299):UNC-86 to DNA is about 11 times that of MEC-3(1–299) alone (Fig. 2). In stability experiments the half-life of MEC-3(1–299) binding is less than 20 s, whereas that of MEC-3(1–299):UNC-86 binding is 5 min, a change that could account for most of the change in affinity.
21. Eleven *mec-3* alleles (*4, 8, 9*) were sequenced. The 2.8-kb *mec-3* genomic fragment that contains the whole *mec-3* coding sequence was amplified from single-mutant animals according to B. D. Williams, B. Schrank, C. Huynh, R. Shownkeen, and R. H. Waterston [*Genetics* **131**, 609 (1992)] and inserted into pBluescript KS(−). We pooled and sequenced DNAs from at least four independent transformants. The mutations found were the following [nucleotide position as in (*4*); amino acid position as in (*6*)]: *e1338*, insertion of an A at position 2762 (W69TAG); *e1498*, C3352T (Q149TAA); *e1612* and *u466*, G3664A (splice donor site abolished); *u6*, C3806T (Q262TAG); *u81*, G3921A (splice donor site abolished); *u99*, G3858A (C279Y); *u184*, G3814A (W264TGA); *u467*, T2082C, G2762A, and deletion of an A at position 3951 (V21A, W69TAG); *u468*, T2036C, C2518T, and G2686A (S6P, Q35TAA, and M44V); and *u469*, G2545A (splice donor site abolished).
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23. To transform TU#55, which encodes the MEC-3(Q266C) protein, and pTU23 (*4*), the wild-type control, we followed previous protocols (*6*) except that we injected tester DNA (10 μg/ml), *dpy-20* plasmid DNA [25 μg/ml; M. Han and P. Sternberg, *Cell* **63**, 921 (1990)], and pBluescript KS(−) plasmid DNA (65 μg/ml) as a carrier into a *mec-3*(*e1338*) *dpy-20*(*e1282*) double mutant.
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34. We generated the glutathione *S*-transferase (GST) fusion proteins GST–MEC-3(217–301), GST–MEC-3(217–293), and GST–MEC-3(217–277) by inserting the appropriate PCR fragments into the pGEX-2T vector (Pharmacia). The resulting proteins made in BL21(DE3)Lys S were purified according to Smith and Johnson (*30*) and cut with thrombin as described in (*31*) to produce the MEC-3 polypeptides.
35. We thank H. Zhu and R. Prywes for advice on gel-shift analysis, M. Finney and G. Ruvkun for providing the *unc-86* antibody and pET-3a–UNC-86, and H. Ingraham for advice on chemical cross-linking. We thank members of our laboratory and department for advice and comments on the manuscript. This work was supported by USPHS grant GM30997 and a McKnight Development Award (M.C.).

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# Prevention of Collagen-Induced Arthritis with an Antibody to gp39, the Ligand for CD40

Fiona H. Durie, Roy A. Fava, Teresa M. Foy, Alejandro Aruffo, Jeffrey A. Ledbetter, Randolph J. Noelle*

**The ligand for the CD40 antigen is a 39-kilodalton protein, gp39, expressed on the surface of activated CD4$^+$ T cells and is essential for thymus-dependent humoral immunity. The role of gp39-CD40 interactions in autoimmune disease was investigated in vivo with the use of an antibody that blocks their interactions (anti-gp39). Arthritis induced in mice by immunization with type II collagen was inhibited by anti-gp39. Anti-gp39 blocked the development of joint inflammation, serum antibody titers to collagen, the infiltration of inflammatory cells into the subsynovial tissue, and the erosion of cartilage and bone. Thus, interference with gp39-CD40 interactions may have therapeutic potential in the treatment of autoimmune disease.**

