Clin Exp Immunol 1996; **103**:89–93

Cytotoxic T lymphocytes specific for murine type II collagen do not trigger arthritis in B10 mice

X.-M. GAO & A. J. MCMICHAEL* *Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, and *Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK*

(Accepted for publication 22 September 1995)

SUMMARY

To investigate the role of cytotoxic T lymphocytes (CTL) in arthritis, we set out to induce CTL specific for murine type II collagen (mCII) in a mouse model. The primary protein sequence of the murine pro-1(II) was screened for fragments bearing H-2 D^b or K^b binding motifs. Six fragments were identified and the corresponding peptides synthesized. One of these peptides, peptide P201 (amino acid 199–208 in the C-propeptide of the murine pro-α1(II)), was found to be a strong binder to H-2 D^b. When used to treat RMA-S cells at 26°C, peptide P201 induced a four-fold increase of surface expression of H-2 D^b. Administration of the P201-treated RMA-S cells into B10 mice (H-2^b) induced strong CTL responses against the immunizing collagen peptide. Despite the high frequencies of mCII-specific CTL precursors in the periphery, however, the immunized mice showed no sign of arthritis up to 16 weeks after immunization. Implications of these data for autoimmunity and arthritis are discussed.

Keywords arthritis collagen peptides autoimmunity CTL

INTRODUCTION

Arthritis of different forms, such as rheumatoid arthritis (RA), juvenile arthritis, ankylosing spondylitis (AS) and reactive arthritis (ReA), are associated with antigens of the MHC [1–5]. For instance, about 40% of patients with RA carry HLA-DR4, a class II MHC product, and 90% of spondylitics possess HLA-B27, a class I product of the human MHC, compared with 13% with DR4 and 7% with B27 in the Caucasian population [1–5]. It is generally accepted that autoimmune T lymphocytes reactive to joint antigens, such as type II collagen (CII) and proteoglycan, may play a central role in the pathogenesis of these disorders [6–9]. Subcutaneous immunization of mice, rats and primates with autologous, or heterologous, CII induces class II MHC-restricted $CD4^+$ helper T cell responses and triggers arthritis [6]. Passive transfer of $CD4^+$ T cell clones specific for cartilage proteoglycan and *Mycobacterium tuberculosis* caused arthritis in recipient rats [10]. Therapeutic vaccination against $CD4^+$ T lymphocytes is now regarded as a possible way of treating RA in the future [11]. It is not clear, however, whether class I MHC-restricted, $CD8^+$ cytotoxic T lymphocytes (CTL) could also trigger inflammatory responses in the joints. The strong AS-B27 association supports this view. Previously we identified CTL specific for a CII peptide in a B27-positive patient with ReA, although attempts to repeat this in another 10 patients were unsuccessful [12]. Here we report results from experiments undertaken to study the role of CTL in arthritis in a mouse model.

Correspondence: X. M. Gao, Department of Biochemistry, Imperial College of Science, Technology and Medicine, Exhibition Road, London SW7 2AZ, UK.

MATERIALS AND METHODS

Mice and monoclonal antibodies

Female B10 mice of 10–12 weeks old were purchased from Olac (Bicester, UK). MoAbs, B22 and Y3, specific for β_2-microglobin-associated H-2 D^b and K^b heavy chains [13,14], respectively, were purified from ascites fluid. The rat hybridomas YTS-169 (anti-murine CD8) and GK1.5 (anti-murine CD4) [15] were grown as ascites in pristane-treated nude mice.

Screening for possible CTL epitopes in murine CII

Mature CII derives from procollagen II composed of three identical polypeptide units—pro-α1(II). The H-2 D^b and K^b-binding motifs (an asparagine at the fifth and a methionine at the ninth position for H-2 D^b; a tyrosine at the fifth and leucine at the eighth position for H-2 K^b) [16–18] were used to screen the primary sequence of murine pro-α1(II) [19,20] for possible CTL epitopes. The six peptides thus identified (Table 1) were synthesized using a Zinsser automatic multiple peptide synthesizer (Zinsser Analytic, Frankfurt, Germany).

