 8, 769.

Zweifach, B.W. (1953), In : The mechanism of inflammation, G. Jasmin and B. Robert (eds.) pp.77, Acta. Inc. Montrol.

Zweifach, B.W. (1966), In : Hypotensive polypeptides, E.G. Erdos.,N. Back and F. Sicuten (eds.) Sprenger Varlag, Berlin.