Experimental evidence has established that the CD40 ligand, gp39, is essential in the development of thymus-dependent immunity (*1*). The expression of gp39 is largely restricted to activated CD4$^+$ T cells (*2–4*), and its receptor, CD40, is a mitogenic receptor expressed on mature B cells (*5*). It has been postulated that expression of gp39 during cognate T cell–B cell interactions is an essential signal in the development of thymus-dependent (TD) immunity. This postulate has been proven with the recognition that X-linked hyper immunoglobulin M (IgM) syndrome (HIM), which results in the severe reduction of thymus-dependent responses and Ig isotype switching, results from point mutations in the gp39 gene (*6–9*). Mutant gp39 proteins from HIM patients cannot trigger B cell activation or bind to CD40 (*6*). Although TD humoral immune responses in HIM patients are impaired, cell-mediated immune responses are intact, suggesting that gp39 is only essential in antibody-mediated immunity. A monoclonal antibody (mAb) specific to murine gp39, MR1, blocks the binding of gp39 to CD40 and also blocks T helper ($T_H$) cell–dependent B cell activation in vitro (*10*). Experiments were designed to test whether a mAb specific to gp39 could be used therapeutically to control the course of humoral immune responses in normal and disease states. The administration of anti-gp39 interferes with the development of primary and secondary humoral immune responses (*11*), apparently by blocking the binding of gp39 to CD40.

The effects of anti-gp39 administration on a murine model of human autoimmune disease, rheumatoid arthritis, are presented herein to determine the role of interactions between gp39 and CD40 in the development of autoimmune disease. Collagen type II–induced arthritis (CIA) is induced in susceptible strains of mice by intradermal injections of heterologous native type II collagen (CII) (*12, 13*). Because the transfer to naïve animals of antibodies to CII leads to a condition histopathologically different from CIA and results in only transient synovitis (*14*), the role of humoral immunity in the development of CIA is not completely understood. In contrast, the transfer of both antibodies to CII and CD4$^+$ T cells from mice immunized with denatured CII completely reconstitutes the symptoms of classical arthritis (*15*). Therefore, synergy between the cellular and humoral arms of the immune response appears to be essential in the development of arthritis.

The kinetics of disease progression that result from the immunization of DBA/1J (*H-2$^q$*) mice with CII are depicted in Fig. 1. Mice were immunized with chick CII in complete Freund's adjuvant (CFA) and then challenged 3 weeks later with soluble CII. After this challenge, a dramatic rise in the rate of disease progression was observed. To quantitate the course of disease progression, we assigned scores to mice depending on the severity of inflammation of the fore- and hindlimbs. Three groups of mice (eight per group) were immunized with CII in CFA, and 1 week later each group received either no mAb, anti-gp39, or irrelevant hamster Ig (HIg) (Fig. 1). Mice were subsequently administered antibody (250 μg per mouse) every 4 days until the end of the experiment. This antibody treatment regime was used because previous titrations showed that this regime of mAb administration inhibited more than 90% of the primary (IgM) anti–sheep red blood cell response (*11*). Antibody half-life is estimated to be 12 days, so that injections every 4

F. H. Durie, T. M. Foy, R. J. Noelle, Department of Microbiology, Dartmouth Medical School, Lebanon, NH 03756.
R. A. Fava, Department of Medicine, Dartmouth Medical School, and Department of Veterans Administration Medical Center, White River Junction, VT 05009–0001.
A. Aruffo and J. A. Ledbetter, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121.
*To whom correspondence should be addressed.

days maintain adequate concentrations to block gp39 function. Because the secondary boost of CII is involved primarily in the induction of arthritogenic antibody, anti-gp39 was administered before the secondary boost to interfere with the development of clinical disease. The HIg-treated mice developed CIA at a more aggressive rate than the control animals, but by 40 days after primary immunization 75 to 80% of the HIg and control animals had developed CIA (Fig. 1A). Anti-gp39 therapy prevented all mice in this group from exhibiting any signs of clinical disease. The mean arthritis index was used as a means of measuring disease severity. This index reflects the extent of clinical disease per animal and the percentage of diseased animals in each group (Fig. 1B). A high percentage of the untreated and HIg-treated mice showed extensive distal joint inflammation; none of the anti-gp39–treated mice exhibited any signs of such inflammation.

