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B AND T-CELL AUTOANTIGENS IN PRISTANE-INDUCED ARTHRITIS

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Short Title: Autoantigens in Pristane-Induced Arthritis

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SUMMARY

Pristane-induced arthritis (PIA) is a murine disease resembling of rheumatoid arthritis (RA) which is characterised by autoimmune responses to joint tissues. To identify the range of potential antigens targeted in PIA, proteins from arthritic or normal joint extracts were fractionated by SDS-PAGE and systematically screened for the ability to react with either serum IgG, or cultured splenic T-cells, obtained from healthy or arthritic mice. Extracts from both normal and arthritic animals contained multiple proteins that were capable of reacting with murine serum IgG in immunoblotting experiments. In healthy controls, more bands were identified in extracts prepared from 30-week old mice than from 8-week old animals, but the widest range of proteins bound were derived from arthritic joints. Furthermore, the sera from PIA-positive mice reacted with more bands from each of the extracts than did normal sera. Fractionated extracts prepared from healthy joints failed to stimulate the *in vitro* proliferation of splenic T-cells from either normal or arthritic animals. When arthritic joint components were screened, T-cells from healthy mice responded weakly to some fractions, but multiple fractions elicited strong proliferation by T-cells from mice with PIA. A band of apparent molecular mass 60kDa was the protein most commonly bound by serum IgG from arthritic mice, and the corresponding fraction stimulated the highest responses by T-cells from PIA-positive animals. These results are consistent with the notion that the 60kDa mammalian heat shock protein is an important antigen in PIA, but that the autoimmune response diversifies with the development of arthritis to target multiple joint components.

INTRODUCTION

The characterisation of target antigens in autoimmune diseases is an important step towards understanding the aetiology of this group of conditions, and in designing specific immunotherapeutic regimes. Although it is now widely accepted that rheumatoid arthritis (RA) is an autoimmune disease [1] the identity of the target autoantigen(s) in the joints remains uncertain. One disease resembling RA which has proved fruitful in the search for candidate autoantigens is pristane-induced arthritis (PIA). PIA, induced by the intraperitoneal injection of mice with the non-antigenic mineral oil 2,6,10,14-tetramethylpentadecane (pristane), shares many histopathological and serological features with rheumatoid arthritis [2-5], and has been demonstrated to be immunologically mediated [2,3,6,7]. To date, attention has focused on the hypothesis that PIA results from the stimulation of T-cells cross-reactive between bacterial heat shock proteins (hsp) and their highly conserved mammalian homologues [7,8]. This proposal is supported by the findings that the development of PIA is associated with increased levels of antibodies, and heightened *in vitro* T-cell responses, against mycobacterial hsp65 and mammalian hsp60 [7,9,10], and that the level of expression of hsp60 in joints parallels susceptibility to arthritis [8]. Furthermore, preimmunisation of mice with hsp65 has been shown to prevent PIA [7] perhaps by limiting the hsp responsive T-cell repertoire to bacterial specific epitopes [9,10]. However, although these results strongly suggest that mammalian hsp60 is an important target autoantigen in PIA, there is no direct evidence as to whether hsp60 from joints or other joint antigens also play a role in the development of the disease. The view that responses to multiple targets in the target tissue may be necessary for disease to develop is supported both by the wide range of candidate autoantigens implicated in the pathogenesis of RA [11-14], and by the identification of multiple autoantigens in other autoimmune diseases, such as autoimmune haemolytic anaemia (AIHA) [15,16] and diabetes [17-20]. The aim of the current work was therefore to determine whether the autoimmune response in PIA is focused solely on hsp, or whether other joint antigens are also targeted. The approach was systematically to screen fractionated murine joint proteins for reactivity with autoantibodies or autoreactive T-cells from normal mice or animals with PIA.

MATERIALS AND METHODS

Animals

Male CBA/Ig[b] mice aged between 8-25 weeks were used in the study. These animals were originally a gift from Professor H.S.Micklem, Department of Zoology, Edinburgh, UK.