Assembly assays

RMA-S is a murine tumour cell line that has a defect in transporting

Table 1. Fragments in murine type II collagen (mCII) bearing H-2 D^b or K^b-binding motifs

ID number	Position	H2 D^b	H-2 K^b
P201	199–208 (C)*	IQGS**N**DVE**M**	
P206	88–95(H)†		GHRG**Y**PG**L**
P207	791–798(H)		GERG**F**PG**L**
P208	931–938(H)		GHRG**F**TG**L**
P209	5–12(C)		DMSA**F**AG**L**
P210	159–166(C)		VQMT**F**LR**L**
P365	Flu-NP‡	ASNE**N**MDA**M**	

* 'C' in parentheses indicates that the peptide is from the C-propeptide of murine pro-α1(II) molecule.

† 'H' in parentheses indicates that the peptide is from the triple helical region of the pro-α1(II) molecule.

‡ P365 is an H-2 D^b-restricted CTL epitope (amino acid 366–374) in the nucleoprotein of influenza A virus [27].

cytosolic peptides into the endoplasmic reticulum (ER). H-2 D^b and K^b molecules expressed in RMA-S cells are thus unfolded and retained in the ER [21]. Addition of appropriate synthetic peptides to live RMA-S cells significantly increases the expression of these class I molecules on cellular surfaces [22,23]; addition of such peptides to the lysate of RMA-S cells helps the H-2 D^b and H-2 K^b molecules to fold, in a dose-dependent fashion, into a wildtype conformation recognized by MoAbs B22 and Y3, respectively [21,24]. RMA-S cells were used for determining the binding affinity of the collagen peptides to H-2 D^b and H-2 K^b molecules in 'assembly assays' as described by Elvin *et al.* [24]. Briefly, RMA-S cells were metabolically labelled with ^{35}S-methionine (Amersham) and then lysed in lysis buffer containing NP-40 (0·5%), NaCl (50 mM), and PMSF (10 μg/ml; Sigma). The precleared lysate was treated with appropriate synthetic peptides, followed by MoAb (B22 or Y3) and protein-A-Sepharose beads (Sigma). The precipitates were thoroughly washed and analysed on SDS–PAGE 10% gel. The gels were fixed, treated with Amplify (Amersham), dried, and protein bands visualized by autoradiography.

Induction of murine CII-specific CTL in vivo

RMA-S cells (10^8) were treated with collagen peptides (5 μM) at 26°C for 18 h. A sample of the treated cells (10^7) was stained sequentially using MoAb B22, or Y3, and FITC-conjugated goat anti-mouse IgG (Sigma) and then analysed on a FACScan IV (Becton Dickson) for surface expression of H-2 D^b or H-2 K^b molecules. If positive, the remaining cells (in 1 ml of saline) were irradiated (60 Gy) and inoculated intraperitoneally into female B10 mice. Mice were killed for spleens 3 weeks later and the spleen cells (10^7) restimulated *in vitro* with feeders, i.e. syngeneic spleen cells (4×10^6) pulsed with the immunizing peptide, for 5 days in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (Sigma) (R10).

^{51}Cr-release assays

EL4 cells, a tumour cell line that express H-2 D^b and K^b molecules, were labelled with ^{51}Cr (Amersham) in the presence, or absence, of synthetic peptides (25 μg/ml) for 1 h. After washes, these cells (10^4 cells) were incubated together with the T cell cultures (effectors) in a total of 200 μl of R10 in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) for 4 h. Supernatant (50 μl) was then harvested from each well and radioactivity determined using a micro-β-counter (Wallac, Milton Keynes, UK). Spontaneous and maximum release of ^{51}Cr by the target cells was determined by adding, to the labelled target cells, medium alone or 1% NP-40, respectively. Experimental wells were dispensed in triplicate. The percentage of specific ^{51}Cr-release was calculated as:

