Clin Exp Immunol 1995; **101**:414–421

Antigen-specific B cells present cartilage proteoglycan (aggrecan) to an autoreactive T cell hybridoma derived from a mouse with proteoglycan-induced arthritis

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(Accepted for publication 3 May 1995)

SUMMARY

Cartilage proteoglycan (aggrecan)-induced polyarthritis in BALB/c mice is characterized by chronic inflammation and destruction of joint tissues similar to that observed in human rheumatoid arthritis. The immunization of mice with fetal human proteoglycan (PG) elicits specific antibodies to the immunizing antigen of which a population cross-reacts with native mouse PG. This (auto)antibody production is immediately followed by an explosive proliferation of autoreactive T cells, suggesting that PG-specific B cells may participate in antigen presentation of PG to autoreactive T cells. We therefore isolated B cells from the spleens and lymph nodes of PG-immunized mice and examined their ability to present PG to a PG-specific T cell hybridoma. The antigen-specific T cell responses elicited by B cells from PG-immunized mice (both arthritic and clinically asymptomatic) were markedly higher than those of non-immune mice and keyhole limpet haemocyanin (KLH)-immunized mice, and these B cells could present low PG concentrations. Levels of B cell presentation corresponded with the serum levels of PG-specific antibodies, implying that these B cells were presenting the PG specifically via their surface immunoglobulin. This B cell–T cell interaction was strongly dependent on MHC class II/T cell receptor (TCR), LFA-1/intercellular adhesion molecule-1 (ICAM-1) and CD28/B7 interactions, as antibodies to Ia, ICAM-1 and B7-2 (but not to B7-1) markedly reduced presentation. These data indicate that PG-specific B cells may play an essential role in governing the development of PG-induced arthritis.

Keywords proteoglycan arthritis animal model B cells T hybridoma antigen presentation

INTRODUCTION

Proteoglycan (aggrecan)-induced progressive polyarthritis in BALB/c mice shows many similarities to human rheumatoid arthritis (RA) [1–4], as indicated by clinical assessments, immunological tests and histopathology of joints. The development of arthritis in BALB/c mice, initiated by i.p. injection with glycosaminoglycan-depleted human fetal cartilage proteoglycan (HFPG), is associated with the expression of both cell-mediated immunity and autoantibody production to the mouse 'self' cartilage PG [1,2]. This is most probably due to cross-reactive epitopes which are present in both mouse and human fetal PG.

We have generated PG-specific T cell hybridomas from mice with PG-induced arthritis [5,6] and used them as tools for studying how PG are presented to T cells by antigen-presenting cells (APC). It was shown previously that macrophages, synovial cells and chondrocytes were effective presenters of PG to these T cell hybridomas, and hence these APC may play important roles in the generation of autoreactive T cells in PG-immunized mice [7]. As the autoantibodies to mouse PG appear before the generation of mouse PG-specific T cell responses [8], and the proliferation of $CD45R^+$ B cells appeared before an increase in $CD4^+$ T helper cell numbers [8,9], we hypothesized that perhaps PG-specific B cells were also involved in the priming of autoreactive T cells [10] and the subsequent development of arthritis. T cell–B cell cooperation has been shown to be important for the development of a number of autoimmune diseases [11,12].

To test this hypothesis, we examined the ability of B cells from arthritic, PG-immunized and non-arthritic and normal mice to present PG to the PG-specific T cell hybridoma, 5/4E8, which induces arthritis when injected into irradiated BALB/c mice [5]. It was shown that only B cells from immunized mice

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(both arthritic and non-arthritic) but not from normal mice could present PG to the T cell hybridoma. Furthermore, this T cell–B cell interaction was inhibited by antibodies to both B7-2 and intercellular adhesion molecule-1 (ICAM-1), whence immunotherapy of arthritic mice with these antibodies may provide a useful therapeutic approach in preventing joint destruction in mice with PG-induced arthritis.

MATERIALS AND METHODS

Preparation of PG antigens

PG from human adult cartilage (HAC) (42–52 years age range), from femoral condyles of fetal human (34–40 weeks of gestation), bovine nasal cartilage (BNC), chick sternal cartilage (CSC), rat chondrosarcoma, dog articular cartilage and femoral condyles of newborn BALB/c mice were prepared as described [13]. Cartilage pieces were frozen immediately and 50-μm cryostat sections were extracted at 4°C with 4 M guanidium chloride in 50 mM sodium acetate pH 5·8, containing a cocktail of protease inhibitors as described [13]. High buoyant density PG monomers (aggrecan) were prepared by dissociative caesium chloride gradient centrifugation. PG was digested with protease-free chondroitinase ABC (Seikagaku America, Inc., Rockville, MD) as described [13]. Cyanogen bromide (CNBr) digestion of HFPG was carried out as described previously [7]. Samples were dialysed against distilled water, lyophilized and sterilized by gamma-irradiation (250 Gy, caesium source).

Immunization of BALB/c mice

Female BALB/c mice (Charles River Colony) weighing 17–18 g were used in all experiments and were injected intraperitoneally with 100 μg of chondroitinase ABC-digested (i) arthritogenic HFPG, or (ii) non-arthritogenic CSC- or BNC-PG (measured as protein) in Freund's complete adjuvant (FCA; Difco Labs, Detroit, MI) followed by three identical booster injections in Freund's incomplete adjuvant (FIA; Difco) on days 7, 28 and 49 [1,2]. In addition, mice injected with adjuvant without antigen, with adjuvant and keyhole limpet haemocyanin (KLH) or non-immunized mice served as controls.

