Cutting Edge: Elimination of an Endogenous Adjuvant Reduces the Activation of CD8 T Lymphocytes to Transplanted Cells and in an Autoimmune Diabetes Model

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CUTTING EDGE

Cutting Edge: Elimination of an Endogenous Adjuvant Reduces the Activation of CD8 T Lymphocytes to Transplanted Cells and in an Autoimmune Diabetes Model

Yan Shi, Shelly A. Galusha, and Kenneth L. Rock[1]

The generation of adaptive immune responses is thought to require the presence of adjuvants. Although microbial adjuvants are well characterized, little is known about what provides the adjuvant effect in responses to transplanted cells or in autoimmune diseases. It had been postulated that, in these situations, injured cells instead released "endogenous adjuvants." We previously identified uric acid as an endogenous adjuvant for coinjected Ags. We now report that elimination of uric acid reduced the generation of CTL to an Ag in transplanted syngeneic cells and the proliferation of autoreactive T cells in a transgenic diabetes model. In contrast, uric acid depletion did not reduce the stimulation of T cells to mature APCs or when endogenous APCs were activated with anti-CD40 Ab. These findings support the concept that danger signals contribute to the T cell responses to cell-associated Ags by activating APCs and identify uric acid as one of these signals. The Journal of Immunology, 2006, 176: 3905–3908.

Adjuvants were first discovered in the 1920s as agents that could augment immune responses to Ags (1). It was subsequently found that these immunostimulatory molecules were essential for generating immune responses to purified proteins, and in their absence, Ags induced tolerance instead of immunity (2). Janeway (3, 4) proposed that adjuvants were required because the immune system had evolved to respond to Ags only in the context of infection. In this model, infections were identified by the presence of adjuvants, i.e., molecules of microbial origin that were molecularly distinct from mammalian structures, that were recognized by receptors on APCs and other cell types. These microbial adjuvants then stimulated the APCs to become immunostimulatory by presenting Ags, expressing costimulatory molecules, secreting cytokines, and migrating to secondary lymphoid tissues.

This model, which is now well accepted, helps to explain why adjuvants are required and how robust immunity is generated to pathogens. However, it does not explain how immune responses are generated to tissue transplants or tumors that lack unique microbial components to provide the required adjuvant effect. In these situations, it was proposed that dying cells release danger signals that function as endogenous adjuvants (5, 6). In support of this concept, dead cells were shown to provide adjuvant activities when coinjected with foreign Ags (7–9).

Autoimmunity is another situation where immune responses are generated to autologous cells that lack microbial components (10). It was suggested that endogenous danger signals also might underlie the triggering of these conditions (5, 6), and there is some experimental evidence that cell death can initiate autoimmunity. For example, injury of pancreatic β islet cells with chemicals (11, 12) or other agents (13, 14) can trigger autoreactive T cells and cause diabetes in genetically susceptible hosts. Moreover, in NOD mice, which spontaneously develop diabetes, there is a developmental wave of islet cell death that precedes the development of autoimmunity (15). Inhibiting this cell death reduces the activation of transferred islet-reactive TCR-transgenic T cells (15).

It is presently unknown whether and to what extent endogenous adjuvants actually play a role in the generation of immunity to host cells. To investigate this issue, it is necessary to identify the endogenous adjuvants and to eliminate their activity. Cells contain more than one endogenous molecule with adjuvant activity (16, 17). We identified one of these endogenous adjuvants as uric acid. This study examines the effect of eliminating uric acid on the generation of immunity to an Ag in transplanted cells and in the activation of autoreactive T cells.

Methods

Mice, cells, and reagents

Rat insulin promoter (RIP)[2] -transmembrane form of OVA (mOVA) (a gift from Dr. William Heath of the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and OT-1 TCR-transgenic mice (obtained from S. Jameson, University of Minnesota, Minneapolis, MN) were bred at University of Massachusetts Medical Animal Facilities. The RIP-mOVA transgene was assayed by PCR with the following primers: RIP OVA 111, CAAGCACATCG CAACCA; and RIP OVA 112, GCAATTGCCTTGTCAGCAT. All other mice were purchased from The Jackson Laboratory. All cells were as previously described (16). All biochemical reagents were purchased from Sigma-Aldrich.

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[2] Abbreviations used in this paper: RIP, rat insulin promoter; mOVA, transmembrane form of OVA; DC, dendritic cell; BM, bone marrow.