$$\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

Precursor frequency analysis for murine CII-specific CTL

Three weeks after the first immunization with peptide/RMA-S cells, groups of B10 mice were given a booster immunization with the same preparations. Spleens were harvested at different time points after the secondary immunization and the splenocytes used for determining the precursor frequencies of murine (m) CII-specific CTL in limiting dilution analysis. The spleen cells were distributed at varying numbers from 500 to 8000 cells/well in 96-well round-bottomed plates in 100 μl of R10. Syngeneic spleen cells (30 Gy irradiated) were then added at 2×10^4 cells/well in 100 μl of R10. Twenty replicate wells were set up at each effector cell number. After 14 days of culture in the presence of IL-2 (10 U/ml) at 37°C, the microcultures were assayed for cytotoxicity against P201-pulsed, ^{51}Cr-labelled EL-4 cells. Wells that showed specific ^{51}Cr-release of 10% or more above the controls were counted as positive. Limiting dilution plots of homocysteine-specific lysis are plotted with the regression line. The P201-specific CTL precursor (CTLp) frequency is estimated from the intercept of the regression line at 37% negative wells. Mice in these groups (20 per group) were also regularly observed for signs of arthritis using the criteria described by Volkman & Collins [25].

RESULTS

Identification of a possible CTL epitope in mCII

Six protein fragments of eight or nine amino acids bearing H-2 D^b or K^b-binding motifs were identified in the primary protein sequence of murine pro-α1(II) (Table 1). These peptides were synthesized and tested for ability to bind to H-2 D^b and K^b molecules in assembly assays using RMA-S cells. As shown in Fig. 1a, peptides P201 and P206 appeared to be able to bind to H-2

