Immunology 1997 **92** 571–576

Differential display analysis of murine collagen-induced arthritis: cloning of the cDNA-encoding murine ATPase inhibitor

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SUMMARY

We used the differential display technique in order to detect a new gene involved in murine type II collagen-induced arthritis (CIA). In this study, we have identified a novel gene, IF_1, whose expression level is increased during the natural course of CIA. Northern blot analyses suggest that IF_1 is involved in the natural course of CIA but is not involved as a trigger of CIA. IF_1 is considered to be the murine ATPase inhibitor gene for several reasons. First, IF_1 shows an extremely high homology to the rat ATPase inhibitor; the highly conserved region between rat and bovine amino acid residues 22–45, which is the minimum sequence showing ATPase inhibitory activities, is also highly conserved in IF_1. Second, IF_1 possesses a histidine-rich region in the same area, which is thought to be important for regulation of mammalian inhibitors. Third, the tissue distribution of IF_1 is very suggestive. The expression of IF_1 was very strong in energetic organs such as the heart, brain and kidney, and the development of arthritis requires great amounts of ATP. As arthritis develops rapidly, the cellular ATP pool may be decreased. Before the ATP pool is exhausted, the ATPase inhibitor may serve as a brake for ATP hydrolysis. If the supply of free energy can be reduced, the inflammation of arthritis may in turn be restored. Our hypothesis is that the ATPase inhibitor is involved in regulating the inflammatory responses.

INTRODUCTION

Murine type II collagen-induced arthritis (CIA) is a useful model for rheumatoid arthritis.[1,2] CIA is characterized by severe swelling and erythema in one or more paws associated with an early massive, predominantly polymorphonuclear and subsequently mononuclear, cell infiltration into the synovium.[3] Both antibody and T-cell responses directed against type II collagen (CII) appear to be a prerequisite for the induction and chronicity of the local inflammatory process.[4,5] Furthermore, the major histocompatibility complex (MHC) appears to influence profoundly the susceptibility to CIA. For example, the induction of murine CIA is limited to strains of H-2^q or H-2^r haplotypes depending on the source of CII used for immunization.[6] As mentioned above, CIA has been investigated extensively, but all of the mechanisms responsible for the development and regulation of CIA have not been determined completely. In order to detect a new factor involved in CIA, we have used the differential display technique developed by Liang & Pardee.[7] In the present study we have identified a novel gene (called IF_1) whose expression level is increased during the natural course of CIA. The gene IF_1 is considered to be the murine ATPase inhibitor.

The ATP synthase–ATPase complex is the primary enzyme responsible for ATP synthesis in most cell types. It is located in the inner mitochondrial membrane of all eukaryotic cells and functions by coupling the energy of a proton electrochemical gradient to the synthesis of ATP. The ATP synthase–ATPase complex can be separated into two distinct units: F_0, a membrane proton channel, and F_1, the moiety that catalyses the synthesis of ATP.[8] A natural inhibitor peptide of the mitochondrial ATP synthase–ATPase complex, so called ATPase inhibitor, was first isolated from bovine heart tissue.[9] It has since been purified from rat liver,[10] yeast,[11] plant[12] and buffalo.[13] But the inhibitor genes have been cloned only from *Saccharomyces cerevisiae*,[14] bovine heart[15] and rat liver.[16] Here we report the process of cloning the murine ATPase inhibitor.

Received 8 July 1997; revised and accepted 18 August 1997.

Abbreviations: CIA, collagen-induced arthritis; CII, type II collagen; RACE, rapid amplification of cDNA ends.

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MATERIALS AND METHODS

Animals

DBA/1 mice were originally purchased from the Seiwa Institute for Experimental Animals (Fukuoka, Japan) and maintained in the Laboratory of Animal Research, at the Nagoya University School of Medicine (Nagoya, Japan).

Induction and evaluation of CIA

Male DBA/1 mice, 7 weeks old, were immunized intradermally with bovine CII (Cosmo Bio Co., Tokyo, Japan) emulsified with complete Freund's adjuvant (CFA) (100 μg CII/100 μl CFA). The animals were subsequently boostered 3 weeks later with CII (100 μg). The mice were carefully monitored three to four times per week for signs of arthritis, beginning 3 weeks after primary CII immunization. The severity of arthritis was evaluated as described previously[17] based on a grading system for each paw as follows: 1 = redness or swelling in paw or toes; 2 = severe swelling and/or joint deformity; 3 = joint ankylosis. A grade 2 mouse was killed for histological examination 2 weeks after the joints had begun swelling. Limbs were dissected, and the joints were decalcified for 3–4 days and then embedded in paraffin blocks. Sections were cut for each joint at different intervals, mounted, and stained with haematoxylin and eosin before analysis.

