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Delayed type hypersensitivity (DTH) to type II collagen (CII) in DBA-1 mice

LYNNE M. FARMER, G. WATT, M. GLATT, A. BLAETTLER, N. LOUTIS & U. FEIGE *Department of Inflammation, Ciba-Geigy Research Labs, Basel, Switzerland*

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SUMMARY

Sensitization of DBA-1 mice with Type II collagen (CII) in complete Freunds adjuvant can cause polyarthritis. A possible link between CII-induced arthritis and delayed type hypersensitivity (DTH) has been suggested, so we decided to investigate the susceptibility of DBA-1 mice to CII induced DTH reactions. The mice were primed with a dose of 10 μg CII i.p. 4 days before challenging with 40 μg CII in the ear. Swelling was measured 48 h later and was found to be reproducible. Responsiveness to CII could be transferred with whole spleen cell populations from primed animals or with enriched spleen T cells, thus confirming the cellular nature of the reaction. Lymph node cells from CII/CFA footpad immunized animals were restimulated with CII *in vitro*. These cells were able to passively transfer DTH sensitivity *in vivo* and exhibited specificity for this antigen *in vitro* in proliferation assays.

Keywords Type II collagen delayed type hypersensitivity arthritis DBA-1 mice.

INTRODUCTION

Autoimmunity to Type II collagen (CII) is considered a possible mechanism in the pathogenesis of human rheumatoid arthritis (RA) and in patients both cellular (Trentham *et al.*, 1978) and antibody mediated (Andriopoulos *et al.*, 1976) immune responses to CII have been found. The development of corresponding CII arthritis animal models both in rats (Trentham, Townes & Kang, 1977) and in mice (Courtenay *et al.*, 1980) has allowed characterization of the infiltrating lymphocytes in joints as mainly $T_{h/dth}$ cells (Klareskog *et al.*, 1982). CII was found to be a potent arthritogen only in its native form (Trentham, Townes & Kang, 1977, 1978; Stuart, Townes & Kang, 1982) and genetic association of susceptibility can be seen (Griffiths *et al.*, 1981; Wooley *et al.*, 1981, 1983). Suggestions have been made that the inflammatory reaction may be a form of self-perpetuating delayed type hypersensitivity (DTH) response to CII (Klareskog *et al.*, 1982; Brackertz *et al.*, 1977) which finally leads to joint destruction.

Several groups working on animal models of autoimmunity have raised antigen specific $T_{h/dth}$ cell lines which are capable of transferring disease symptoms (Holoshitz *et al.*, 1983; Ben-Nun, Wekerle & Cohen, 1981a, 1981b; Ben-Nun & Cohen, 1981; Hohlfeld *et al.*, 1984; Maron *et al.*, 1983). In a model of experimental autoimmune enchephalitis (EAE) these cells were also found to mediate an antigen specific DTH in the naive recipients (Holoshitz *et al.*, 1984) suggesting a possible link between these two immunological phenomenon.

In this study the nature of the DTH response to CII in DBA-1 mice was examined. The results show that DBA-1 mice can be made susceptible to CII DTH; that the preparation of the CII solution is critical to the response; that the responsiveness is transferable with spleen T cells and also

Correspondence: Dr U. Feige, Ciba-Geigy AG, R-1056. 2.17, CH-4002 Basel, Switzerland.

with lymph node cells from footpad primed animals and that these cells show in-vitro specificity for CII.

MATERIALS AND METHODS

DBA-1/J mice. Mice were obtained from the Jackson Laboratory, Bar Harbour, Maine, USA. Age and sex matched animals were used in all experiments.

Media. RPMI 1640 (Gibco, Paisley, UK) was used throughout. For use in cell culture, supplements were added as follows: 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, 1% (× 100) BME amino acids, 1% (× 100) BME vitamins, 1% (× 100) MEM non essential amino acids (all supplied by Gibco). Gentamycin, 0·14 mg/ml (SEROMED, Biochrom KG, FRG) was added along wth 0·005 mg/ml minocyclin (SEBIO GmbH).

Preparation of collagen II solutions. Bovine Type II Collagen was extracted from cartilage by methods previously described (Strawich & Nimmi, 1971) and dissolved for use in DTH experiments as follows. Lyophilized CII was dissolved overnight at 4°C at 2 mg/ml in sterile 0·1 M acetic acid (No. 62, Merck). For ear injections, only 2 ml CII solution was dialysed at 4°C for 20 h against 500 ml RPMI 1640 with $NaHCO_3$. The medium was changed once during this time.