Another consequence of immunization with CII is a precipitous rise in serum IgG titers to CII. In CII-immunized mice treated with anti-gp39, the serum IgG anti-CII titers were inhibited compared to the anti-CII titers found in mice untreated or treated with HIg (Fig. 2). In fact, titers of anti-CII in mice treated with anti-gp39 were similar to titers found in nonimmune control sera (*16*). Furthermore, during the course of the experiments (40 to 60 days), the administration of anti-gp39 did not alter the concentrations of total serum IgM or IgG1, as might have been anticipated from the HIM studies (*17*). In addition, we have not detected antibodies to the anti-gp39 reagent in treated mice (*11*). Finally, CII-immunized mice treated with anti-gp39 for 40 days and not given additional anti-gp39 had not developed clinical signs of CIA at 70 days after initial immunization. Therefore, anti-gp39 does not have to be given continuously to prevent disease onset (*18*).

Histologically, joints from all of the untreated and HIg-treated mice were severely damaged by the rapidly expanding synovial pannus. Mononuclear cell infiltration, thickening of the synovial membrane, and bone erosion by osteoclasts were apparent as well as exudate and polymorphonuclear cell accumulation in the synovial space (Fig. 3A). In contrast, there were no signs of inflammatory processes in four of the five joints examined from mice treated with anti-gp39 (Fig. 3B). In one joint from such a mouse, only rare polymorphonuclear leukocytes were present. Thus, the lack of clinical arthritis in these mice was not simply the result of a low level of inflammation but of a complete lack of initiation of synovial inflammation.

The data in this study establish gp39 as a potential target for therapeutic intervention. In vitro studies have shown that

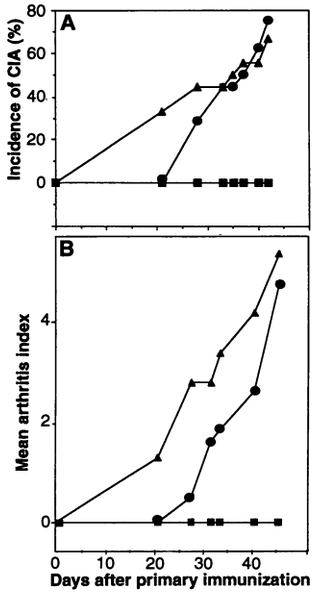


**Fig. 1.** (**A**) The prevention of CIA by anti-gp39 treatment. Male DBA/1J mice 6 weeks old were anesthetized and immunized with chick CII emulsified in a complete adjuvant, prepared by the combination of Freund's incomplete adjuvant (Difco, Detroit, Michigan) and mycobacterium tuberculosis (2 mg/ml; Ministry of Agriculture and Fisheries, Weybridge, Surrey, England). Type II collagen (200 μg) was injected in a single intradermal site at the base of the tail and then challenged with soluble CII (100 μg) 21 days later. The mAb to gp39 (MR1) is a hamster mAb to mouse gp39 and was prepared as described in (*10*). Antibody treatment (DEAE-purified MR1 and HIg) was initiated 7 days after the primary immunization and maintained throughout the study (250 μg per mouse every 4 days). Mice (eight per group) were then routinely monitored for the development of clinical symptoms of CIA by inspection of distal joint inflammation. (**B**) Anti-gp39 treatment inhibits the severity of CIA. The arthritic index (0 to 4) was determined by monitoring of hind- and forepaws and scored on a subjective scale, with 4 as intense swelling and erythema (*21–23*). The mean arthritis index was then determined by summation of the total score of each joint in each group of mice and dividing by the total number of animals in each group: control (circles), HIg-treated (triangles), and anti-gp39–treated mice (squares). These results are representative of two such experiments.

