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Pinealectomy ameliorates collagen II-induced arthritis in mice

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SUMMARY

To extend our previous findings that exposure to constant darkness (stimulation of endogenous melatonin release) as well as treatment with exogenous melatonin magnifies the severity of collagen-induced arthritis in mice, we have examined the effects of melatonin cutback by removing the pineal gland. Two strains of mice, DBA/1 and NFR/N, were subjected to surgical pinealectomy. The melatonin levels in sera were reduced by approximately 70% by the pinealectomy compared with the corresponding sham-operated controls. After 3–4 weeks of rest the mice were immunized with rat type II collagen to induce autoimmune arthritis, and the animals were kept in constant darkness during the experiments. In comparison with the controls, all groups of pinealectomized mice showed reduced severity of the arthritis by means of (i) a slower onset of the disease, (ii) a less severe course of the disease (reduced clinical scores), and (iii) reduced serum levels of anti-collagen II antibodies. These effects were not significant in all experiments, but the trends were always the same. Thus, the present results strengthen the hypothesis that high physiological levels of melatonin (which can be induced by exposure to darkness) stimulate the immune system and cause exacerbation of autoimmune collagen II arthritis, while inhibition of melatonin release (pinealectomy or exposure to light) has a beneficial effect.

Keywords pinealectomy collagen II arthritis mice melatonin

INTRODUCTION

The pineal gland is now recognized as an important neuroendocrine transducer organ. It is involved in the regulation of many vital processes such as seasonal reproduction, and also affects thyroid, pituitary and adrenocortical function. These effects are mainly controlled by the hormone melatonin, which is synthesized and released in a circadian pattern by the pineal gland. Melatonin is physiologically regulated by length and intensity of photic stimuli, and has its highest concentrations during the dark period of the day.

The pineal gland is not the only source of melatonin production. It has also been found in the retina [1], Harderian glands, [2], intestine [3,4] and brain [5,6], but about 80% of the melatonin produced is of pineal origin.

Several investigators have also proposed an immunoregulatory role of the pineal gland. Maestroni and coworkers [7–9] have observed an enhancement of T cell-mediated humoral immune responses by melatonin in antigen-primed mice, and pharmacological pinealectomy (β-blockade) has been found to suppress the same response [7,10]. It has also been reported that surgical pinealectomy inhibits humoral immune function in mice, and causes a depression of bone marrow progenitors for granulocytes and macrophages and inhibits humoral immune function in mice [11]. In addition, IL-2 production and natural killer (NK) cell activity is reduced in C57Bl/6 mice after pinealectomy [12]. On the other hand, neonatal removal of the pineal gland in rats appears to have no effect on skin graft rejection or mitogen-induced lymphocyte proliferation [13].

Our earlier findings that constant darkness as well as administration of exogenous melatonin enhance the course of collagen-induced arthritis (CIA) in DBA/1 mice [14,15] has led us to examine if CIA is affected by melatonin depression through pinealectomy. We have chosen to perform a surgical pinealectomy to avoid too much interference with other physiological functions, which are common side effects when using β-blockers (propranolol) or enzyme inhibitors. The experiments have been performed by using two different mouse strains, DBA/1 and NFR/N, both of haplotype H-2^q, which are susceptible to the induction of CIA.

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MATERIALS AND METHODS

Animals

DBA/1 and NFR/N mice, both of haplotype H-2^q, 8 weeks old, were kept in our animal unit under normal lighting conditions (LD 12:12) before surgery, and were placed in constant darkness (LD 0:24) during the experiment. They were maintained at a temperature of 22 ± 1°C and given standard food pellets and drinking water *ad libitum*.

Surgical procedure
The animals were anaesthetized with a mixture containing 4·25 g chloral hydrate, 0·97 g sodium pentobarbital, 2·1 g magnesium sulphate, 42·8 g propylene glycol and 9 g ethanol in 100 ml solution and were given 4·5 ml/kg body weight. The skull was opened by using a dental drill (for details, see the method of Waynforth [16]). The pineal gland was immediately removed with a pair of forceps. In sham-operated animals the skull was opened but the pineal gland was left intact. The flap of bone was then replaced and dusted with an antibiotic powder (Terramycin, Pfizer) and the skin incision was closed with stitches. After surgery, the animals were left to recover for 3–4 weeks before the experiments were started.