Uricase was further purified by gel filtration and anion ion exchange chromatography. Allopurinol suspension was first adjusted to pH 11 for sterilization by filtration and then reduced to pH 8.5 for injection. All Abs were purchased from BD Pharmingen, except for anti-CD40 FGK45 (a gift from Dr. R. Novell, Dartmouth College, Hanover, NH). To generate uric acid degradation products, uric acid (2 mg/ml) in borate buffer (pH 8.5) was incubated with 100 μg/ml uricase overnight at 37°C, and the mixture was then passed through a 10-kDa cutoff filter to remove uricase; under these condition, uric acid was completely hydrolyzed.

Immunization

BALB/c mice were injected i.p. with 500 μg of allopurinol and 10 μg of uricase on the day before, the day of, and the day after the immunization. Freeze-thaw-treated 15.12 cells (gp120-transfected 3T3 cells) were trypsinized, incubated for 30 min with 2 μg of uricase, and injected i.p. into BALB/c mice at the indicated cell numbers. After 14 days, splenocytes (4×10^7) were cultured in 10 ml of hybridoma culture medium (HCM), which were stimulated with 10^{-8} M of the HIV gp120 peptide RGPGRAFVTI. CTL assays were then performed with indicated target cells, as described (16). Controls were identical except for the omission of uricase and allopurinol in all steps.

Adoptive transfers and uric acid depletion

C57BL/6 or RIP-mOVA mice were treated to eliminate uric acid with the following schedule: day −2, day −1, and day +2, 500 μg of allopurinol (100 μl) i.p.; day 0 and day +1, allopurinol plus 10 μg of uricase (in 100 μl of PBS), i.p. Single-cell suspensions of lymph nodes from OT-1 mice were collected and labeled with 1 μM CSFE (Molecular Probes) for 20 min at 37°C. A total of 500,000 (unless indicated otherwise) unfractionated cells in 0.5 ml of PBS was i.v. infused from the tail vein on day 0. In some control experiments, splenocytes or bone marrow (BM)-derived dendritic cells (DCs) from C57BL/6 mice were incubated with the indicated concentrations of SIINFEKL peptide for 30 min, washed extensively, and injected i.p. on day −1. In other control experiments, 25 μg of FGK45 Ab in 100 μl of PBS was injected i.p. on day 0. In yet other control experiments, RIP-mOVA mice were i.p. injected with a daily dose of uric acid degradation products equivalent to 200 μg of initial uric acid (estimated amount of extracellular uric acid in mice) from day −2 to day +2.

Flow cytometric analysis

Mice were sacrificed on day +3, and pancreatic and inguinal draining lymph nodes were harvested. Single-cell suspensions were then stained with anti-CD8 (53-6.7, PerCP; eBioscience) and anti-Thy1.1 (HIS51, allophycocyanin; eBioScience). CD8$^+$ and Thy1.1$^+$ cells were gated and analyzed for their CFSE profiles. Data were analyzed with FlowJo software (Tree Star). Division index is the average number of divisions that a cell (that was present in the starting population) has undergone. Percentage divided is the percentage of the cells of the original sample that divided.

Results and Discussion

Role of uric acid in the generation of CTL responses to Ags in transplanted cells

When 3T3 cells expressing the HIV gp120 Ag are injected into syngeneic mice, gp120-specific CTL responses are generated in the absence of any microbial adjuvant (Fig. 1). The danger hypothesis postulates that responses to such cellular Ags are stimulated by endogenous adjuvants released from the injected cells. We have shown previously that 3T3 cells contain endogenous adjuvants, one of which is uric acid, that can augment responses to coinjected Ags. To test whether endogenous adjuvants actually play a role in the generation of CTL responses to cell-associated Ags, we depleted the mice of uric acid by reducing its synthesis with allopurinol and removing extracellular uric acid with uricase and then immunized them with limiting numbers of gp120-transfected cells. These treatments can reduce the endogenous adjuvant activity of cell lysates (16). Depletion of uric acid reduces the CTL responses stimulated by gp120-transfected cells by ∼80% (Fig. 1) (*p* = 0.0006). Therefore, uric acid depletion reduces the generation of immunity to an Ag made by transplanted cells.
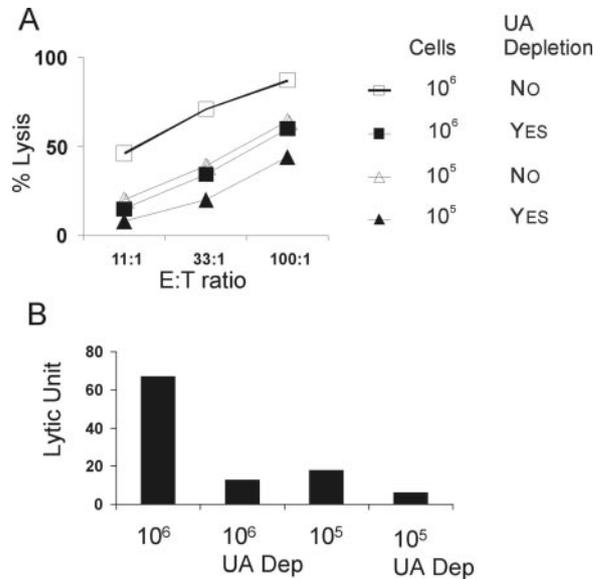