Culture media

The complete medium for culture of all cell types was Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented (sDMEM) with 10 mM HEPES (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0·5 mM 2-mercaptoethanol (2-ME; Sigma Chemical Co., St Louis, MO), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Gibco), 100 μg/ml gentamicin (Sigma) and 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS; Hyclone, Logan, UT). Conditioned medium, as a source of IL-2, consisted of supernatants of rat spleen cells that had been stimulated for 48 h with 5 μg/ml concanavalin A (Con A) (ICN, Costa Mesa, CA) as we described [7]. All tissue culture experiments, unless indicated, were performed in a humidified atmosphere of 5% CO_2 in air.

Cell lines and PG-specific T cell hybridoma

The cell lines described (CTLL-2 and BW5147) were obtained from American Type Culture Collection (ATCC; Rockville, MD). The PG-specific T cell hybridoma 5/4E8 was obtained following fusion of *in vitro* stimulated lymphocytes from arthritic mice with BW5147 thymoma cells [14] as previously described [5,6]. Hybridoma 5/4E8 expressed T cell receptor (TCR) $\alpha\beta^+$ (Vβ4), CD4$^+$ and CD8$^-$ phenotypes and secreted IL-2 but not IL-4 following stimulation with cartilage PG [5,7].

Measurement of anti-PG antibodies

Serum antibodies were measured by solution radioimmunoassay (RIA) as described elsewhere [1,13]. Serial dilutions of sera were incubated with ^{125}I-labelled HAPG, HFPG, BNCPG, rat chondrosarcoma or mouse PG and the antigen–antibody complex pelleted either with protein A bearing *Staphylococcus aureus* (Zysorbin; Zymed Labs, San Francisco, CA) for IgG or with pig anti-mouse IgM followed by Zysorbin [15]. Radioactivity of pellets was measured using a gamma counter (Beckman, Model Gamma 5500B).

Detection of PG-specific IL-2 production

Mouse spleen and lymph node cells (2×10^5/well) were cultured with PG in triplicate wells in 96-well microtitration plates for 24 h and the supernatants were then harvested and assayed in a CTLL-2 proliferation assay as described [8]. The result for PG-stimulated cells was expressed as Δct/min (net counts/min shown as mean ct/min in figures), calculated by subtracting the counts obtained with unstimulated cells.

T cell hybridoma 5/4E8 (1×10^5 cells/well) was tested with PG or PG fragments in 96-well microtitration plates in the presence of syngeneic APC (either 2×10^5/well unseparated mouse spleen cells, purified B cells or purified T cells) in triplicate wells at 37°C for 18 h and the supernatants were then tested for IL-2 content as described above.

Isolation of B cells using nylon wool

Scrubbed nylon fibre (≈600 mg) (three denier, 3·81 cm; Polysciences, Inc., Warrington, PA) was packed into a 10-ml syringe. The column was washed through and filled with 50 ml of sDMEM and incubated at 37°C for 2 h in air with 5% CO_2. After incubation, 10^7 spleen mononuclear cells purified on Lympholyte M (Cedarlane Laboratories, Westbury, NY) or lymph node cells (plastic-adherent cells were removed in both cases) in 2 ml of sDMEM at 37°C were layered gently onto the column, followed by 1 ml warm medium. The column was sealed and returned to the incubator for 45–60 min. The T cells were then eluted with 20 ml medium, spun down, counted and resuspended to a concentration of 2×10^6 cells/ml in sDMEM. The column was then washed through with 50 ml of warm medium to remove the non-adherent 'null' cells. B cells were eluted by adding a further 10 ml warm medium, rapidly inserting the plunger, and compressing the wool as hard as possible. This process was repeated twice; the collected B cells were washed twice in DMEM and resuspended to a concentration of 2×10^6/ml in sDMEM. The enriched B cell populations from both spleen and lymph nodes were shown to be ≈85% CD45R$^+$, < 10% CD3$^+$, and a mean of 7·4% mac-1$^+$ by flow cytometry (described below; Table 1, spleen cells shown).

Magnetic separation of B cells

To try to reduce the percentage of mac-1$^+$ cells in the B cell population, splenic B cells were also isolated using miniMACS magnetic separation columns (Miltenyi Biotech Inc, Sunnyvale, CA). Briefly, lymphocytes, after removing plastic-adherent cells

Table 1. Phenotypes of separated splenic B cell populations

	Nylon wool		Magnetic beads	
	B cells	Non-B cells	B cells	Non-B cells
CD45R	81·1 ± 4·0	17·2 ± 2·3	97 ± 1·9	1·8 ± 0·23
CD3	9·5 ± 1·2	72·5 ± 4·1	0·9 ± 0·2	87·2 ± 3·4
mac-1	7·4 ± 0·3	16·4 ± 2·4	1·8 ± 0·3	10·9 ± 1·1
IL-2 (ct/min)	58 000 ± 63 243	421 546 ± 21 394	563 ± 256	438 560 ± 27 351

B cells were isolated from mononuclear spleen cells (previously purified by Lympholyte M-sedimentation and plastic adherence for 1 h at 37°C) either by adherence to nylon wool or by magnetic separation using an anti-CD45R MoAb. Cell surface phenotypes of B cell populations were determined by flow cytometry. Numbers indicate the mean percentage of cells positive for CD45R, CD3 or mac-1 from six mice. Levels of IL-2 are shown as mean ct/min of CTLL-2 cells cultured with a conditioned media from cells stimulated with 2·5 μg/ml concanavalin A.

from Lympholyte M-separated spleen cells, were incubated with a biotinylated MoAb to CD45R (clone RA3–6B2; GIBCO) for 30 min on ice. This MoAb recognizes an isoform of CD45R present only in murine B cells [17]. After three washes in PBS, the cells were then incubated with MACS colloidal superparamagnetic microbeads (Miltenyi) conjugated with streptavidin for 20 min and then with streptavidin-R-PE (GIBCO) for 30 min on ice. After a further two washes, the cells were separated on a type A2 steel wool column (Miltenyi) in the presence of a high gradient magnetic field. Cells recovered from the column were shown to be ≈97% $CD45R^+$, < 1% $CD3^+$, and < 2% mac-1^+ (Table 1). Cells that passed through the column were < 2% $CD45R^+$, 11% mac-1^+, and > 87% $CD3^+$.