Priming and challenging of animals for DTH. Animals were primed with 10 μg CII solution i.p. Four days later primed and unprimed controls were challenged. The mice were first anaesthetized by 0·25 ml by 10 mg/ml Evipan-Natrium (Bayer) i.p. then 40 μg CII was injected subcutaneously into the left ear in a 20 μl volume using a 5–50 μl Hamilton syringe and Acufirm 1400 LL22 needles. Swelling was measured 24, 48 and 72 h later using an engineers micrometer accurate to 0·01 mm (Oditest, H.C. Kroeplin GmbH, Switzerland). Results are expressed as per cent increase in thickness of left ear compared to the right ear. The volumes and concentrations of CII were as described above unless otherwise stated.

Preparation of spleen and T cells for DTH transfer. Spleens were removed aseptically from mice and single cell suspensions may be pressing the spleens through a metal sieve. The cells were then washed twice in Dulbecco's Phosphate Buffered Saline (PBS) pH 7·4 and either injected into animals directly or the T cell fraction was enriched using nylon wool columns (Julius, Simpson & Herzenberg, 1973). Animals were challenged for DTH 1–2 h later along with primed and unprimed controls as described above.

Depletion of T-cell subsets. This was performed by panning cells incubated with anti-Lyt1 or anti-Lyt2 antibodies on plastic Petri dishes coated with affinity purified goat anti-mouse IgG (Mage, McHugh & Rothstein, 1977). Briefly spleen cell suspensions were depleted of B-lymphocytes and macrophages by passage over nylon wool columns. The cells were washed twice in PBS/FCS and incubated at 4°C for 30 min with the appropriate dilution of anti-Lyt1 or anti-Lyt2 monoclonal antibodies. The cells were spun, resuspended at 10^7 cells/ml and 3 ml of each suspension added per Petri dish. The plates were incubated for 1 h at 4°C, the non-adherent cells collected, washed twice in PBS and one spleen equivalent of cells per animal was injected i.v. in a volume of 0·25 ml.

In vitro restimulation of lymph node cells from footpad primed mice. Animals were injected with 50 μg CII in CFA in each hind footpad on Day 0, Day 7 and the popliteal lymph nodes were harvested on Day 16 (Maron *et al.*, 1983). The lymph nodes were teased apart and the cell suspension was washed twice. The cells were maintained in RPMI 1640 complete medium containing 1% syngeneic serum and additives (see Media) with 4 μg/ml CII at 34°C, 5% CO_2 for 3 days. The cells were washed once in PBS before reinjecting i.v. in a volume of 0·25 ml. Animals were challenged for DTH 1–2 h later along with primed and unprimed controls.

Proliferation assays. Lymph node cells from primed mice (see above) were plated into 96-well flat-bottomed plates (Falcon) at 5×10^5 cells per well with various dilutions of Type II collagen (CII), Type I collagen ((CI), Bovine Achilles, Sigma) and the mitogen Concanavalin A (Con A, Sigma). The cells were pulsed with 2 μCi of tritiated thymidine 3 days later ([6-^{3}H] thymidine, Amersham, UK) per well.

Table 1. Time course of DTH responses to CII in DBA-1 mice

	Priming dose i.p.	Challenging dose s.c. ear	% Ear swelling ± s.d. 24 h	48 h	72 h	*n*
Experiment 1	—	PBS	12±11	5±3	12±8	5
	—	40 μg CII	10±8	10±10	11±11	5
	10 μg CII	40 μg CII	27±7	47±13	29±4	5
Experiment 2	—	PBS	8±5	6±4	3±2	5
	—	40 μg CII	15±18	20±9	10±9	5
	10 μg CII	40 μg CII	22±10	25±7	23±6	5
Experiment 3	—	PBS	4±6	8±7	6±6	5
	—	40 μg CII	31±9	22±5	16±7	5
	10 μg CII	40 μg CII	40±14	45±14	37±19	5

Table 2. Challenge only control ear swelling is dependent on the age of the dialysed CII solution

Age of CII solution after dialysis	1 day	2 days	7 days
% Ear swelling in challenge only controls	15±4	26±11	70+14
n	41	5	5

RESULTS

Time course of DTH responses to CII in DBA-1 mice. For DTH to CII in DBA-1 mice a priming dose of 10 μg CII in 0·1 M acetic acid i.p. followed 4 days later by a challenging dose of 40 μg dialysed CII in the ear was found to be optimal (data not shown). The time course of ear swelling followed the pattern expected for a normal DTH (Table 1) with maximal responses at 48 h after challenge. In eight experiments the average swelling reached 36 ± 14% ($n=41$) whereas in challenge only controls the response averaged 15 ± 7% ($n=41$). Animals challenged with PBS alone gave a response of 9 ± 7% ($n=44$).