RNA isolation

Grade 2 CIA mice and age-matched control mice were prepared for RNA isolation. Total RNA was isolated from the joint tissues of diseased and control paws by the guanidium–thiocyanate–phenol–chloroform extraction procedure, as described previously.[18] RNA collection for differential display and Northern blot confirmation was performed 10 days after the joints had started swelling. RNA for use in differential display was subjected to DNase treatment to remove contaminating DNA.

Differential display

Differential display was performed as described by Liang & Pardee,[7] using a RNAimage™ kit (GenHunter Co., Brookline, MA). DNA-free total RNA (0·2 μg) was reverse-transcribed using oligo-dT primers, followed by polymerase chain reaction (PCR) with an arbitrary upstream primer in the presence of [α-^{32}P]dATP. The arbitrary primer used was H-AP-6, 5′-AAGCTTGCACCAT-3′. The downstream oligo-dT primer used was H-T_{11}C, 5′-AAGCTTTTTTTTTTTC-3′. The amplification was performed for 40 cycles under the following conditions: 95° for 30 seconds, 40° for 2 min, and 72° for 30 seconds. A variety of PCR products, representing the 3′ ends of numerous expressed cDNAs, were visualized by autoradiography on a standard denaturing polyacrylamide sequencing gel.

All of the differentially expressed bands were precisely extracted from the gel and reamplified for 40 cycles. The amplified products were used for Northern blot confirmation as a probe or directly ligated into the pCR™3 TA cloning vector (Invitrogen, San Diego, CA). DNA sequencing was performed in order to obtain the sequence information to generate the gene-specific primers for rapid amplification of cDNA ends (RACE).

Northern blot analysis

Equal amounts of total RNA (20 μg) were subjected to electrophoresis in 1% agarose gels containing 6% formaldehyde and 1 × morpholine propanesulfonic acid (MOPS) buffer (1 × MOPS buffer = 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA). RNA was transferred onto Hybond-N nylon membranes (Amersham, Braunschweig, Germany) by capillary blotting with 10 × SSC (1 × SSC = 0·15 M NaCl and 0·015 M sodium citrate) and cross-linked by UV-irradiation. For the analysis of the expression of RNAs, cDNA probes were labelled with [α-^{32}P]dCTP by random priming and hybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0·5% sodium dodecyl sulphate (SDS), and 100 μg/ml denatured salmon sperm DNA at 42° for 14 hr. After hybridization, filters were washed twice in 2 × SSC–0·1% SDS for 10 min at room temparature, then twice in 1 × SSC–0·1% SDS for 30 min at 65°. The washed filters were exposed to X-ray films or scientific imaging films (Fuji Film Co., Tokyo, Japan).

RACE

RACE was performed by using a commercially available kit (Marathon™, Clontech Inc., Palo Alto, CA) to amplify the full-length mRNA. The reactions were carried out according to the protocol given by the manufacturer. For RACE, we created two gene-specific primers based on DNA sequence information derived from the cDNA fragment we had subcloned after differential display. The mRNA isolated from the grade 2 CIA mouse was reverse-transcribed using an oligonucleotide primer with the sequence 5′-TTCTAGAATTCAGCGGCCGC $(T)_{30}N_{-1}N$-3′ (N_{-1} = G, A or C; N = G, A, C, or T), which was provided in the kit. An adaptor with the sequence 5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGC-CCGGGCAGGT-3′, which was also provided in the kit, was ligated to the cDNA. 5′-RACE was performed using the adaptor primer and one of the gene-specific primers (5′-GGGGAGTGATACGGGCCACTCTGTGA-3′). 3′-RACE was performed using the adaptor primer and the other gene-specific primer (5′-GACCACCATTCGAAGGAGATAGA-GCG-3′). After 30 cycles in a thermocycler, an aliquot of the reaction was analysed on a 1% agarose gel. The DNA fragments were purified from the agarose gel and cloned into the pCR™3 TA cloning vector (Invitrogen) followed by DNA sequencing in both directions.