Pristane-Induced Arthritis (PIA)

Arthritis was induced in mice by two i.p. injections of pristane (Aldrich Chemical Co., Gillingham, Dorset, UK) 50 days apart. The incidence of arthritis in the ankle joints was determined by visual assessment as previously reported [2,7].

Preparation of Joint Extracts

Stifle (knee) joints were dissected from freshly sacrificed mice and homogenised individually in SDS-PAGE gel sample buffer containing 8M urea and 5% v/v 2-mercaptoethanol. After low speed centrifugation, any insoluble material was discarded, and the extracts were stored at -20°C until use.

SDS-PAGE

SDS-PAGE was performed in 12% polyacrylamide gels according to the method of Laemmli (1970) using the Mini Protean II (Bio-Rad, Hemel Hempstead, UK).   Samples were analysed at a concentration of approximately 2μg protein per lane. Apparent molecular masses were calculated from migration relative to the standard protein markers (SDS-6H, Sigma, Poole, Dorset, UK).

Silver Staining of Polyacrylamide Gels

Gels were stained for protein as previously described [21].

Immunoblotting to Detect Joint Proteins Reactive with Murine Serum IgG

Joint extracts fractionated by SDS-PAGE were transferred to nitrocellulose membranes by Western blotting using a semi-dry apparatus (Pharmacia LKB, Uppsala, Sweden) with a

continuous buffer system as described by the manufacturer. Blots were blocked overnight at 4°C

in PBS containing 5% w/v bovine albumin (PBS-BSA). The membranes were successively

incubated with murine sera and peroxidase conjugated sheep antibody specific to murine IgG

(Sigma), diluted 1/50 and 1/1000 respectively.   Both reagents were made up in PBS-BSA and

each incubation lasted for 1 hour at 37°C, followed by 6x5 minute washes in PBS containing

0.05% Tween 20 (PBS/Tween) A luminographic technique was used to develop the blots [22].

Briefly, membranes were soaked in luminescent substrate solution, sandwiched between polythene

sheets, and exposed to film in an X-ray cassette without intensifying screens.

Preparation of Blotted Joint Antigens for T-cell Proliferation Assay

Blotted antigens were prepared for stimulation of T-cells cultures using a modification of a

technique described previously [15,16]. Briefly, joint extracts containing approximately 300μg

protein were analysed by SDS-PAGE and blotted onto nitrocellulose membranes under aseptic

conditions. The blots were extensively washed before the outer lanes were removed and stained

with Ponceau S (Sigma) to enable the protein bands corresponding to particular fractions to be cut

from the membrane in strips. Cultures were stimulated by placing 100mm^2 strips on the bottom of

wells.

T-cell Proliferation Assay

T-cell proliferation *in vitro* was measured by the method previously described [23]. T-cells were

obtained aseptically from individual mice by panning single cell suspensions of macerated spleens

over plastic Petri dishes coated with mouse IgG/rabbit anti-mouse IgG immune complexes. A

purity of >85% was achieved as assessed by anti-Thy 1.2 staining and flow cytometry (FACScan,

Becton Dickinson Ltd., Oxford, GB). Unselected spleen cells, irradiated with 1000 rads

(Gravatom Industries caesium source, Gosport, GB) to prevent their division, were used as the

source of syngeneic antigen presenting cells (APC). T-cells and APC were each cultured together

in 2ml wells at 1.25 x 10^6 cells/ml, in the presence or absence of antigen, using the alpha

modification of Eagle's medium (Flow) supplemented with 4mM L-glutamine (Flow), 100U/ml

benzyl penicillin (Glaxo Ltd., Greenford, GB), 100μg/ml streptomycin sulphate (Evans Medical

Ltd., Greenford, GB), 5×10^{-5}M 2-mercaptoethanol (Sigma), 20 mM HEPES and 0.5% fresh normal mouse serum. All cultures were incubated at 37°c in a humidified atmosphere of 5% CO_2 and 95% air. Proliferation was estimated from the incorporation of tritiated (^{3}H) thymidine (Amersham International Ltd., Amersham, UK) in triplicate 100μl samples withdrawn from the wells over the period 4-7 days after stimulation, using a 1450 Microbeta Liquid Scintillation Counter (LKB Wallac). All results are expressed as delta counts per minute (CPM), representing the mean CPM of triplicate samples minus the mean CPM in unstimulated control cultures.