Induction of arthritis
Native rat type II collagen (CII), prepared from a rat chondrosarcoma [17], was dissolved in 0·1 M acetic acid, emulsified in an equal volume of Freund's complete adjuvant (FCA; Difco, Detroit, MI) and injected intradermally in the skin around the base of the tail (50 μg in each mouse in a volume of 100 μl).

Evaluation of arthritis
Animals were regularly observed for signs of arthritis. The day when the first clinical signs can be observed has been defined as the onset day of the disease (normally 4–6 weeks after immunization). The median day of onset for a group of animals gives information about the speed of disease development. All observations were made under a red dark-room lamp. The degree of arthritis was evaluated by using a scoring system where each paw is scored from 1 to 3 (1 = detectable swelling of one joint, 2 = severe swelling of more than one joint, 3 = severe arthritis of the entire paw [18]). The onset day was set as the day of the first appearance of arthritis, and any mouse without clinical symptoms at the end of an experiment was given the day of sacrifice as onset day. The best trend curve showing development of arthritis is normally obtained by showing the mean of the clinical score within the group. Such graphs are not useful for statistical analyses because of the non-parametric nature of the data, but are valuable in providing a comprehensive picture of the development of the disease. Therefore, all statistical conclusions are based on a comparison of the median values of the clinical scores.

Measurement of antibodies to CII
Quantification of antibodies to CII in serum was performed by using ELISA as described elsewhere [18].

Quantification of melatonin in serum
Levels of serum melatonin were measured by using high performance liquid chromatography (HPLC) with electrochemical detection. The serum sample (100 μl) was extracted with 10 volumes of diethylether by vortex-mixing for 1 min. The sample was allowed to freeze and the ether phase was decanted and evaporized under a nitrogen stream, redissolved in 25 μl of the mobile phase (see below) and injected into the column. The HPLC system consisted of a Milton-Roy Minipump, model 396 (Laboratory Data Control, Riviera Beach, FL) and a Nucleosil RP-18 column, 3 μm particle size, 0·4 cm i.d. × 7·5 cm length (Machery-Nagel, Duren, Germany). The detector was a model LC-3 electrochemical detector (Bioanalytical Systems, Lafayette, IN) with a glass carbon electrode set at +0·9 V *versus* an Ag/AgCl reference electrode. The mobile phase consisted of 0·1 M sodium acetate, 0·1 M citric acid, 25% methanol (v/v) and 0·008% EDTA (w/v). The flow rate was 1 ml/min.

Evaluation of pinealectomy
When killed, each mouse was inspected to assure the completeness of surgery. Any mouse with possible remnants of the pineal gland was excluded.

Statistical analysis
The significance of the data was evaluated by using the Mann–Whitney *U*-test (scoring data and day of first appearance) or Student's *t*-test (levels of antibodies to CII).

RESULTS

Pinealectomy of DBA/1 mice
In both experiments, surgical pinealectomy caused similar effects: (i) a delay of the onset of the disease; (ii) a less severe arthritis (reduced median clinical scores); and (iii) lower levels of antibodies to CII compared with sham-ectomized controls. The delay of the onset of the disease (increased median of first day of clinical signs of arthritis) was significant ($P = 0·027$) in experiment 2 (Table 1). The difference in median clinical scores was not significant (Fig. 1, Table 1), while the reduction of the levels of anti-CII antibodies in serum of pinealectomized mice was significant ($P = 0·034$) in experiment 1 (Table 1).

Pinealectomy of NFR/N mice
Pinealectomy of NFR/N mice caused similar effects as in DBA/1 mice: (i) a delay of the onset of the disease; (ii) a less severe arthritis (reduced clinical scores) (Fig. 2); and (iii) reduced serum anti-CII levels compared with sham-ectomized animals (Table 1). The delay of the onset of the disease (increased median day for first clinical signs of arthritis) was significant (Table 1). The reduced severity of the ongoing disease (median clinical scores) was only significant on one occasion. The difference in levels of anti-CII antibodies was not significant (Table 1).