DNA sequencing and gene database searches

DNA sequencing was performed in both directions using the Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Weterstadt, Germany) and analysed on the Applied Biosystems DNA Sequencing System (model 373A). Sequences were compared to the GenBank™ database at the National Centre for Biotechnology Information (NCBI, Bethesda, MD), using the BLAST alignment algorithm Network Service.

RESULTS

Histological examination confirmed that severe inflammation occurred in the CIA mice

Histological examination was performed 2 weeks after the joints had begun swelling, to confirm the development of CIA. The histopathology of joints from the grade 2 CIA mouse is shown in Fig. 1. Synovial proliferation with pannus formation, cartilage erosion and bone degradation was observed. In addition, the filtration of neutrophils and mononuclear cells (macrophages and lymphocytes) was observed with exudation of cells into the joint space. These data confirmed that severe inflammation had occurred in the CIA mice.

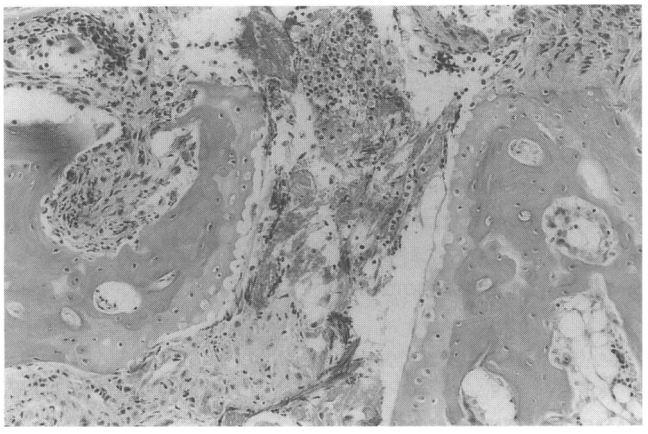

Figure 1. Histological confirmation of CIA. The section from the arthritic joints demonstrates synovial proliferation with infiltration of neutrophils and mononuclear cells, as well as cartilage destruction and bone erosion (haematoxylin and eosin stain, magnification × 150). Histological examination was performed to confirm the development of CIA. A grade 2 CIA mouse was killed 2 weeks after the joints had begun swelling. Limbs were dissected and the joints were decalcified for 3–4 days and then embedded in paraffin blocks. Sections were cut for each joint at different intervals, mounted and stained with haematoxylin and eosin before analysis.

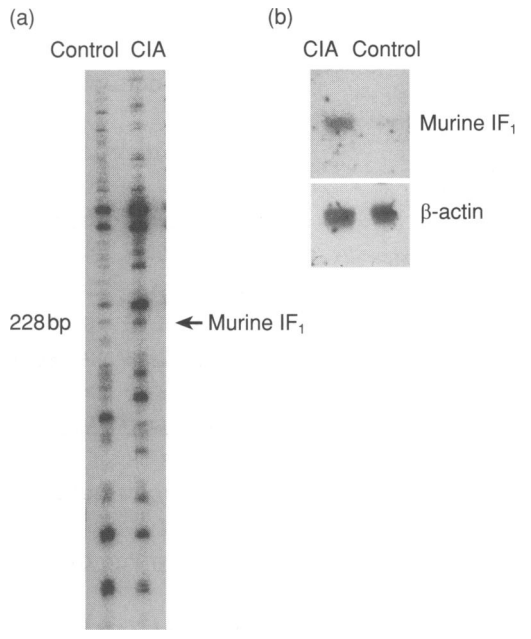

Figure 2. Differential display analysis and Northern blot confirmation of the candidate cDNA clone (called IF_1). (a) Differential display analysis. Total RNA was isolated from the joint tissues of CIA (grade 2) and control mice. The RNA was then subjected to differential display analysis, using the anchor primer, H-T_{11}C (5′-AAGCTTTTTTTTTTC-3′) and the arbitrary primer, H-AP-6 (5′-AAGCTTGCACCAT-3′). All of the differentially expressed bands were excised, reamplified by PCR and random prime-labelled for use in probing Northern blots. Here we discuss only one of those clones (IF_1). The position of IF_1 is indicated by an arrow. (b) Northern blot analysis. Total RNA (20 μg) isolated from the joint tissues of CIA (grade 2) and control mice was subjected to Northern transfer and then sequentially hybridized with ^{32}P-labelled IF_1 and β-actin-specific cDNA probes.