FIGURE 1. Removal of uric acid significantly reduces the CTL response against cell-associated Ag: *A*, Uric acid-depleted and control BALB/c mice were immunized i.p. with 15.12 cells at indicated numbers for 14 days, and CTL assays were performed as described in *Methods*. Data are expressed as percentage of specific lysis at the indicated E:T ratios. *B*, Data from *A* calculated as LU (at cytolysis of 20%).

Role of uric in the activation of autoreactive T cells.

To examine the role of uric acid in the activation of autoreactive T cells, we used a model developed by Heath and colleagues (18) where mOVA is expressed as a transgene under the control of RIP. In this model, transgenic mice express the mOVA as a self Ag in the insulin-producing (β) cells in the islets of Langerhans. BM-derived APCs acquire OVA from the islets, migrating to the draining lymph nodes where they cross-present the OVA-derived peptide SIINFEKL on their surface H-2K^b class I molecules. When these mice are injected with TCR-transgenic cells (OT1) specific for SIINFEKL bound to H-2K^b (which are "autoreactive" to the transgenic Ag), the T cells are stimulated to proliferate in the pancreatic lymph nodes and can then destroy islet cells and cause diabetes. Thus, this is an experimental model of autoimmune disease and one that occurs in the absence of microbial adjuvants. The question is what provides the adjuvant signal that promotes the ability of the APCs presenting the autoantigen to stimulate the autoreactive T cells.

To test the hypothesis that uric acid might help initiate the activation of autoreactive T cells to cellular Ags, we depleted it from RIP-mOVA mice. Autoreactive OT1 transgenic T cells were labeled with CFSE, and limited numbers (5×10^5) were transferred into uric acid-depleted or control transgenic mice. After 72 h, lymph nodes were harvested, and the proliferation (CFSE dilution) of transgenic T cells was analyzed by flow cytometry. In the draining pancreatic lymph nodes of control mice, a substantial number of OT1 T cells have undergone multiple rounds of division (Fig. 2). In these experiments, the proliferating OT1 T cells were initially present primarily in the draining lymph node, because this is the site of Ag presentation. At later time points (on or after day 4), OT1 cells that have proliferated emigrate from this site and are found in other lymphoid organs (data not shown).

When limited numbers of OT1 T cells were injected into mice depleted of uric acid, there was a substantial reduction in
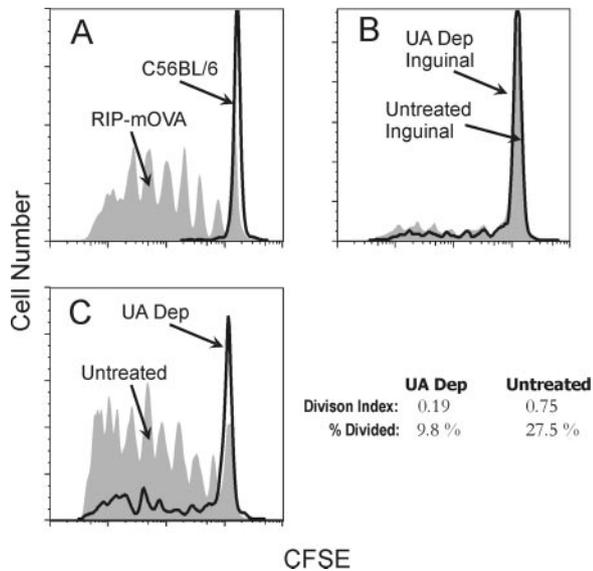
FIGURE 2. Removal of uric acid reduces autoreactive OT-1 T cell division in RIP-mOVA mice. B6 and RIP-mOVA mice were treated and infused with CFSE labeled OT-1 cells as described in *Methods*. *A*, OT-1 division is Ag driven and seen in the pancreatic lymph nodes in RIP-mOVA (shaded area), but not in C57BL/6 control (heavy line), mice. *B*, Similar to *A*, OT-1 division is minimal in inguinal lymph nodes of RIP-mOVA mice with (shaded area) or without uric acid depletion (heavy line). *C*, Uric acid depletion (shaded area) reduces OT-1 division in pancreatic draining lymph nodes, compared with control (uric acid-sufficient) mice (heavy line). The assay is representative of eight independent experiments. The inserted table is the comparison of division index with the percentage of cells divided for *C*.
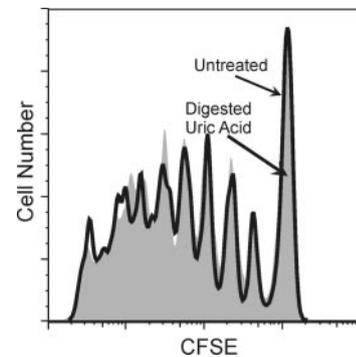