RESULTS

SDS-PAGE Analysis of Joint Extracts

The proteins present in murine knee joint extracts were analysed by SDS-PAGE. A typical gel is depicted in Figure 1, Lanes A-C. It can be seen that the extraction technique resulted in the solubilization of multiple proteins from joints, ranging from over 200kDa to less than 29kDa in apparent molecular mass. There were no consistent differences between the banding patterns obtained using either normal mice aged 8 (Lane A) or 30 (Lane B) weeks, or 30 week old arthritic animals (Lane C). Note that although none of the major stained bands corresponds to the molecular mass of the 60kDa mamalian hsp, this protein has previously been demonstrated in similar joint extracts by immunochemical techniques [8].

Detection of Joint Autoantigens Reactive with Serum IgG by Immunoblotting

Joint extracts obtained from 30 week old arthritic, age-matched normal or 8 week old normal mice were screened in immunoblotting experiments to identify protein bands that reacted with serum IgG from individual animals in each of the 3 groups. Examples of the results, obtained by probing extracts from arthritic joints with a serum from each group, are shown in Figure 1 (Lanes D-F), and illustrate that more than one protein band was reactive with IgG from typical sera. Figure 2 summarises the data from the experiments testing all 3 types of extract against the complete panels of individual sera. It is evident that multiple proteins from each of the extracts were capable of

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binding serum IgG, but that the number of reactive bands from the joints increased with age in normal animals and was highest in mice with PIA.

It can also be seen from Figure 2 that the patterns of the bands detected varied with the source of the sera used to probe the blots. Thus, the sera from 8 week old mice reacted with fewest protein bands from each of the extracts, whilst more bands were detected by sera from older animals, and the sera of PIA cases showed the widest spectrum of reactivity. In addition, the band profile recognised varied between sera from different individuals within each group, raising the question of which proteins are most frequently bound by serum IgG. Figure 2 shows that a band with an apparent molecular mass of 60kDa, which was present in extracts from the joints of all 3 groups of mice, was recognised by sera from the majority of animals.

Stimulation of T-cells by Fractionated Joint Autoantigens

Fractionated joint extracts from normal or arthritic mice were tested for the ability to stimulate the *in vitro* proliferation of splenic T-cells from healthy or PIA-positive animals. Typical results (n=3) are shown in Figure 3, which demonstrates that extracts from normal animals failed to provoke responses by T-cells from mice in either group. It can also be seen that, when proteins from the joints of arthritic mice were screened, a minority of the fractions stimulated weak proliferation by normal murine T-cells. In contrast, multiple fractions from arthritic joints elicited strong responses by T-cells from PIA-positive individuals: T-cells from arthritic mice responded to proteins of 20-30kDa, 30-40kDa, 40-50kDa, 50-80kDa and 80-175kDa, with the greatest proliferation being stimulated by the 50-80kDa fraction.