Differential display analysis and Northern blot confirmation

We used the differential display method in order to clone genes involved in CIA. Total RNA were prepared from control and CIA mice (grade 2), which were converted to cDNA. The cDNA were amplified by the 5′-primer (H-AP-6) and the 3′-primer (H-T_{11}C) for 40 cycles. Amplified products were analysed on sequencing gels (Fig. 2). All of the differentially expressed bands were eluted from the display gel and reamplified by PCR. Using the reamplified fragment, Northern blot analyses were performed to confirm whether the difference observed by PCR reflected a real difference in mRNA expression or not. In this paper, however, only one of the candidate clones (termed IF_1) was characterized further because the remaining clones were not successful in Northern blot analyses or DNA sequencing (data not shown). In fact, some clones failed to detect any signal in Northern blot, presumably due to very low mRNA levels or due to very short probes. Other clones were false positive. Some of the candidate clones failed in DNA sequencing. Only IF_1 showed a distinct difference between CIA and control mice in Northern blot analysis. As shown in Fig. 2, the expression of IF_1 was increased in CIA compared with that of the control. Once this result was obtained, we could proceed with further analysis of IF_1.

Cloning and sequencing of murine IF_1

An aliquot of the reamplified product was subcloned into pCR™3 (Invitrogen) and subjected to DNA sequencing. The subclone of IF_1 was 228 bases in length (Fig. 3a, underlined). RACE was used to amplify the full-length mRNA. For RACE, we created two gene-specific primers based on DNA sequence information derived from the subclone of IF_1. RNA from the CIA mouse (grade 2) was reverse-transcribed to make a cDNA library. The adaptor provided in the RACE kit was ligated to the cDNA library. RACE was performed in the 5′ and 3′ directions using gene-specific primers and the adaptor primer. After 30 cycles in a thermocycler, an aliquot of the reaction was analysed on a 1% agarose gel. The 5′-RACE product revealed a single band of *c.* 450 bases. The 3′-RACE product showed a single band of *c.* 300 bases. Each band was purified from the gel and cloned into pCR™3. Both clones were analysed by DNA sequencing in both directions and hence we could determine the complete nucleotide sequences of IF_1.

Figure 3 shows the complete nucleotide and deduced amino acid sequences of IF_1. Sequence comparison of IF_1 with the NCBI data bank showed 92% homology with the rat ATPase inhibitor. The rat ATPase inhibitor protein is composed of 107 amino acids and IF_1 encoded a protein of 106 amino acids.

Northern blot analysis for characterization of IF_1

Northern blot analysis was performed in order to characterize the IF_1 gene. We prepared RNAs from control and CIA mice from different stages. RNA was collected from CIA mice 2, 7 and 13 days after the joints had started swelling. Figure 4 shows the Northern blot analysis. The expression of IF_1 mRNA was strong at 7 and 13 days but at 2 days it was similar to that of the control.