Serum levels of melatonin
Serum levels of melatonin in pinealectomized mice were reduced to about 30% of the levels in normal or sham-ectomized mice (Fig. 3).

DISCUSSION

We have earlier reported on the enhancement of CIA in response to (i) exposure to darkness (physiological stimulation of endogenous melatonin release) [14], and (ii) treatment with exogenous melatonin [15], which is in agreement with the present finding that low melatonin levels reduce the severity of arthritis.

By using surgical pinealectomy one can not eliminate melatonin production completely, since there are some other minor sources of melatonin synthesis in the organism (see Introduction). Melatonin can also be reduced by using pharmacological agents that either block adrenergic impulses or inhibit melatonin precursors. The use of adrenergic blockers, like propranolol, is bound to affect not only pineal adrenergic responses but all other adrenergic responses as well, while they

Table 1

Treatment	Days after immunization	*n*	Median day of onset	Arthritic mice (%)	Median score	Mean anti-CII (μg/ml)
DBA/1 Px (I)	35	9	63	0	0	
	45			33	0	
	60			44	0	
	80			55	1	253±144†
DBA/1 ShX (I)	35	19	45	21	0	
	45			37	0	
	60			58	2	
	80			60	5	496±309
DBA/1 Px (II)	35	22	79*	9	0	
	45			14	0	
	60			32	0	
	80			54	1·5	271±227
ShX (II)	35	32	51	22	0	
	45			22	0	
	60			53	3	
	80			63	4·5	298±252
NFR/N Px	35	10	68*	10	0	
	45			40	0	
	60			60	1·5	
	70			60	1	293±146
ShX	35	11	37	36	0	
	45			80	3	
	60			80	3	
	70			90	4	386±205

* Significantly higher than the corresponding control ($P < 0{\cdot}05$).
† Significantly lower than the corresponding control ($P < 0{\cdot}05$).
CII, Type II collagen; Px, pinealectomy; ShX, sham-ectomy.

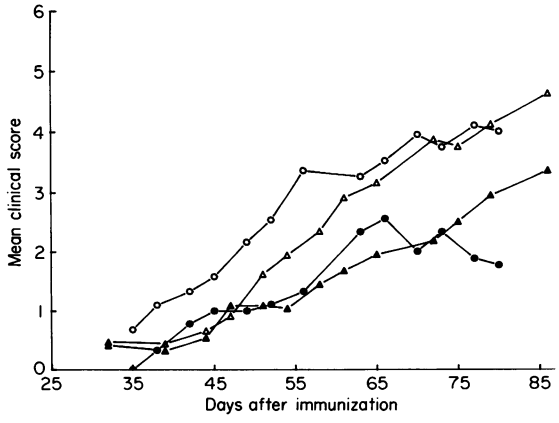

Fig. 1. Trend curve (mean clinical scores) showing severity of arthritis in pinealectomized (PX) and sham-operated (ShX) DBA/1 female mice kept in constant darkness (two experiments); medians and statistics in Table 1. ●, PX, experiment 1; ▲, PX, experiment 2; ○, ShX (experiment 1); △, ShX (experiment 2).

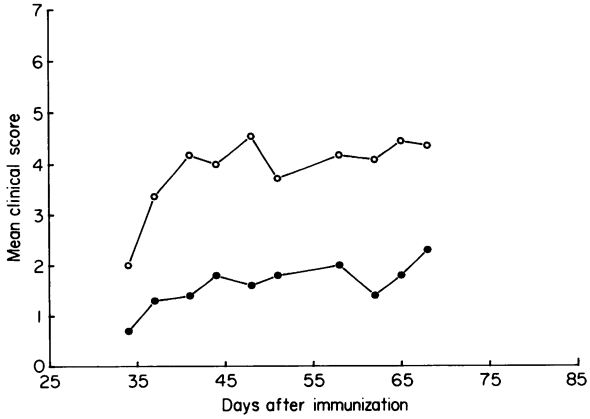

Fig. 2. Trend curve (mean clinical scores) showing severity of arthritis in pinealectomized and sham-operated NFR/N female mice kept in constant darkness (experiment 3); medians and statistics in Table 1. ●, Pinealectomized; ○, sham-ectomized.