Magnetic separation of B cells yielded the most pure B cell population with no T cell contamination, as stimulation with 2·5 μg/ml Con A resulted in no IL-2 production (Table 1). Nylon wool-separated B cells, however, produced IL-2 to Con A stimulation, suggesting the presence of small numbers of T cells (Table 1).

Flow cytometry

The purified B cells were examined for CD45R, CD3, mac-1, MHC class II, ICAM-1, B7-1 and B7-2 expression by flow cytometry as previously described [8]. Rat MoAbs specific for the mouse Ia region ($I\text{-}A^{b,d,q}$-, $I\text{-}E^{d,k}$-reactive) were purified from the ascitic fluid of nude mice intraperitoneally injected with hybridoma M5/114.15.2 by affinity chromatography on protein-A columns (Pierce, Rockford, IL). The biotinylated goat anti-rat IgG was obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). The murine ICAM-1-, B7-1- and B7-2-specific biotinylated MoAbs were obtained from Pharmingen (San Diego, CA). The biotinylated MoAbs to CD45R, CD3 and mac-1 were obtained from GIBCO.

Effect of specific monoclonal antibodies on B cell presentation of PG

The purified mouse B cells (from magnetic separation) were cultured with the 5/4E8 hybridoma cells in the presence of 50 μg/ml HAPG and MoAbs for 18 h at 37°C. Non-biotinylated MoAbs specific for ICAM-1, B7-1 and B7-2 were obtained from Pharmingen. The anti-Ia MoAb was the same as the one described above. The MoAbs were added to the B cells and hybridomas at a concentration of 50 μg/ml and the effect on IL-2 production was measured as described above. The ICAM-1, B7-1 and B7-2-specific MoAbs (all IgG2a) were immobilized to the wells of a 96-well plate by incubation for 18 h at 37°C in sterile sodium carbonate buffer, pH 9·6. Immobilized rat IgG2a (Pharmingen) was used as an isotype control in all experiments.

Statistical analysis

Student's unpaired *t*-test was used to detect significant differences ($P < 0.05$) between groups.

RESULTS

PG-specific antibodies and T cells in immunized mice

In HFPG-immunized mice, PG-specific antibodies to immunizing antigen appeared after 2 weeks and autoreactive antibodies to native mouse PG became detectable in sera ≈21–28 days after the first injection of PG (Fig. 1). Antibodies that cross-reacted with HACPG, BNCPG and rat chondrosarcoma PG could also be detected at this time (Table 2). In immunized non-arthritic (BNCPG- or CSCPG-injected mice) the same antibody profiles were obtained, but antibodies to mouse PG were not detected, or were detected at very low level, in these animals (Table 2). No PG-specific antibodies were detected in KLH-immunized mice, in mice given adjuvant alone or in unimmunized mice (Table 2). Levels of PG-specific antibodies in HFPG- (Fig. 1) and BNCPG- or CSCPG-immunized mice (not shown) continued to increase over the next 2–3 weeks, plateauing at weeks 9–11 of immunization. PG-specific T cell responses in the lymph nodes and spleen appeared in both HFPG- (Fig. 1) and BNCPG- or CSCPG-immunized mice (not shown) after the appearance of PG-specific antibodies (usually around the onset of arthritis in HFPG-immunized mice) and increased as joint inflammation progressed (Fig. 1). T cells from lymph nodes of all immunized mice generally responded more strongly than splenic T cells.

Purified B cells from the spleen and lymph nodes of immunized mice present PG (aggrecan) to a PG-specific T cell hybridoma

Lympholyte M-purified spleen and lymph node mononuclear cells (from either immunized or non-immune mice) were

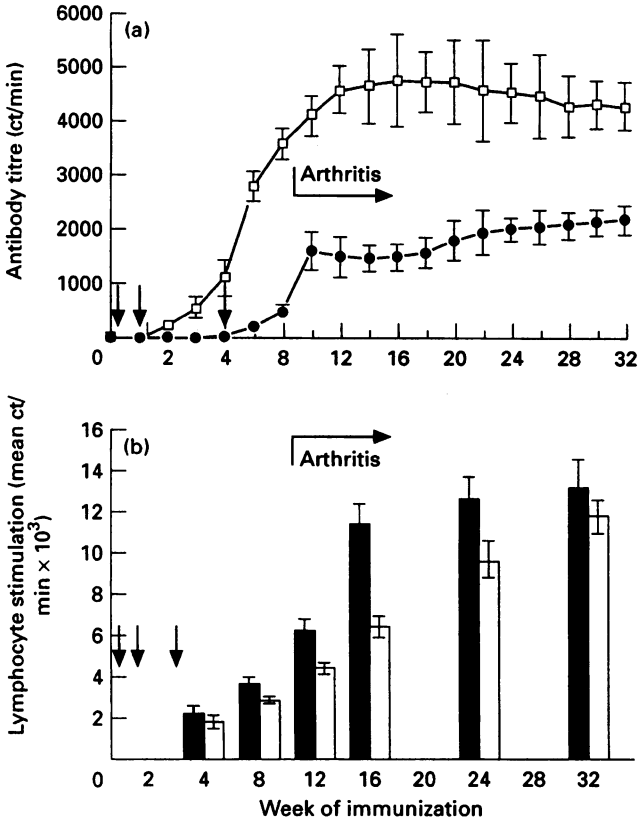

Fig. 1. Measurement of anti-proteoglycan (PG) antibodies in the sera of human fetal PG (HFPG)-immunized BALB/c mice (a) and lymphocyte proliferation in the presence of chondroitinase ABC-digested HFPG (b). Antibody titres (lines) were measured at 1:62 500 (□ for HFPG) and 1:2500 (● for mouse PG) serum dilutions using solution radioimmunoassay (RIA) [2,13]. Proliferation of T lymphocytes from spleen (□) and joint draining lymph nodes (■) are shown as Δct/min in response to chondroitin sulphate-depleted HFPG (columns). Proliferation of T cells from spleen and lymph nodes is shown as mean ct/min in response to chondroitin sulphate-depleted HFPG (columns).