For further analysis, Multiple Tissue Northern Blot™

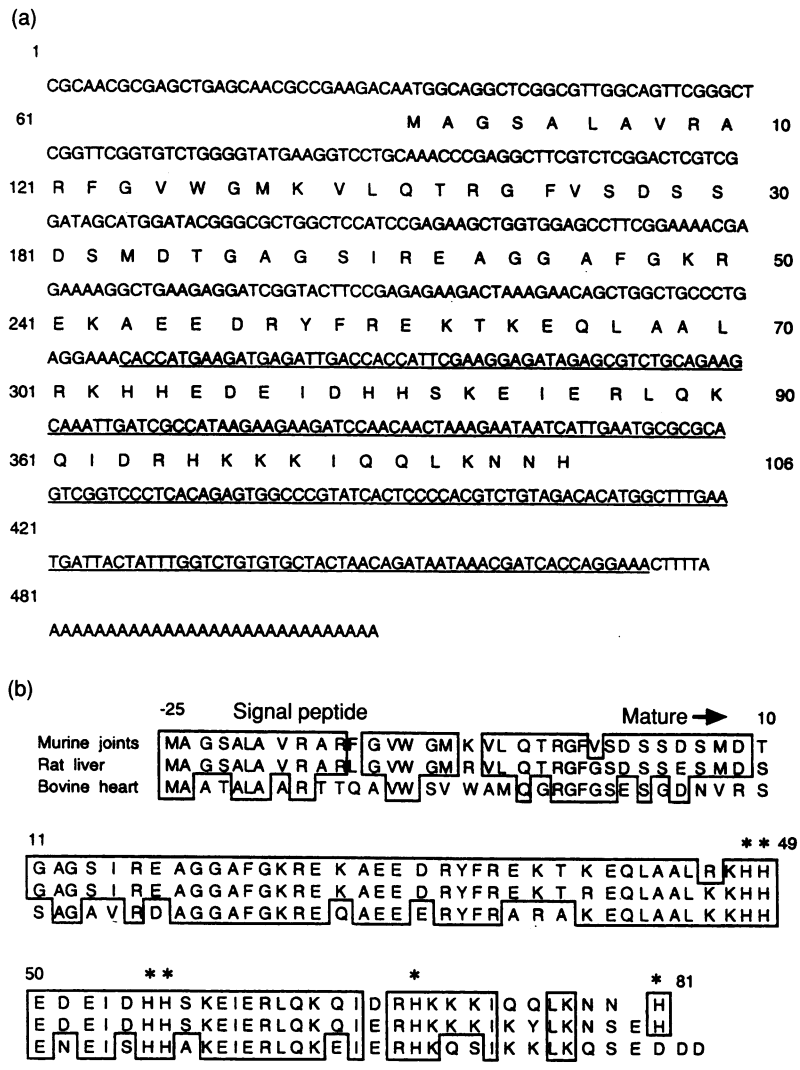

Figure 3. Primary structure of the murine IF_1. (a) Nucleotide and deduced amino acid sequences of the murine IF_1. Deduced amino acid residues are given under the nucleotide sequence and the positions are indicated on the right. The positions of nucleotide residues are indicated on the left. The subclone of the murine IF_1, which was directly derived from differential display, is underlined. (b) Comparison of known inhibitor protein sequences. The sequences from murine joints, rat liver[16] and bovine heart[15] are shown. The first 25 residues of rat and bovine sequences are considered as putative mitochondrial signal sequences[16] and are numbered −25 to −1. The mature ATPase inhibitors are numbered 1–82 (rat liver) or 1–84 (bovine heart). The conserved amino acids are boxed. Histidine residues are asterixed.

(Clontech) was used to investigate the tissue distribution of IF_1 mRNA. As shown in Fig. 5, all tissues examined had distinct expressions of IF_1. Especially strong expressions of IF_1 were detected in the heart, brain, kidney and testis.

DISCUSSION

CIA has been investigated extensively as a useful model for rheumatoid arthritis.[1,2] However, all of the mechanisms involved in the development and regulation of CIA have not been clarified completely. Therefore we tried to detect a new gene involved in CIA, using the differential display technique developed by Liang & Pardee.[7] Differential display is a useful technique for making a direct comparison of gene expression in normal and diseased tissue. We identified a novel gene, IF_1, whose expression level is increased in CIA mice. As shown in Northern blot analysis, at 2 days after the joints had started swelling, the expression of IF_1 was similar to the control. At 7 and 13 days, the expression increased. Therefore we suggest that IF_1 is involved in the natural course of CIA but is not involved as a trigger of CIA.

We believe that IF_1 could be the murine ATPase inhibitor gene for several reasons. The cDNAs encoding ATPase inhibitors have been cloned only from *Saccharomyces cerevisiae*,[14] bovine heart[15] and rat liver.[16] Murine ATPase inhibitor has not yet been cloned. IF_1 shows extremely high homologies with these ATPase inhibitors, especially to the rat ATPase inhibitor (92%). The protein encoded by IF_1 is 106 amino acids in length, only one amino acid smaller than the rat ATPase inhibitor. The highly conserved region between rat and bovine amino acid residues 22–45, which is the minimum sequence showing ATPase inhibitory activities,[8] is also highly