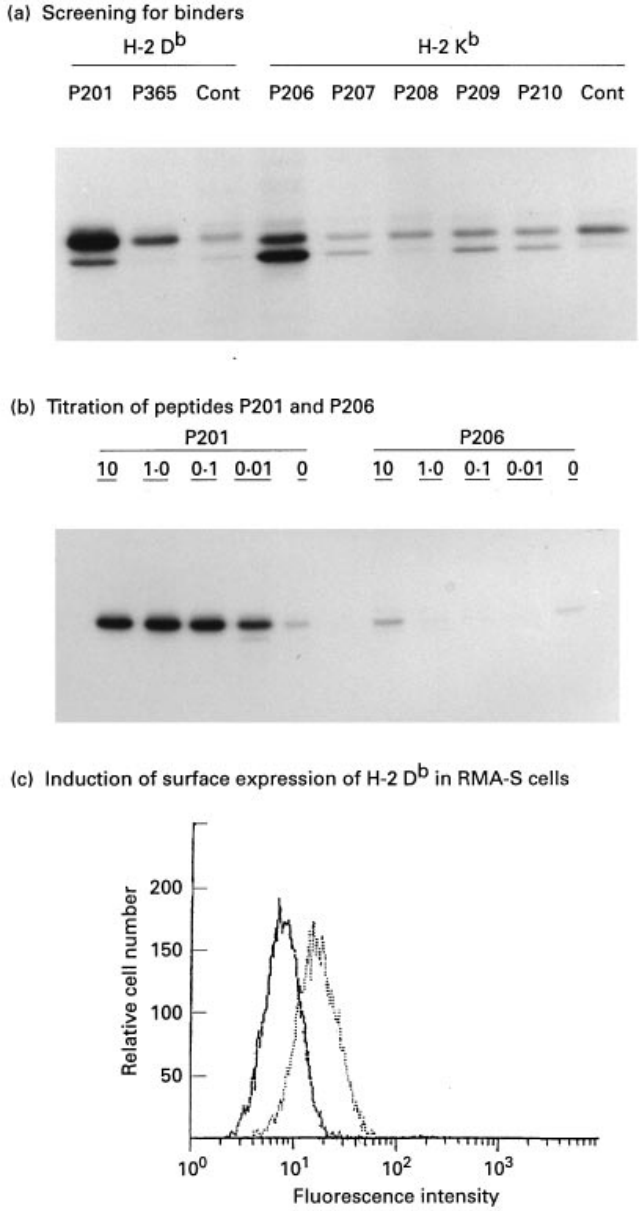

Fig. 1. Binding affinity of murine type II collagen (mCII) peptides to H-2 D^b and K^b molecules. (a) Assembly assay to test the ability of synthetic peptides to bind to H-2 D^b and K^b molecules of RMA-S cells. Peptide concentration in the lysate was 50 μM. (b) Titration of peptides P201 and P206 in assembly assay using RMA-S cells. The binding affinity of P201 to H-2 D^b is at least 1000-fold higher than that between P206 and the H-2 K^b molecules. (c) FACS analysis of RMA-S cells that had been treated with either peptide P201 (dotted line) or P206 (solid line) at 26°C. The cells were stained sequentially with MoAb B22 and FITC-conjugated goat anti-mouse IgG. These cells were also stained with MoAb Y3; only background level of staining was observed.

D^b and H-2 K^b molecules, respectively. Titration of P201 and P206 in subsequent assembly assays showed that P201 was a very strong binder to H-2 D^b (definite binding at 10 nM or less), whilst the binding affinity between P206 and H-2 K^b was at least 1000-fold lower (Fig. 1b). When used to treat RMA-S cells (26°C for 18 h), P201 induced a four-fold increase of D^b antigen expression on the cells (Fig. 1c).

Induction of mCII-specific CTL in vivo

RMA-S cells were treated with peptide P201 at 26°C for 18 h. A portion of the treated cells were stained for H-2 D^b expression (as shown in Fig. 1c) and the rest washed, irradiated (60 Gy) and inoculated intraperitoneally into female B10 mice. Peptide P206 was also included as a negative control. Spleen cells from these mice were harvested 3 weeks later and tested for lysis activity against appropriate peptide-pulsed EL-4 cells. As demonstrated in Fig. 2a, spleen cells from mice immunized with P201, but not P206, showed significant specific CTL activity against P201-treated EL4 cells. Chinese hamster ovary (CHO) cells transfected with either H-2 D^b or HLA-B27 gene [26] were pulsed with P201 and used as target cells in ^{51}Cr-release assays. Only H-2 D^b-expressing CHO cells were able to present P201 to these CTL (not shown). Lysis of the target cells by mCII-specific CTL was inhibited by MoAb against murine CD8, but not CD4 (not shown), indicating that the killer cells were $CD4^-$ and $CD8^+$.

mCII-specific CTL do not cause arthritis in B10 mice

Three weeks after the first immunization with peptide-pulsed RMA-S cells, groups of mice were boosted intraperitoneally with the same preparations. The immunized mice were monitored for frequencies of mCII-specific CTLp in the spleen and also regularly observed for signs of polyarthritis. As shown in Fig. 2c, the frequencies of P201-specific CTLp in mice immunized with P201 maintained at no less than 1/5000 for at least 5 weeks after booster immunization (Fig. 2c), but none of them developed significant polyarthritis (not shown). Histological examination was performed on joint sections from these mice, and no apparent synovitis was observed (not shown).

DISCUSSION

Class I MHC molecule can present not only endogenously produced protein fragments but also exogenous peptides to specific CTL [21,27]. The articular chondrocytes that express CII are buried in the cartilage and not normally accessible to T lymphocytes [28]. However, it may be possible for class I MHC molecules expressed on synovial membrane cells to present degraded fragments of CII or other proteins in the joints and stimulate an autoimmune CTL response [12]. A sustained CTL response in the joints would cause local inflammation and ultimately arthritis.