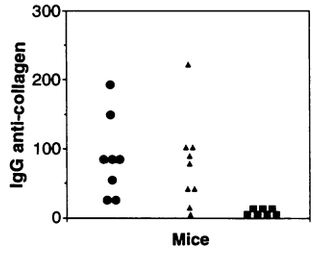


**Fig. 2.** Inhibition by anti-gp39 of the secondary humoral immune response to CII. Serum samples were analyzed for the presence of mouse CII–reactive IgG by a modification of the previously described method (*18*). Each well of a polyvinyl microtiter plate was coated overnight at 4°C with 100 μl of chick CII (5 μg/ml) and then blocked by incubation with phosphate-buffered saline containing 1% fetal calf serum and 0.02% azide. After the plates were washed, a 100-μl aliquot of each serum diluted in phosphate-buffered saline was added and the plates were incubated for 2 hours at 37°C, washed again, and incubated for two more hours with 100 μl of alkaline phosphatase–conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, Alabama). The plates were thoroughly washed and phosphatase substrate (Sigma) was added, resulting in the appropriate color change. Readings were determined by an enzyme-linked immunosorbent assay reader (Dynatech Labs) at an absorbance of 410 nm. We quantitated IgG1 anti-CII on the basis of a standard curve that was generated using a hyperimmune antisera to CII. The values are represented in arbitrary units; titers of antibody were determined for control mice, HIg-treated mice, and anti-gp39–treated mice (symbols as in Fig. 1).

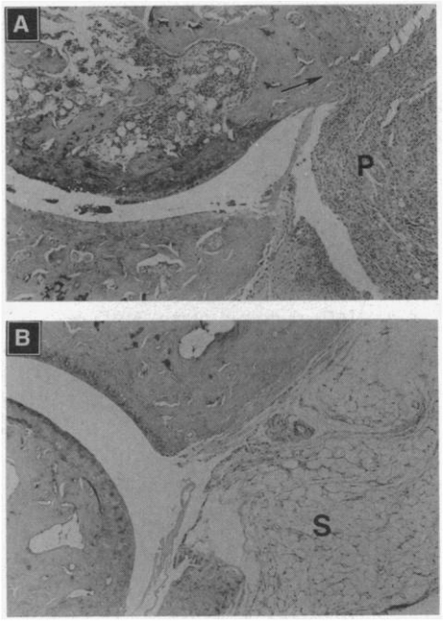


**Fig. 3.** The prevention by anti-gp39 of histological changes associated with CIA. Five mice from each group were killed 8 weeks after immunization, and their hindpaws were formalin-fixed, decalcified in formic-hydrochloric acid with electrolysis, embedded in paraffin and stained for 2 min in 0.1% Mayer's hematoxylin, 10 s in saturated lithium carbonate, and 1 min in 0.025% eosin. Tissue sections were examined and the overall results are illustrated with a representative example from (**A**) Hamster Ig–treated and (**B**) anti-gp39–treated groups. In (A), a fibrous and highly cellular pannus (P) composed of mono- and polynuclear cells was apparent surrounding most articulations of hindpaws and was often seen extending into sites of bone erosion (arrow), where numerous osteoclasts were also seen. The surface of articular cartilage was extensively damaged, and exudate and polymorphs were present within the synovial space. In (B) the synovial membrane appeared virtually identical to normal synovium (S), with no apparent thickening of the synovial membrane, exudation, or other signs of inflammation.

anti-gp39 blocks both the ability of the $T_H$ cell to trigger B cell activation (9) and the binding of CD40 to its ligand (*10*). This effect suggests that anti-gp39 binds at or near the CD40 binding site (*10*). Unlike treatments with other mAbs to T cells or fusion proteins (*19, 20*), it does not appear that treatment of mice with anti-gp39 prevents the priming of antigen-specific $T_H$ cells or causes the cytotoxic or functional deletion of these cells. The results of experiments showing that unaltered $T_H$ function can be adoptively transferred from mice treated with anti-gp39 (*11*) suggest that anti-gp39 administration does not result in $T_H$ tolerance. The blockade of gp39 function has potential therapeutic benefits for affecting the onset of autoimmune disease.

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# Synthesis and Degradation of Cyclic ADP–Ribose by NAD Glycohydrolases


Hyuntae Kim, Elaine L. Jacobson, Myron K. Jacobson*



**Cyclic adenosine diphosphoribose (cADPR), a recently discovered metabolite of nicotinamide adenine dinucleotide (NAD), is a potent calcium-releasing agent postulated to be a new second messenger. An enzyme that catalyzes the synthesis of cADPR from NAD and the hydrolysis of cADPR to ADP-ribose (ADPR) was purified to homogeneity from canine spleen microsomes. The net conversion of NAD to ADPR categorizes this enzyme as an NAD glycohydrolase. NAD glycohydrolases are ubiquitous membrane-bound enzymes that have been known for many years but whose function has not been identified. The results presented here suggest that these enzymes may function in the regulation of calcium homeostasis by the ability to synthesize and degrade cADPR.**