effective presenters of HACPG to the T cell hybridoma (Fig. 2a,b). However, nylon wool-adhered B cells from the spleens and lymph nodes of immunized mice (either arthritic or non-arthritic) could present HACPG to the T cell hybridoma at a relatively low (12·5 μg/ml) concentration (Fig. 2a,b). In most cases the responses of arthritic mice were slightly higher than those of immunized but non-arthritic mice, but these differences were not statistically significant. B cells from lymph nodes were better at presenting the PG than splenic B cells. Presentation of HACPG by B cells from non-immune normal mice was significantly ($P < 0{\cdot}01$) lower than that of PG-immunized mice (Fig. 2a,b).

Magnetically separated splenic B cells from immunized mice could also present HFPG, HAPG and BNCPG very strongly to the hybridoma over a wide range of antigen concentrations (2·5–50 μg/ml) (Fig. 3a–c). Responses to human PG were always significantly higher ($P < 0{\cdot}05$) than those to BNCPG, independent of whether the B cells derived from HFPG, HACPG or BNC-immunized mice. Again, the responses of immunized mice were significantly higher ($P < 0{\cdot}01$) than those of both non-immune and KLH-immunized control animals at all antigen concentrations. Unimmunized and KLH-immunized animals could present the PG only at higher concentrations of antigen, suggesting that they were presenting the PG non-specifically, probably by phagocytosis [18–21]. B cells from KLH-immunized mice presented PG significantly ($P < 0{\cdot}05$) better than normal mice, perhaps representative of an increased activation state of these B cells, as activated B cells have a higher affinity for antigen and increased rates of phagocytosis over resting cells [18]. The ability of B cells from PG-immunized mice, but not from KLH-immunized or normal animals, to present the PG at low concentrations suggests that these B cells are presenting the antigen specifically via their surface immunoglobulin receptor.

We have previously shown that CNBr peptides of PG (but not native PG) can be presented by fixed APC in the absence of immune processing, implying that these peptides bind directly to MHC class II molecules on the surface of APC [7]. In support of this, CNBr peptides of PG were presented equally

Table 2. Proteoglycan (PG)-specific serum antibody titres in PG-immunized and control mice

Groups	Arthritic (HFPG-immunized)	Immunized non-arthritic (BNCPG-immunized)	KLH-immunized	Adjuvant only	Normal (non-immunized)
Antibodies to					
Mouse PG	1116 ± 137	260 ± 245	ND	ND	ND
HFPG	3862 ± 1041	2993 ± 849	ND	ND	ND
HACPG	3842 ± 977	3686 ± 566	ND	ND	ND
BNCPG	2284 ± 266	2668 ± 442	ND	ND	ND
CSCPG	1228 ± 228	1116 ± 327	ND	ND	ND

Mean serum antibody titres (Δct/min ± s.d.) measured in sera of five animals of each group are shown at the time of antigen presentation experiments. ^{125}I-labelled chondroitinase ABC-digested PG were calibrated to have a specific activity of 10·5–12 μCi/μg PG. Serum dilutions of immunized mice (both arthritic and non-arthritic) were 1 : 2500 for measuring antibodies to mouse PG (first line) and 1 : 62 500 in all other cases. No antibody to PG was detected (ND) in keyhole limpet haemocyanin (KLH)-immunized, adjuvant-injected or normal mice up to 1 : 100 serum concentrations. HFPG, Human fetal PG; HACPG, human adult cartilage PG; BNCPG, bovine nasal cartilage PG; CSCPG, chick sternal cartilage PG.

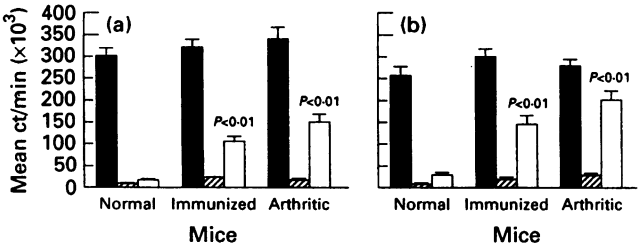

Fig. 2. Unseparated (■) lymphocytes or nylon wool-separated B (□) or T cells (▨) from spleen (a) and lymph nodes (b) of normal, proteoglycan (PG)-immunized non-arthritic and arthritic mice (six mice/group) were cultured together with 20 μg/ml human adult (HA) PG and 5/4E8 T cell hybridoma for 18 h. CTLL-2 proliferation is expressed as Δct/min in the presence of conditioned medium from the three cell types.

well by B cells from both immunized and non-immune mice (results not shown).

The ability of B cells to present PG correlates with the levels of PG-specific serum antibody

There is ≈ 10–50% individual difference in antibody production and 20–30% in T cell response among animals immunized with PG antigens. Higher anti-PG antibody level was associated with a higher level of T cell response, whereas the source of PG (human, canine, bovine) seemed to be less important. The levels of PG-specific antibodies in the arthritic and immunized non-arthritic groups of mice are shown in Table 2. All PG-immunized groups of mice had high serum titres to HACPG, HFPG and BNCPG (both native and chondroitinase ABC-digested), and similarly the B cells from these mice could effectively present these PG to the hybridoma (Figs 2 and 3). However, no IL-2 production, i.e. no antigen-specific response, by the hybridoma was obtained using rat chondrosarcoma and canine PG which are not recognized by hybridoma 5/4E8 [5,7]. In summary, the PG specificity of B cells was an absolute requirement for PG presentation to the T cell hybridoma.