DISCUSSION

The main finding reported here is that B and T-cells respond to a complex range of joint proteins in PIA. Similar diversity in the identities of target antigens has previously been described in models of other autoimmune diseases such as AIHA [15,16] and diabetes [17,18,20]. The results indicate that at least 2 distinct processes are responsible for such complexity. First, the range of potential autoantigens expressed in the joints appears to increase with age, and also with the development of PIA, since immunoblotted joint extracts from older normal mice contained more serum IgG-reactive bands than extracts from younger animals, and the highest number of bands were detected in blots from arthritic joints. The finding that fractionated extracts from the joints of arthritic, but not normal, mice stimulated the proliferation of murine splenic T-cells *in vitro* indicates that changes in the levels of putative B-cell autoantigens associated with the onset of PIA are also paralleled by increases in the expression of autoreactive T-cell epitopes. Further evidence that the expression of joint autoantigens is increased in PIA is provided by our previous study on the levels hsp60 [8]. We reported that hsp60, which is strongly implicated as an important target for autoreactive B and T-cells, is present at higher concentration in joints from mice with arthritis than from normal animals [8].

The second process responsible for the complex pattern of joint antigens in PIA is that the specificities of the autoimmune response broaden with age and the onset of disease. Thus, serum IgG binds to more immunoblotted protein bands from each of the joint extracts when the serum is obtained from arthritic rather than healthy animals, and the spectrum of proteins bound by normal sera widens as the mice grow older. The variation between the band patterns reactive with individual sera indicates that the bands represent specific autoantigens recognised by IgG autoantibodies. The results also show that, compared to normal splenic T-cells, T-cells from animal with PIA respond *in vitro* more strongly to a wider range of arthritic joint fractions; a phenomenon which may be similar to the diversification of the autoimmune T-cell response to several pancreatic islet antigens that follows the onset of disease in the non-obese diabetic mouse. It is envisaged that initial tissue damage leading to increased antigen availability and presentation are crucial for the observed increase in T cell recognition [17,18].

Given that the autoimmune responses in PIA are specific for a wide range of joint antigens, the question arises as to which of these potential targets is/are important in the pathogenesis of the arthritis. It could be argued that there may be only one dominant, disease inciting autoantigen, and that the stimulation of B and T-cells responsive to other joint components is merely a consequence of the inflammatory process and irrelevant to the development of arthritis. In support of this contention, evidence reported elsewhere [7-10] strongly suggests that hsp60 may be the key antigen in PIA. The current work is also consistent with such a role for hsp60, since a band with an apparent molecular mass of 60kDa was the immunoblotted joint protein most commonly recognised by sera from arthritic mice, and the strongest *in vitro* proliferation of T-cells from PIA-positive animals was elicited by the 50-80kDa fraction of arthritic joint proteins. An alternative point of view is that, rather than only a minority of potential autoantigens being relevant to PIA, the development of disease requires autoimmune responses to multiple targets in the joints. A similar mechanism, involving B and T-cell responses to a range of autoantigens has also been suggested in AIHA [15]. However, it would be expected that all autoantigens capable of initiating PIA are present in normal, as well as arthritic, joints and are targeted by immune responses in most, if not all, affected animals. Crucially, these expectations are fulfilled by only a minority of the potential autoantigens identified in the present study. Overall, we propose that the alternative hypotheses outlined above are not mutually exclusive, and that whilst the induction of PIA is dependent on autoimmune responses to dominant antigens such as hsp60, the expression of a wide range of other autoantigens in arthritic joints may be important for the perpetuation and exacerbation of disease. This proposition is supported by the finding that, despite the apparent immunodominant role of hsp60 in PIA, the development of disease can be prevented in a proportion of animals by the induction of oral tolerance to type II collagen [24].

Another question raised by the results is whether the joint destruction in PIA can be directly attributed to either the autoantibodies or the autoreactive T-cells that were detected in arthritic animals. It has been clear for some time that PIA is an immune-mediated disease [2,3,6,7], and it has now been demonstrated that CD4 T-cells play a pivotal role in the pathogenesis of the arthritis [25]. However, the importance of antibodies reactive with joint autoantigens has yet to be ascertained. Recent work has also indicated that the quality of the autoimmune responses to joint

antigens may be a key factor in the determining whether mice develop PIA Thus, immunity hsp in arthritic animals is biased to a T-helper (Th) 1 dominated response, characterised by predominantly IgG2a autoantibodies and γ-interferon production by antigen-stimulated T-cells *in vitro* [26, Beech et al manuscript in prep.], whilst the corresponding reaction in PIA-resistant mice is deviated towards a Th2 response, with higher levels of IgG1 antibodies and increased interleukin-4 & interleukin-5 secretion by cultured T-cells [26, Beech et al manuscript in prep.].