Dependency of challenge only control swelling on the age of the dialysed CII used. In early attempts to optimize the system it was noted that the responses in the challenge only controls increased considerably if the collagen preparation used was not freshly prepared. Table 2 shows that solutions which had been dialysed overnight and then kept for some time at 4°C caused an increase in challenge only control swelling. It was also noted that the dialysis buffer or medium used was critical to obtaining a low background response (data not shown). RPMI 1640 was found to be the most suitable.

DTH transfer with spleen (T) cells from primed animals. All classical DTH responses are known to be mediated by cells of the $T_{h/dth}$ phenotype, so the ability of spleen T cells from primed animals to transfer susceptibility to DTH was tested. Mice were primed as previously described and spleens removed after 4 days; spleen T cells were enriched on nylon wool. Both whole spleen suspensions

Table 3. Induction of susceptibility to DTH by transfer of spleen (T) cells from primed DBA-1 mice

				% Ear swelling ± s.d. at 48 h		
					Controls*	
Experiment No.	Cell type	Cell no. $\times 10^{-6}$	*n*	Cell transfer + challenge	Prime + challenge	Challenge only
1	Spleen	100	5	33 ± 16	32 ± 15	16 ± 4
2	Spleen	100	5	23 ± 13	36 ± 16	10 ± 4
3	Spleen T	50	4	33 ± 13	ND	18 ± 6
4	Spleen	100	5	21 ± 14	32 ± 18	12 ± 17
		50	5	25 ± 7		
		25	5	13 ± 6		
	Spleen T	50	5	41 ± 5		
		25	5	31 ± 6		
		12	5	20 ± 7		
	Normal spleen	100	5	3 ± 3		

* 5 animals per group.
ND = not done

Table 4. Induction of susceptibility to DTH by transfer of Lyt1$^+$ but not Lyt2$^+$ spleen T cells from footpad primed DBA-1 mice

	% Ear swelling ± s.d. at 48 h*		
		Controls	
Cells transferred	Cell transfer + challenge	Prime + challenge	Challenge only
Spleen T	26 ± 10	28 ± 7	5 ± 4
Spleen T Lyt1$^+$ depleted	7 ± 8		
Spleen T Lyt2$^+$ depleted	21 ± 6		
Normal spleen T cells	11 ± 7		

* 10 animals per group.

and the T cell enriched fraction were able to transfer susceptibility to CII DTH to naive DBA-I mice (Table 3). By depletion of the splenic T cell population from either Lyt1$^+$ or Lyt2$^+$ cells it was shown that the DTH transferring capacity was within the Lyt1$^+$ population (Table 4).

Transfer of DTH with lymph node or spleen cells from footpad primed mice. T cells capable of transferring susceptibility to DTH were also found in the popliteal lymph nodes of footpad immunized DBA-I mice. The in-vitro restimulated cells enabled the recipient to make an excellent

Table 5. Induction of DTH susceptibility by transfer of spleen (T) or popliteal lymph node cells from primed DBA-1 mice

				% Ear swelling ± s.d. at 48 h		
					Controls*	
Experiment No.	Cell type	Cell no. $\times 10^{-6}$	*n*	Cell transfer + challenge	Prime + challenge	Challenge only
1	Lymph node	10	10	54 ± 15	32 ± 8	15 ± 4
		5	10	45 ± 15		
		2·5	10	35 ± 15		
	Spleen	10	10	35 ± 6		
		5	10	25 ± 6		
		2·5	10	27 ± 9		
2	Lymph node	10	10	36 ± 16	37 ± 15	8 ± 4
		5	10	31 ± 8		
		2·5	10	29 ± 11		
	Spleen T	10	10	36 ± 11		
		5	10	34 ± 14		
		2·5	10	35 ± 12		