Cell surface phenotype of purified mouse B cells

There were no significant differences in the levels of MHC class II, ICAM-1 or B7-1/B7-2 on the surface of magnetically separated B cells from the three groups of mice (Fig. 4a). All these cell surface markers were clearly detectable on B cells, except that B7-1 was detectable only at low levels or absent (Fig. 4a).

Effect of MoAbs on B cell presentation

Incubation of highly purified magnetically separated B cells from arthritic and immunized non-arthritic mice with antibodies to ICAM-1 significantly ($P < 0{\cdot}01$) reduced presentation of HACPG (25 μg/ml) to the T cell hybridoma compared with the rat isotype control. Antibodies to B7-1 had no effect on presentation, whereas B7-2-specific MoAb reduced B cell presentation by ≈70% ($P < 0{\cdot}01$), and an anti-Ia antibody completely abrogated presentation (Fig. 4b).

DISCUSSION

We have previously demonstrated the abilities of a B lymphoma cell line (A20), macrophages, synoviocytes and chondrocytes to present PG to a PG-specific T cell hybridoma [7], capable of inducing arthritis when injected into BALB/c mice [5]. In this present study we now highlight the importance of B cells in the priming of cartilage PG (aggrecan)-specific T cells. B cells from PG-immunized arthritic and non-arthritic, as well as from KLH-immunized mice, were examined for their abilities to present different species of PG to a PG-specific T cell hybridoma. We used two (nylon wool and magnetic) methods of B cell separation and both methods, particularly magnetic separation, yielded pure populations of B cells with little or no macrophage or T cell contamination. Nylon wool-separated B cells contained a mean of 7·4% mac-1$^+$ cells, although in some cases this number was as low as 3·5% without affecting the results. These mac-1$^+$ cells could be either macrophages or CD5$^+$ B cells, which also express mac-1. However, when the B cell populations were irradiated with 30 Gy, antigen presentation was reduced by > 90%, implying that it was due to radiosensitive B cells [20], that were mainly responsible for the observed PG presentation to the T cell hybridoma. However, to clarify this point further, we isolated B cells by magnetic separation to yield CD45R$^+$ B cell populations which contained a mean of only 1·8% mac-1$^+$ cells (range 0·9–2·1%), which surely must have precluded PG presentation by non-specific macrophages. Furthermore, the CD45R$^+$ B cell

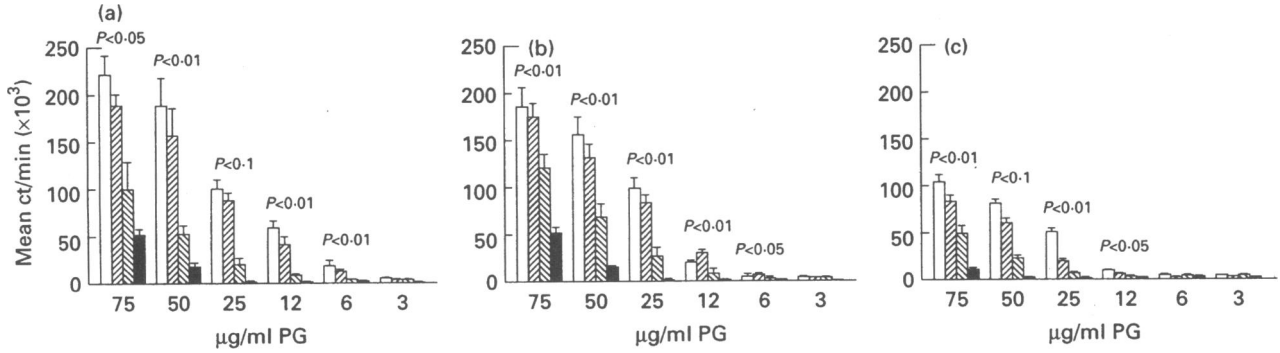

Fig. 3. Magnetically separated splenic B cells from non-immune normal (■), keyhole limpet haemocyanin (KLH)-immunized (▧), proteoglycan (PG)-immunized non-arthritic (▨) and arthritic mice (□) (six mice/group) were cultured with decreasing dilutions (from 75 to 3 μg/ml) of chondroitinase ABC-digested human fetal (HF) PG (a), human adult (HA) PG (b), and bovine nasal cartilage (BNC) PG (c) together with 5/4E8 hybridoma cells for 18 h. CTLL-2 proliferation is expressed as Δct/min in the presence of conditioned medium.