Our results demonstrate that CTL specific for autologous CII can exist *in vivo*. This is in agreement with our earlier finding of B27-restricted CTL specific for human CII in a patient with ReA [12]. Interestingly, the collagen peptide (P109, amino acid sequence: SRFTYTALK) recognized by the human CTL is also from the C-propeptide region of the pro-α1(II) molecule [12]. Chiocchia *et al.* have recently made $CD8^+$ cytotoxic T cell hybridomas specific for mCII by fusing hybridoma cells with splenocytes from C3H.Q (H-2^q) mice immunized with CII in adjuvant [29]. Vaccination with irradiated CII-specific hybridoma cells attenuated CII/Freund's complete adjuvant (FCA) arthritis in (C3H.Q $\times$ AKR) F_1 mice [29], implying that CTL could play an important role in arthritis. However, their data did not show how these CTL were induced *in vivo*; normally immunization with CII in FCA elicits $CD4^+$ helper T cell, rather than CTL, responses [6,26,30]. Furthermore, it was not clear whether these putative CTL would play a protective or pathological role *in vivo*.

Genetic make-up of animals and humans can determine their susceptibility to autoimmune diseases [1,6,31,32]. In the mouse

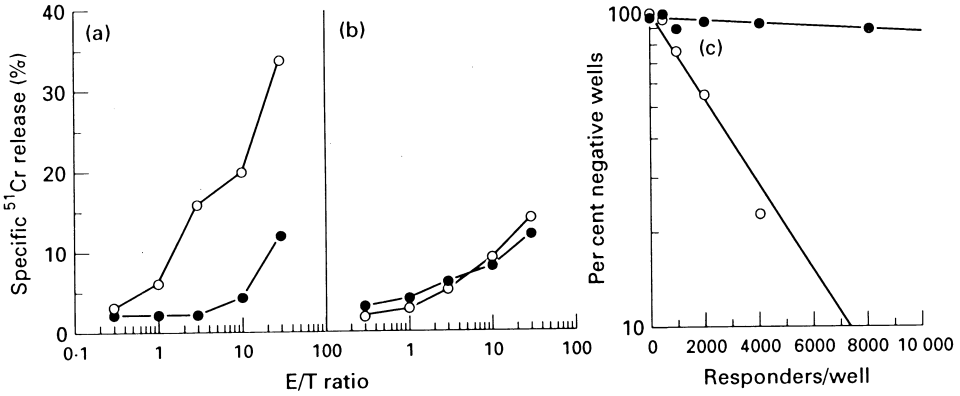

Fig. 2. Induction of murine type II collagen (mCII)-specific cytotoxic T lymphocytes (CTL) in B10 mice. ^{51}Cr-release assay using splenocytes, from B10 mice immunized with P201- (a), or P206- (b) treated RMA-S cells, as effectors and EL-4 cells pulsed with either P201 or P204 as target cells. Spontaneous release of ^{51}Cr by EL-4 cells was < 15%. (c) Limiting dilution analysis using splenocytes from B10 mice 5 weeks after the booster immunization with peptide-pulsed RMA-S cells**.** The P201-specific CTLp frequency, estimated from the intercept of the regression line at 37% negative wells, was 1/2500, whilst that for P206 was less than 1/10^7. ○, P201; ●, P206.