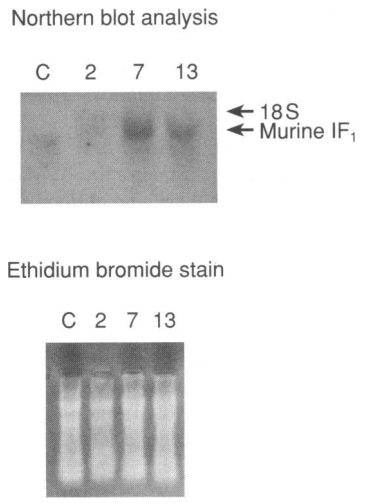

Figure 4. Northern blot analysis showing an increase of murine IF_1 mRNA at 7 and 13 days after the joints had started swelling. Total RNA was isolated from the joint tissues of control and CIA (2, 7 and 13 days after the joints had started swelling) mice. Twenty μg of total RNA was loaded on each lane. Each blot was hybridized with ^{32}P-labelled murine IF_1 probes. The ethidium bromide stain of the RNA samples served as a loading control.

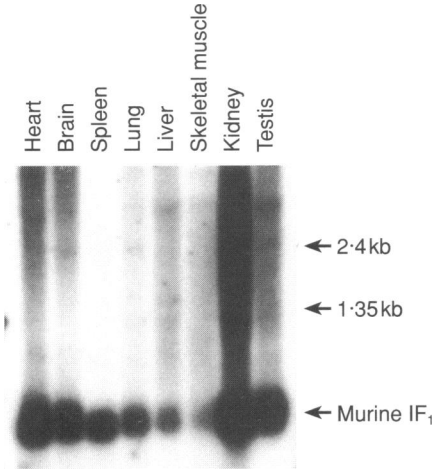

Figure 5. Tissue-specific expression of murine IF_1 mRNA. Northern blot of poly $(A)^+$ RNA from various murine tissues. We used murine IF_1 cDNA to probe a commercial tissue blot (Clontech) on which 2 μg of poly $(A)^+$ RNA from different tissues is displayed.

conserved in IF_1. In particular, the rat minimum sequence showing ATPase inhibitory activities is conserved perfectly in IF_1. Furthermore, IF_1 possesses a histidine-rich region in the same area, which is thought to be important for regulation of mammalian inhibitors.[8,16] These data confirmed to us that IF_1 was the murine ATPase inhibitor gene.

Unexpectedly, we cloned the murine ATPase inhibitor from the differential display analysis of CIA. Why is the expression of ATPase inhibitor increased in the joint tissues of CIA? An intrinsic inhibitor of mitochondrial F_1F_0–ATPase has been found in bovine heart tissue,[9] rat liver,[10] buffalo,[13] yeast[11] and plant mitochondria.[12] The inhibitor binds to the F_1 portion of the enzyme complex, completely inhibiting its ATP hydrolysing activity and ATP-driven energy-transfer reactions, but not oxidative phosphorylation.[9,19] The inhibitor is considered to bind rapidly to F_1F_0-ATPase when the membrane potential is lost, in order to preserve the cellular ATP pool;[16] moreover, it is thought to be released from its binding site upon energization of the membrane.[20–22]

As shown in Northern blot analysis, in early stages of CIA the expression of IF_1 was similar to that of the control. But in later stages, IF_1 was expressed more strongly than in the control. IF_1 is considered to be involved in the natural course of CIA, but is not involved as a trigger of CIA. In other words, there is a possibility that the IF_1 plays a role in trying to restore the diseased joint tissues to normal. It means that IF_1 may work as an anti-inflammatory protein. The tissue distribution of IF_1 was very suggestive. The expression of IF_1 was very strong in energetic organs such as the heart, brain and kidney, which means that ATPase inhibitors are abundant in places where a great deal of free energy is spent to maintain homeostasis. It is known that the development of arthritis requires great amounts of free energy, which is derived from ATP hydrolysis. As the arthritis develops rapidly, the cellular ATP pool may be decreased. Before the ATP pool is exhausted and the membrane potential is completely lost, the ATPase inhibitor may serve as a brake for ATP hydrolysis. If the supply of free energy can be reduced, the inflammation of arthritis may in turn be restored.

This is the first report that shows that the ATPase inhibitor works in tissues with inflammation. Further analysis is essential in order to clarify the whole mechanism, including whether the up-regulation of the ATPase inhibitor is a general feature of inflammation. But it is suggested here that the ATPase inhibitor may be involved in the regulatory mechanisms of inflammation.

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