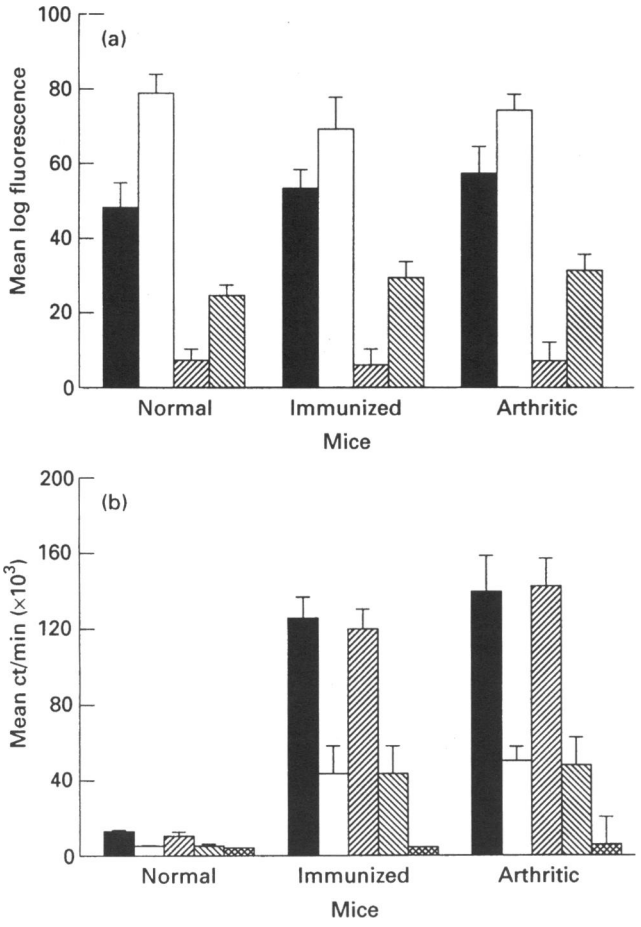

Fig. 4 (a) Ia (■), intercellular adhesion molecule-1 (ICAM-1) (□), B7-1 (▨), and B7-2 (▧) expression by B cells from normal, immunized and arthritic mice (five mice/group). Levels are expressed as mean log fluorescence intensity. (b) Effect of MoAbs on proteoglycan (PG) presentation. B cells from normal, immunized non-arthritic and arthritic mice (six mice/group) were cultured with human adult (HA) PG (50 μg/ml) in the presence of 50 μg/ml MoAbs specific for Ia (▩), ICAM-1 (□), B7-1 (▨) and B7-2 (▧), and the effect on IL-2 production of hybridoma 5/4E8 determined. Rat IgG was the control (■). CTLL-2 proliferation is expressed as Δct/min.

population was visualized as a single sharp peak when examined by flow cytometry, confirming that it consisted of a single cell type and presentation was abolished following irradiation (results not shown).

We found that only B cells (from both separations) from the spleens and lymph nodes of immunized mice (whether they were arthritic or clinically asymptomatic) but not from normal unimmunized mice, could present low concentrations of PG to the T cell hybridoma. Non-immune and KLH-injected mice could only present high concentrations of PG, probably non-specifically by phagocytosis [18,20]. The abilities of the B cells to present HACPG, HFPG and BNCPG to the T cell hybridoma, when each immunized mouse was analysed individually, correlated strongly with the levels of antibodies specific for these PG that were present in the mouse serum. Lymph node B cells were generally stonger presenters of PG than splenic B cells. This agrees with our earlier findings, where T cells from lymph nodes were generally stronger responders to PG than splenic T cells in an *in vitro* lymphocyte proliferation assay [8], suggesting that priming of both B and T cells is higher in the lymph nodes, particularly in the joint draining lymph nodes.

PG that were not recognized by the hybridomas such as canine and rat chondrosarcoma PG did not result in IL-2 production when presented by mouse B cells. Ideally a relevant negative control would be to examine the ability of the B cells from immunized mice to present a PG that does not elicit serum antibody, but is recognized by the hybridoma. Unfortunately such a control is not available, as mice injected with PG (of any species) produce antibodies, predominantly against carbohydrate components, which are present in all species of PG tested. Hence any PG-immunized mouse has antibodies recognizing all species of PG tested. However, as B cells from PG-immunized mice could present low concentrations of PG when responses of B cells from KLH-immunized mice were undetectable, it would suggest that B cells from PG-immunized mice can present the PG specifically to the T cells via their surface immunoglobulin antigen receptor, and that this effective presentation is not solely a consequence of an increased activation state.

Although the presence of PG-specific antibodies in the sera and inflamed joint tissues of arthritic mice is well documented [1,2,22,23], this is indirect evidence for the functional role of PG-specific B cells in PG-induced arthritis. We have previously been unable to demonstrate *in vitro* antibody production to PG, but demonstration of *in vitro* antibody production is notoriously difficult and strongly dependent on the stock of FCS used [18]. These are therefore the first data to demonstrate direct evidence of the crucial role of PG-specific B cells in PG-immunized mice. It was originally thought that only activated B cells and B lymphoma cells, but not resting B cells, could present antigen to T cell hybridomas and T cell clones [18]. However, these studies used irradiated resting B cells, and it was subsequently shown that the APC function of resting B cells was lost following irradiation [20]. We have shown that B cells from non-immunized mice (presumably resting B cells) can present antigen to T cells, which is in agreement with either data showing that non-irradiated resting B cells at relatively high cell numbers (identical to the numbers used in this study) can present antigen to both T cell hybridomas and T cell clones [20,21]. Furthermore, we have shown that B cells from immunized (PG and KLH) mice, which are presumably activated to some degree, were considerably better APC, which is also in agreement with earlier work [18]. Finally, B cells from PG-immunized mice could present PG better than B cells from KLH-immunized mice, emphasizing the ability of PG-specific B cells to present antigen to T cells via their specific receptor for antigen.

For maximal stimulation of T cells, a co-stimulatory signal provided by the APC is required in addition to triggering of the TCR by interaction with MHC class II and antigen. In the case of B cell presentation to T cells, the interaction of members of the B7 family on the B cell with CD28 on the surface of the T cell is particularly important for maximum IL-2 production, and to a lesser extent interferon-gamma (IFN-γ) production, by T helper cells. We therefore examined the importance of the co-stimulatory molecules B7-1 and B7-2, which were both shown to be present to varying degrees on the isolated B cells, in PG presentation to the T cell hybridoma. ICAM-1 is an adhesion molecule which we have shown previously to be important in PG presentation by macrophages, synoviocytes