model, some strains (e.g. H-2^q and H-2^r haplotypes) of mice are much more susceptible than others (e.g. H-2^b haplotype) to collagen-induced arthritis (CIA) [6]. Most previous studies on T cell-mediated arthritis have been carried out in susceptible strains of mice or rats [6]. It is equally important, however, to understand the mechanisms of natural resistance of resistant strains. Our results suggest that B10 mice are not tolerant to autologous CII. Despite the strong anti-mCII CTL responses induced by immunization with P201/RMA-S cells, however, the immunized B10 mice did not develop significant polyarthritis or synovitis. Both CD4$^+$ and CD8$^+$ T lymphocytes are found in synovial fluid from healthy joints, although their numbers may be small. It is therefore reasonable to suggest that P201-specific CTL could also enter the joints (at least they should not be specifically excluded from the joints). Why then did the potentially autoreactive CTL not cause immunopathological reactions *in vivo*? The following possibilities may be considered: (i) class I MHC antigens may be expressed at a lower level by the synovial membrane cells of B10 mice compared with that in susceptible strains; (ii) potentially autoreactive T lymphocytes may be kept under control by the regulatory (or suppressive) T cells *in vivo*. In this regard, it would be of importance to compare the nature of mCII-specific CTL responses in B10 mice and that in mice of H-2^q or H-2^r haplotypes. Unfortunately, our approach relies on the knowledge of motifs of the epitopes presented by class I MHC antigens. Once the epitope motifs presented by H-2^q or H-2^r class I molecules becomes available, we should be able to address this question directly; (iii) unlike most proteins, collagen molecules are heavily glycosolated and rich in hydroxyl lysine residues. Michaelsson *et al.* and Haurum *et al.* have shown that carbohydrates of glycopeptides of CII can be recognized by specific CTL [32,33]. Thus, differences between naturally produced and synthetic collagen peptides (e.g. P201) might also be responsible for the inability of the P201-specific CTL to trigger inflammatory reactions *in vivo*. We consider this unlikely, however, because peptide P201 contains neither a glycosylation site nor a lysine residue (Table 1); (iv) peptide P201 is in the C-propeptide region of mCII which is cleaved off during the maturation process of the molecule. The turn-over of CII in adult mice is very slow (K. Chia, personal communication), and the concentration of C-propeptide fragments in the joints may therefore be too low to attract P201-specific CTL to the joints.

Taken together, the results of our successful induction of mCII-specific CTL in mice have opened up new prospects in investigating the role of autoimmune CTL in arthritis. The methodological approach that we have described could be conveniently adapted to the study of other potential autoantigens.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust and Medical Research Council, UK.

REFERENCES

1 Bennet PH, Burch TA. Population studies of rheumatic diseases. Amsterdam: Exerpta Medica Foundation, 1968: 456–7.
2 Nepom GT, Erlich H. MHC class-II molecules and autoimmunity. Annu Rev Immunol 1991; **9**:493–525.
3 Schlosstein L, Terasaki PI, Bluestone PDR, Pearson CM. High association of HL-A antigen, w27, with ankylosing spondylitis. N Eng J Med 1973; **288**:704–5.
4 Brewerton DA, Caffrey M, Hart FD, James DCO, Nicholls A, Sturrock RD. Ankylosing spondylitis and HL-A 27. Lancet 1973; **1**:904–7.
5 Brewerton DA, Caffrey M, Nichollos A, Walter D, Ocates JK, James DCO. Reiter's disease and HL-A 27. Lancet 1973; **2**:996–8.
6 Holmdahl R, Anderson M, Goldschmidt TJ, Gustafsson K, Jansson L, Mo L. Type II collagen autoimmunity in animal and provocations leading to arthritis. Immunol Rev 1990; **118**:193–232.
7 Londei M, Savill CM, Verhoef A *et al.* Persistence of collagen type II-specific T-cell clones in the synovial membrane of patient with rheumatoid arthritis. Proc Natl Acad Sci USA 1989; **86**:636–40.
8 Hermann E, Yu DTY, zum Buchenfelde KHM, Bernhrd F. HLA-B27-restricted CD8 T cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis. Lancet 1993; **342**:646–50.
9 van Eden W, Thole JER, van der Zee R *et al.* Cloning of the mycobacterial epitope recognised by T lymphocytes in adjuvant arthritis. Nature 1988; **331**:171–3.
10 van Eden W, Holoshitz J, Nevo Z *et al.* Arthritis induced by a T-lymphocyte clone that responds to *Mycobacterium tuberculosis* and to cartilage proteoglycans. Proc Natl Acad Sci USA 1985; **82**:5117–20.