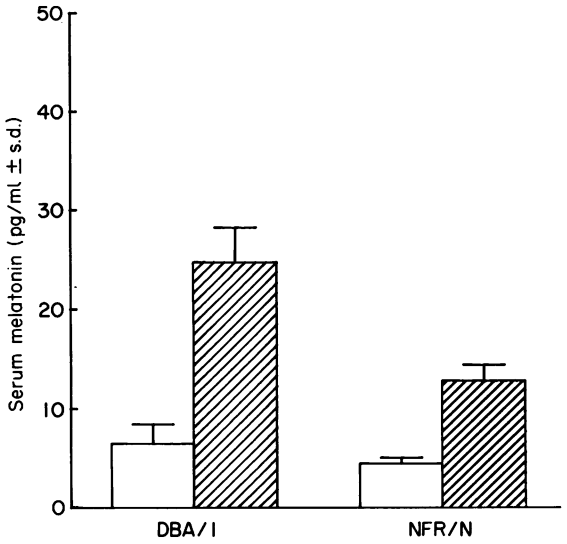

Fig. 3. Serum levels of melatonin at day (11.30–12.30) in pinealectomized (□) and sham-operated DBA/1 and NFR/N mice (▨) kept in constant darkness for 70 days. Each bar represents the mean of five observations. Error bars indicate s.d.

have been shown to have little or no effect on human rheumatoid arthritis [19]. The use of p-CPA (para-chlorophenylalanine) inactivates tryptophan-hydroxylase, thereby depleting the serotonin content of the central nervous system. However, certain immune reactions can be affected by manipulating the metabolism of serotonin [20,21]. Despite the fact that some melatonin release will remain in pinealectomized mice, similar ectomy-effects on the course of the arthritis (not significant in all groups) were seen in all three experiments performed: (i) a delay of the onset of the disease; (ii) reduced severity by means of clinical scores (ongoing arthritis); and (iii) reduced serum levels of CII antibodies. The delay of the onset of the disease was significant in two out of three experiments, and the medians of the clinical scores were very close to significance (significant on scattered days only; not shown in the figures). The decrease in serum levels of CII antibodies was significant in one out of three experiments.

We consider the present results to be important, since they strengthen the following earlier observations: (i) mice subjected to constant darkness (a physiological up-regulation of melatonin secretion), develop a much more severe arthritis than mice kept in constant light or under normal lighting conditions [14]; (ii) mice injected with melatonin develop a more severe CIA [15].

Taken together, all results obtained so far indicate that pineal melatonin is the major hormone responsible for the darkness-induced exacerbation of collagen arthritis. Reversed, it could be postulated that inhibition of melatonin will slow down the course of CIA and reduce the frequency and/or delay the onset.

It still remains unclear by which mechanism melatonin exerts its effects in CIA. CIA is known to be dependent on both T and B cell autoimmune reactions to CII [22]. In this, as well as in earlier studies [14,15], we have shown that melatonin stimulates, and lack of melatonin decreases, anti-CII B cell activity. Since this B cell response is T cell-dependent [22], melatonin may interact with lymphocyte activation, involving autoreactive T or B cells, or both. This possibility is in accordance with the findings of Maestroni and coworkers [9] that melatonin affects the activation of T cells directly, via specific or non-specific receptors. However, melatonin may also affect lymphocyte activation indirectly, via the release of various other hormones, such as pituitary prolactin, adrenocortical steroids, ovarian sex steroids and endogenous opiates, all well known immunoregulatory hormones. Thus, it seems reasonable to believe that the effect of melatonin on CIA is caused by a complex combination of direct and indirect effects of various immunoregulatory hormones.

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