and chondrocytes [6], and has also been touted as a potential co-stimulatory molecule. Antibodies to both ICAM-1 and B7-2 were strongly inhibitory, whereas B7-1-specific MoAbs had no effect. This supports earlier data which showed that B7-2 antibodies were more efficient inhibitors of T cell co-stimulation by activated B cells, splenocytes and dendritic cells than anti-B7-1 antibodies. These differences may simply be related to the fact that B7-2 is expressed at higher levels on mouse B cells (both immunized and normal) than B7-1, or that B7-2 has a higher affinity for CD28 than B7-1. We have used T cell hybridomas in our experiments, which may have co-stimulatory requirements different from untransformed T cells. Indeed, there are reports of T cell hybridomas which could recognize peptides presented by MHC molecules incorporated into artificial membranes where no co-stimulation by APC was required. However, we have shown that hybridoma 5/4E8 does have co-stimulatory requirements, since antibodies to B7-2 reduce antigen presentation by mouse B cells (shown in this study) and the mouse macrophage cell line J774 A.1. Thus, if the interaction between the B cells and T cells *in vivo* is comparable to that between the B cell and our hybridoma *in vitro*, antibodies to B7-2 may provide a useful immunotherapy to reduce B–T cooperation and therefore joint destruction in PG-induced arthritis. Indeed, inhibition of the CD28/B7 interaction *in vivo* prevents rejection of xenogeneic pancreatic islets and heart grafts, probably by reducing allogeneic T cell co-stimulation.

Although T cells probably play a critical role in the amplification of cross-reactive (auto)antibody production, the data presented here also suggest that B cells elicited to foreign PG may initiate an autoimmune T cell response *in vivo*. It is likely that these PG-specific B cells *in vivo* take up the autoantigen (mouse PG) via their surface immunoglobulin receptor or non-specifically by phagocytosis in the lymph nodes and spleen, process the large PG molecule and present self-peptides to T cells. Autoreactive T cells subsequently migrate to and proliferate in the synovium and joint-draining lymph nodes [33], where self-peptides are present in relatively high concentrations as a result of the normal turnover of the cartilage matrix and, even more, as a consequence of increased PG degradation in inflammatory joint diseases [34]. Activated autoreactive B and T cells in the synovium then release various cytokines which promote proliferation of autopathogenic T cells and provoke the release of proteolytic enzymes to degrade cartilage matrix components [35–39]. Activated T cells may also produce IFN-γ, which induces the expression of cell adhesion molecules [40], hence facilitates further the invasion of inflammatory cells and up-regulates MHC class II expression on synoviocytes [7] and promotes further generation of autoreactive T cells.

ACKNOWLEDGMENTS

This work was supported by the NIH (AR 40310), the National and Illinois Arthritis Foundations (USA) and by the National Science Foundation of Research (OTKA F12912), Hungary.

REFERENCES

1 Glant TT, Mikecz K, Arzoumanian A, Poole AR. Protoglycan-induced arthritis in BALB/c mice. Clinical features and histopathology. Arthritis Rheum 1987; **30**:201–12.

2 Mikecz K, Glant TT, Poole AR. Immunity to cartilage proteoglycans in BALB/c mice with progressive polyarthritis and ankylosing spondylitis induced by injection of human cartilage proteoglycan. Arthritis Rheum 1987; **30**:306–18.

3 Mikecz K, Glant TT, Buzás E, Poole AR. Cartilage proteoglycans as potential autoantigens in humans and in experimental animals. Agents Actions 1988; **23**:63–66.

4 Glant TT, Mikecz K, Thonar EJA, Kuettner KE. Immune responses to cartilage proteoglycans in inflammatory animal models and human diseases. In: Woessner JF, Howell DS, eds. Cartilage degradation: basic and clinical aspects. New York: Marcel Dekker, Inc. 1992: 435–73.

5 Buzás EI, Brennan FR, Negroiu G, Holló K, Mikecz K, Glant TT. Characterization of proteoglycan (aggrecan)-specific T cell hybridomas. Arthritis Rheum 1994; **37**:S358.

6 Brennan FR, Mikecz K, Buzás E, Glant TT. Interferon-gamma but not granulocyte/macrophage colony-stimulating factor augments proteoglycan presentation by synovial cells and chondrocytes to an autopathogenic T cell hybridoma. Immunol Letters 1995; **45**:87–91.

7 Brennan FR, Negroiu G, Buzás EI *et al.* Presentation of cartilage proteoglycan to a T cell hybridoma derived from a mouse with proteoglycan-induced arthritis. Clin Exp Immunol 1995; **100**:104–10.

8 Buzás E, Mikecz K, Brennan FR, Glant TT. Mediators of autopathogenic effector cells in proteoglycan-induced arthritic and clinically asymptomatic BALB/c mice. Cell Immunol 1994; **158**:292–304.

9 Brennan FR, Mikecz K, Buzás EI, Glant TT. Humoral immune responses to self-proteoglycan (PG) triggers the activation of autoreactive T cells in PG-induced arthritis in BALB/c mice. Arthritis Rheum 1994; **37**:S396.

10 Lin R-H, Mamula MJ, Hardin JA, Janeway CA Jr. Induction of autoreactive B cells allows priming of autoreactive T cells. J Exp Med 1991; **173**:1433–9.

11 Male DK, Pryce G, Cooke A, Hutchings P, Marshall-Clarke S, Roitt IM. T and B cell connections in experimentally induced autoimmunity. Ann NY Acad Sci 1986; **475**:94–105.

12 Ando DG, Sercarz EE, Hahn BH. Mechanisms of T and B cell collaboration in the *in vitro* production of anti-DNA antibodies in the NZB/NZW F_1 murine SLE model. J Immunol 1987; **138**:3185–90.

13 Glant TT, Mikecz K, Poole AR. Monoclonal antibodies to different protein-related epitopes of human articular cartilage proteoglycans. Biochem J 1986; **234**:31–41.

14 Kappler JW, Skidmore B, White J, Marrack P. Antigen-inducible, *H-2*-restricted, interleukin-2-producing T cell hybridomas. J Exp Med 1981; **153**:1198–214.