11 Breedveld FC, Struyk L, van Laar JM *et al.* Therapeutic regulation of T cells in rheumatoid arthritis. Immunol Rev 1995; **144**:5–16.
12 Gao XM, Wordsworth P, McMichael AJ. Collagen-specific cytotoxic T lymphocyte responses in patients with ankyosing spondylitics and reactive arthritis. Eur J Immunol 1994; **24**:1665–70.
13 Hammerling GJ, Hammerling U, Lemke H. Qat-4 and Qat-5, new murine T-cell antigens governed by the Tla region and identified by monoclonal antibodies. J Exp Med 1979; **150**:108–16.
14 Hammerling GH, Rush E, Tada N *et al.* Localisation of allodeterminants on H-2K^b antigens determined with monoclonal antibodies and H-2 mutant mice. Proc Natl Acad Sci USA 1982; **79**:4727–32.
15 Cobbold SP, Jayasuriya A, Nash A. Therapy with monoclonal antibodies by elemination of T cell subsets *in vitro*. Nature 1984; **312**:548–52.
16 Zhang W, Young AC, Imarai M *et al.* Crystal structure of the major histocompatibility complex class I H-2Kb molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. Proc Natl Acad Sci USA 1992; **89**:8403–7.
17 Matsumura M, Fremont DH, Peterson PA *et al.* Emerging principles for the recognition of peptide antigens by MHC class I molecules. Science 1992; **257**:927–34.
18 Rammensee HG, Falk K, Rotzschke O. Peptides naturally presented by MHC class I molecules. Annu Rev Immunol 1993; **11**:213–44.
19 Metsaranta M, Toman D, de Crombrugghe B *et al.* Mouse type II collagen: complete nucleotide sequence, exon structure, and alternative splicing. J Bio Chem 1991; **266**:16862–9.
20 Metsaranta M, Toman D, de Crombrugghe B *et al.* Mouse type II collagen gene. J Biol Biochem 1989; **266**:16862–9.
21 Townsend A, Ohlen C, Bastin J *et al.* Association of class I major histocompatibility heavy and light chains induced by viral peptides. Nature 1989; **340**:443–8.
22 Ljunggren HG, Stam NJ, Ohlen C *et al.* Empty MHC class I molecules come out in the cold. Nature 1990; **346**:467–80.
23 De Bruijin ML, Schumacher TN, Nieland JD *et al.* Peptide loading of empty MHC molecules on RMA-S cells allows the induction of primary CTL responses. Eur J Immunol 1991; **21**:2963–70.
24 Elvin J, Cerundolo V, Elliott T *et al.* A quantitative assay of peptide-dependent class I assembly. Eur J Immunol 1991; **21**:2025–3.
25 Volkman A, Collins FM. Role of hos factors in the pathogenesis of Salmonella-associated arthritis in rats. Infect Immun 1976; **13**:1154–60.
26 Gao XM, Tite JP, Lipscombe M *et al.* Recombinant *Salmonella typhimurium* strains that invade nonphagocytic cells are resistant to recognition by cytotoxic T lymphocytes. Infect Immun 1992; **60**:3780–9.
27 Townsend AMR, Rothbard J, Goteh FM *et al.* The epitopes of influenza nucleoprotein recognised by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 1986; **62**:285–95.
28 Gradially FN. Fine structure of joints. In: Sokoloff L, ed. The joints and synovial fluid. New York: Academic Press, 1978: 140–56.
29 Chiocchia G, Boissier MC, Manoury B *et al.* T cell regulation of collagen-induced arthritis in mice. II. Immunomodulation of arthritis by cytotoxic T cell hybridomas specific for type II collagen. Eur J Immunol 1993; **23**:327–52.
30 Holmdahl R, Karlsson M, Andersson ME *et al.* Localisation of a critical restriction site on the I-A^b chain that determines susceptibility to collagen-induced arthritis in mice. Proc Natl Acad Sci USA 1989; **86**:9475–9.
31 Griffiths MM. Immunogenetics of collagen-induced arthritis in rats. Intern Rev Immunol 1988; **4**:1–15.
32 Michaelsson E, Malmstrom V, Reis S *et al.* T cell recognition of carbohydrates on type II collagen. J Exp Med 1994; **180**:745–9.
33 Haurum JS, Arsequell G, Lellouch *et al.* Recognition of carbohydrate by major histocompatibility complex class I-restricted glycopeptide-specific cytotoxic T lymphocytes. J Exp Med 1994; **180**:739–44.