  Having shown that autoimmune responses to a wide range of targets other than hsp60 can be detected in PIA, future work will concentrate on further characterising these additional joint antigens. Many clearly represent minor components, since variations in their expression were not paralleled by any changes in the intensities of silver-stained protein bands after electrophoretic analysis of joint extracts. Identification of these components will allow their role in PIA to be clarified, by determining whether either the induction of specific tolerance, or the deviation of the immune response, can influence the development or progression of PIA. Such elucidation of the mechanisms involved in the pathogenesis of the experimental arthritides may be crucial to the eventual development of specific immunotherapy in human RA.

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Figure 1 SDS-PAGE of murine joint extracts. Material prepared from normal animals aged 8 (Lanes A) or 30 (Lanes B) weeks, or from arthritic mice (Lanes C-F) were analysed. Lanes A-C were developed with a silver protein stain. Bands from Lanes D-F that were reactive with serum IgG were developed by immunoblotting. Immunoblots were probed with sera from individual normal animals aged 8 (Lanes D) or 30 (Lanes E) weeks, or from an arthritic mouse (Lanes F). The position and molecular mass of the standard protein markers is indicated for each gel.
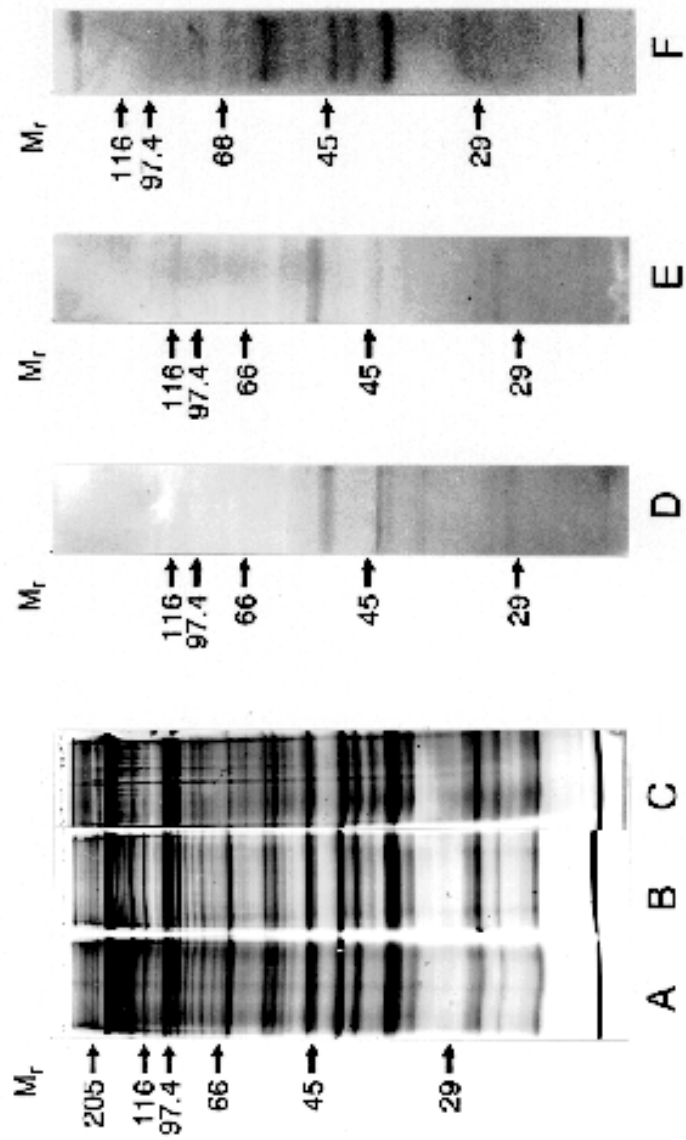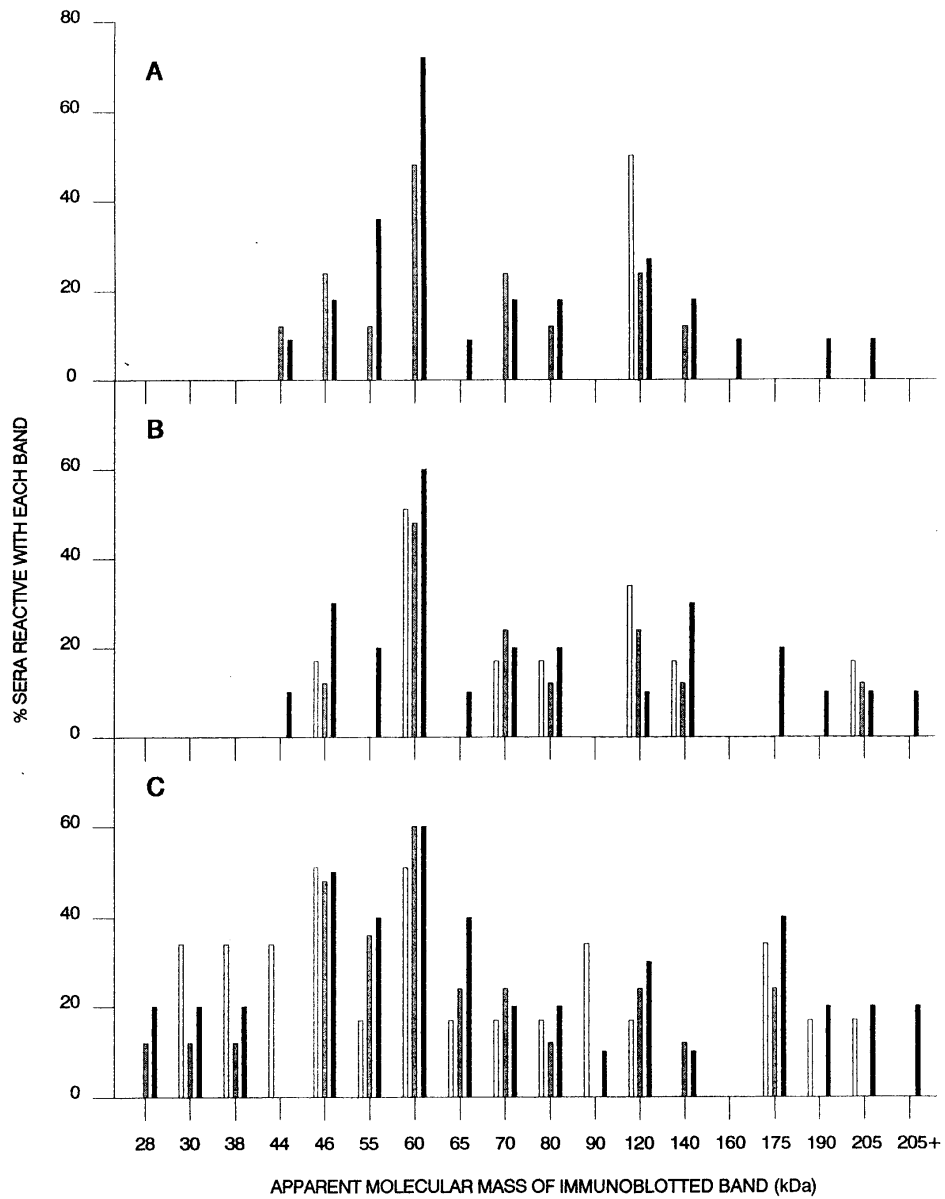
Figure 1