15 Glant TT, Mikecz K, Roughley PJ, Buzás E, Poole AR. Age-related changes in protein-related epitopes of human articular-cartilage proteoglycans. Biochem J 1986; **236**:71–75.

16 Miltenyi S, Müller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. Cytometry 1990; **11**:231–8.

17 Coffman RL, Weissman IL. B220: a B cell-specific member of the T200 glycoprotein family. Nature 1981; **289**:681–3.

18 Chesnut RW, Colon SM, Grey HM. Requirements for the processing of antigens by antigen-presenting B cells. I. Functional comparison of B cell tumors and macrophages. J Immunol 1982; **129**:2382–8.

19 Glimcher LH, Kim K-J, Green I, Paul WE. Ia antigen-bearing B cell tumor lines can present protein antigen and alloantigen in a major histocompatibility complex-restricted fashion to antigen-reactive T cells. J Exp Med 1982; **155**:445–59.

20 Ashwell JD, DeFranco AL, Paul WE, Schwartz RH. Antigen presentation by resting B cells. Radiosensitivity of the antigen-presenting function and two distinct pathways of T cell activation. J Exp Med 1984; **159**:881–905.

21 Frohman M, Cowing C. Presentation of antigen by B cells:

functional dependence on radiation dose, interleukins, cellular activation, and differential glycosylation. J Immunol 1985; **134**:2269–75.

22 Dayer E, Mathai L, Glant TT, Mikecz K, Poole AR. Cartilage proteoglycan-induced arthritis in BALB/c mice: antibodies recognize human and mouse cartilage proteoglycan and can cause depletion of cartilage proteoglycan with little or no synovitis. Arthritis Rheum 1990; **33**:1394–405.

23 Glant TT, Fülöp C, Mikecz K, Buzás E, Molnár G, Erhardt P. Proteoglycan-specific autoreactive antibodies and T-lymphocytes in experimental arthritis and human rheumatoid joint diseases. Biochem Soc Trans 1990; **18**:796–9.

24 Mondino A, Jenkins MK. Surface proteins involved in T cell costimulation. J Leuk Biol 1994; **55**:805–15.

25 Jenkins MK, Pardoll DM, Mizuguchi J, Chused TM, Schwartz RH. Molecular events in the induction of a nonresponsive state in the IL-2 producing helper T lymphocyte clones. Proc Natl Acad Sci USA 1987; **84**:5409–13.

26 Van Seventer GA, Shimizu Y, Shaw S. Roles of multiple accessory molecules in T-cell activation: bilateral interplay of adhesion and costimulation. Curr Opin Immunol 1995; **3**:294–303.

27 Lenschow DJ, Su H-T, Zuckerman LA *et al.* Expression and functional significance of an additional ligand for CTLA-4. Proc Natl Acad Sci USA 1993; **90**:11054–8.

28 Boussiotis VA, Freeman GJ, Gribben JG, Daley J, Gray G, Nadler LM. Activated human B lymphocytes express three CTLA-4 counterreceptors that costimulate T-cell activation. Proc Natl Acad Sci USA 1993; **90**:11059–63.

29 Azuma M, Ito D, Yagita H *et al.* B70 is a second ligand for CTLA-4 and CD28. Nature 1993; **366**:76–79.

30 Brennan FR, Finnegan A, Mikecz K, Buzas EI, Glant TT. IL-10 inhibits antigen presentation by a mouse macrophage line to a T cell hybridoma by mechanisms independent of the downregulation of B7–1. Eur J Immunol 1995; submitted.

31 Lenshow DJ, Zeng Y, Thistlewaite JR *et al.* Long term survival of xenogeneic pancreatic islet grafts induced by CTLA-4 Ig. Science 1992; **257**:789–91.

32 Turka LA, Linsley PS, Lin H *et al.* T cell activation by CD28 ligand B7 is required for cardiac allograft rejection *in vivo*. Proc Natl Acad Sci USA 1992; **89**:11102–5.

33 Mikecz K, Glant TT. Migration and homing of lymphocytes to lymphoid and synovial tissues in proteoglycan-induced murine arthritis. Arthritis Rheum 1994; **37**:1395–403.

34 Pettipher ER, Higgs GA, Henderson B. Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint. Proc Natl Acad Sci USA 1986; **83**:8749–53.

35 Kresina TF, Yoo JU, Goldberg VM. Evidence that a humoral immune response to autologous cartilage proteoglycan can participate in the induction of cartilage pathology. Arthritis Rheum 1988; **31**:248–57.

36 DiPasquale G. Interleukins and metalloproteinases in arthritis. In: Kresina TF, ed. Monoclonal antibodies, cytokines, and arthritis. Mediators of inflammation and therapy. New York: Marcell Dekker, Inc. 1991:173–98.

37 Greenwald RA. Cartilage degradation in animal models of inflammatory joint disease. In: Woessner JF, Howell DS, eds. Joint cartilage degradation. New York: Marcel Dekker, Inc. 1993:385–408.

38 Nietfeld JJ, Wilbrink B, Helle M *et al.* Interleukin-1-induced interleukin-6 is required for the inhibition of proteoglycan synthesis by interleukin-1 in human articular cartilage. Arthritis Rheum 1990; **33**:1695–701.

39 Shinmei M, Masuda K, Kikuchi T, Shimomura Y. Interleukin 1, tumor necrosis factor, and interleukin 6 as mediators of cartilage destruction. Semin Arthritis Rheum 1989; **18** (Suppl. 3):27–32.

40 Mikecz K, Brennan FR, Kim JH, Glant TT. The role of adhesion molecules in the development of autoimmune arthritis. Scand J Rheumatol 1995; **24** (Suppl. 101): 91–98.