The concentration of cytosolic free $Ca^{2+}$ is a key factor in cellular regulation (*1*). Many extracellular signals stimulate the synthesis of the second messenger inositol 1,4,5-trisphosphate, which activates membrane $Ca^{2+}$ channels resulting in $Ca^{2+}$ release from internal stores (*2*). Reports of $Ca^{2+}$ mobilization that appear to be unrelated to inositol polyphosphate metabolism suggest the presence of other second messengers involved in the regulation of $Ca^{2+}$ homeostasis (*2*). A likely candidate for such a second messenger is cADPR, a metabolite of NAD. Cyclic ADP-ribose is a potent mediator of $Ca^{2+}$ release in sea urchin egg microsomes (*3, 4*) and mammalian cells (*5, 6*). A saturatable site for binding cADPR exists in sea urchin egg microsomes (*7*), and cADPR has been implicated in $Ca^{2+}$-induced $Ca^{2+}$ release (*8–10*) and insulin secretion (*6*).

Low amounts of cADPR have been found in all mammalian tissues tested (*11*); however, little is known about the enzymes that synthesize and degrade this nucleotide. A molluscan enzyme that catalyzes the conversion of NAD to cADPR has been purified and cloned (*12–14*). In mammalian tissues an activity that generates cADPR has been detected (*7, 15*), but no enzyme has been isolated. Although the presence of enzymes that degrade cADPR has been postulated (9), the occurrence of such an activity has not been demonstrated. We detected cADPR degradative activity in a variety of animal tissue homogenates. We report here the isolation and characterization of an enzyme from canine spleen microsomes that efficiently catalyzes the hydrolysis of cADPR to ADPR. This enzyme also catalyzes the synthesis of cADPR from NAD. The ability of the enzyme to catalyze the net conversion of NAD to ADPR identifies it as an NAD glycohydrolase (NADase). These results suggest the possibility of a new function for these membrane-bound enzymes that have been known for many years, yet whose function has remained an enigma.

We developed a rapid assay in which [$^{32}$P]cADPR serves as substrate (*16*). The


H. Kim, Department of Biochemistry and Molecular Biology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX 76107, and Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Lexington, KY 40536.

E. L. Jacobson, Department of Medicine and Department of Biochemistry and Molecular Biology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX 76107, and Department of Clinical Sciences, College of Allied Health Professions, and Lucille Parker Markey Cancer Center, University of Kentucky, Lexington, KY 40536.

M. K. Jacobson, Department of Biochemistry and Molecular Biology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX 76107, and Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, and Lucille Parker Markey Cancer Center, University of Kentucky, Lexington, KY 40536.

*To whom correspondence should be addressed at the Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky.


**Table 1.** Purification of cADPR hydrolase from canine spleen.

| Fraction | Protein (mg) | Total activity (U)* | Specific activity (U/mg) | Fold purification | Yield (%) | NADase/ cADPRase† | ADPR cyclase/ cADPRase† |
|---|---|---|---|---|---|---|---|
| Tissue extract | 7310 | 164 | 0.022 | 1 | 100 | 2.81 | 0.049 |
| Crude microsome | 2300 | 125 | 0.054 | 2.4 | 76 | 2.75 | 0.054 |
| Blue A Sepharose | 79.9 | 33.2 | 0.416 | 18.6 | 20.3 | 3.59 | 0.071 |
| Con A–Sepharose | 24.3 | 24.4 | 3.36 | 150 | 14.9 | 2.72 | 0.050 |
| Red A Sepharose | 0.036 | 12.8 | 357 | 15900 | 7.8 | 2.87 | 0.057 |

*One unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 μmol of cADPR to ADPR per min at 37°C under the assay conditions described in (*16*). †Ratios of activities of NADase or ADPR cyclase to cADPR hydrolase (cADPRase). One unit of activity is defined as the amount of enzyme that results in the net formation of 1 μmol of ADPR (NADase) or cADPR (ADPR cyclase) per min at 37°C under the assay conditions described in (*35, 36*).