Figure 2

Figure 2 Reactivity of IgG in murine sera with immunoblotted joint extracts prepared from normal animals aged 8 (A) or 30 (B) weeks, or from arthritic mice (C). Sera used to probe the extracts were obtained from individual normal animals aged 8 (open square) or 30 (crossed square) weeks, or form arthritic mice (solid square). Each extract was probed with at least 17 individual sera.
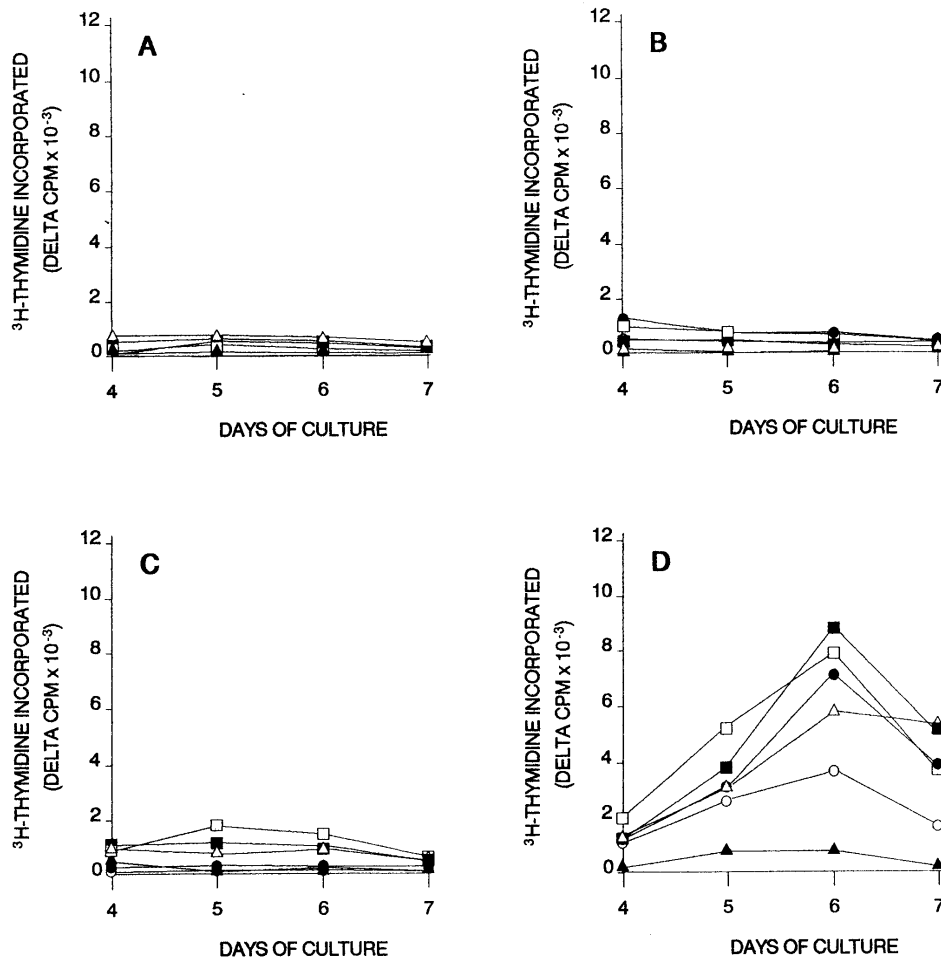
Figure 3

Figure 3

Proliferative responses of splenic T-cells from normal animals aged 8 weeks (A & C) or arthritic

mice (B & D) stimulated with joint fractions prepared from normal animals aged 8 weeks (A & B)

arthritic mice (C & D). The joint fractions used to stimulate cultures were 20-30kDa (open

circle), 30-40kDa (closed circle), 40-50kDa (open square), 50-80 kDa (closed square), 80-175

kDa (open triangle) or 175+kDa (closed traingle). In all experiments the background cpm was <

500 and variation of replicates was always less than 15%.