* 10 animals per group

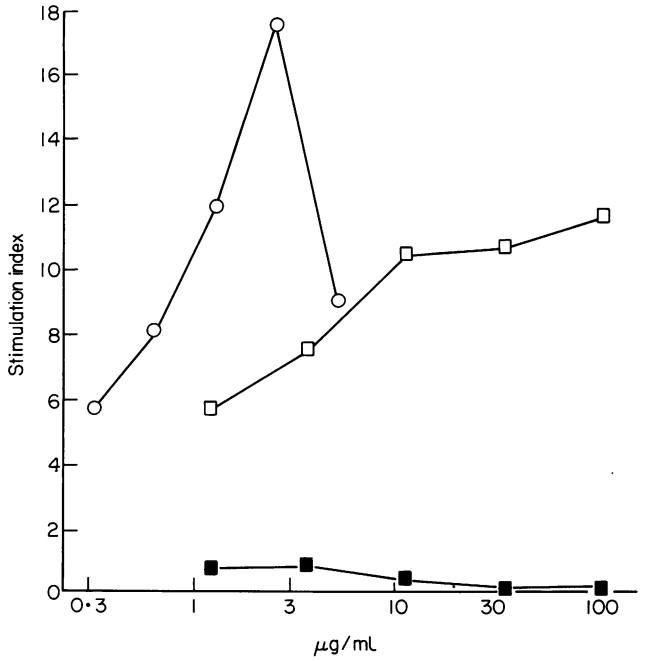

Fig. 1. In-vitro response of lymph node cells to (□) Type II collagen, (■) Type I collagen and (○) Concanavalin A

DTH response upon challenge to CII. The measurements obtained were as high or higher than those of the actively primed and challenged animals of the positive control group (Table 5). Spleen cells from the same animals also transferred susceptibility to DTH. When spleen T cells are enriched the response increases to approximately the same as that of lymph node cells (Table 5). The in-vitro specificity of the lymph node cell preparations used above was tested by their proliferative response to CII, CI and Con A at various concentrations (Fig. 1). The response to CII was high (max. stimulation index (SI) = 12,229,924 ct/min) when compared to a relatively crossreactive antigen CI (max. SI = 1). Stimulation of the cells with the T cell mitogen Con A shows maximal proliferative responses (SI = 18) at 2·5 μg/ml.

DISCUSSION

In several animal models of experimental autoimmune disease, T cell lines have been established which can transfer disease symptoms, e.g. thyroiditis (Maron *et al.*, 1983), experimental autoimmune encephalomyelitis (EAE) (Ben-Nun *et al.*, 1981b), myasthenia gravis (Hohlfeld *et al.*, 1984) and arthritis (Holoshitz *et al.*, 1983). Holoshitz *et al.* have shown that T cell lines responsive to basic protein of myelin (BP) can not only transfer EAE but, if attenuated, can protect against actively induced disease (Ben-Nun, Wekerle & Cohen, 1981a). These lines have recently also been found to transfer susceptibility for a DTH skin reaction in an antigenspecific manner (Holoshitz *et al.*, 1984). This transfer of susceptibility to DTH was similar to transfer of EAE in that in both cases the cells had to be activated, *in vitro*, by BP or Con A. T cells mediating DTH have also been associated with cells responsible for graft rejection in mice (Loveland *et al.*, 1981). Both the graft rejection mechanism and DTH sensitivity were found to be contained in the Lyt1$^+$ cell population.

The linkage of EAE and graft rejection with cells involved in DTH responses suggested the possibility that DTH reactions could be used as a primary in-vivo screen for potentially autoreactive T cell lines. In order to screen for T cell lines which are capable of inducing CII arthritis we have set up this DTH model to CII in DBA-1 mice. CII has been suggested by many workers as one of the target antigens in RA although the initiating antigen of the disease is not yet known (Andriopoulos *et al.*, 1976; Trentham *et al.*, 1978). Normally CII arthritis takes 4–8 weeks to manifest itself in DBA-1 mice so in-vivo screening of the arthritogenic potential of cell lines is time-consuming. In order to get a first insight into whether a cell line with in-vitro CII specificity has any potential activity *in vivo*, CII DTH may be a quick and useful method.

We have shown here that DBA-1 mice can indeed be made susceptible to CII DTH. A reproducible swelling was found to be critically dependent on the use of fresh CII solution for unknown reasons (Table 2).

The true DTH nature of the response has been demonstrated by both time course experiments (Table 1) and by the ability of Lyt1$^+$ cells to transfer susceptibility, thus confirming the cellular nature of the response (Table 4).

In preliminary attempts to raise CII specific T cell lines, the optimal method was found to be the classical footpad injection with CII/CFA emulsion (data not shown). Both spleen cells and lymph node cells from these animals have been shown to be capable of transferring in-vivo reactivity to CII using the DTH model (Table 5). In-vitro responses of lymph node cells showed marked specificity for CII compared to CI as a stimulating antigen (Fig. 1). From these results we conclude that lymph node cells from animals immunized in this way could provide a source of potentially in-vivo reactive T cell lines which could be used to study the role of CII specific T cells in arthritis.

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