FIGURE 3. Uric acid metabolites produced by uricase do not alter the OT-1 activation. Identical to Fig. 2*C*, except that digested uric acid (heavy line) was injected in place of uricase/allopurinol (see *Methods*). The shaded area represents untreated RIP-mOVA receiving only OT-1 cells. The dark line represents control RIP-mOVA mice.

the number of proliferating autoreactive T cells, compared with control mice. The number of cells stimulated to proliferate was reduced by more than 2-fold, although the ones that entered cell cycle underwent a similar number of division cycles as controls (Fig. 2*C*). This reduction in proliferation was seen consistently in eight experiments (percentage divided, 30.5 ± 9.7% for control and 10.9 ± 4.7% for uric acid-depleted mice; $p = 0.0002$), but only under conditions where there were low initial precursor frequencies. When larger numbers of OT1 T cells were transferred into these mice, the number of cells that proliferated and the number of times that they divided increased substantially (data not shown), and under these conditions, uric acid depletion did not reduce responses. We speculate that the increased proliferation may be due to the increased frequency of responding cells and their autocrine and paracrine amplification of responses; we presume that this amplification reduces or obviates the need for the endogenous adjuvant. Alternatively, perhaps the increased number of T cells is causing more damage and releasing additional endogenous adjuvants.

Uricase catalyzed the oxidation of uric acid into allantoin and water. To test whether these uric acid degradation products were somehow immunosuppressive, we injected mice with the same amount of these reactants as would be generated in vivo through treatment with uricase. The end products of uric acid oxidation had no effect on the activation of the autoreactive OT1 T cells in RIP-mOVA mice (Fig. 3).

We have shown previously that uric acid can stimulate DCs to mature, and this is the presumed basis for its adjuvant effects. If this model is correct, then OT1 T cells should be activated normally in uric acid-depleted mice, when activated DCs are present. We tested this hypothesis in two ways. In one set of

experiments, we pulsed APCs ex vivo with SIINFEKL peptide and then injected them into uric acid-depleted or control mice. As a source of APCs, we used the resident cells in either spleen or BM-derived DCs; under the conditions of our experiments, these APC populations contained mature DCs. Both of these sources of APCs stimulated OT1 T cells to proliferate equivalently in both control and urate-depleted mice, even under limiting conditions of stimulation observed with the splenic APCs (Fig. 4). The activation of the OT1 T cells is Ag specific, because these cells did not proliferate in animals injected with APCs pulsed with a control peptide, as expected (data not shown). In another set of experiments, we activated the endogenous APCs in mice in situ by injecting an agonistic CD40 Ab. This treatment, unlike a control Ab, also restored the proliferation of the OT1 T cells in the uric acid-depleted mice (Fig. 5).

These control experiments establish several important points. First, they demonstrate that the OT1 T cells themselves are fully responsive to activated APCs in the absence of uric acid. Therefore, uric acid depletion is not affecting the T cells directly. Second, uric acid depletion is not inhibiting the ability of DCs or T cells to migrate and interact, because this occurs normally with the exogenously supplied DCs or with CD40 stimulation, even under limiting conditions. Third, the observation that activated exogenous or endogenous APCs stimulate responses in the absence of uric acid, whereas endogenous APCs do not, implies that uric acid is acting at the level of the endogenous APCs. This is consistent with current models of how
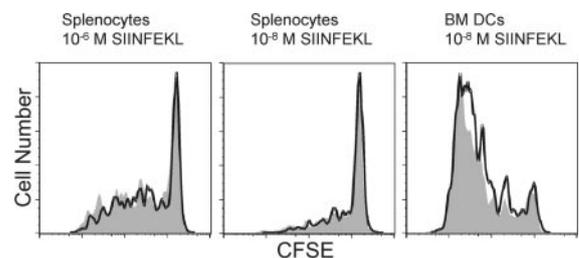

FIGURE 4. OT-1 T cells in uric acid-depleted mice are fully responsive to SIINFEKL-pulsed syngeneic APCs. A total of 10^6 splenocytes or BM DCs from C57BL/6 mice were pulsed with the indicated concentration of SIINFEKL peptide for 1 h, washed three times, and were then injected i.p. into control (heavy line) or uric acid-depleted (shaded area) C57BL/6 mice (see *Methods*) and assayed as in Fig. 2*C*.
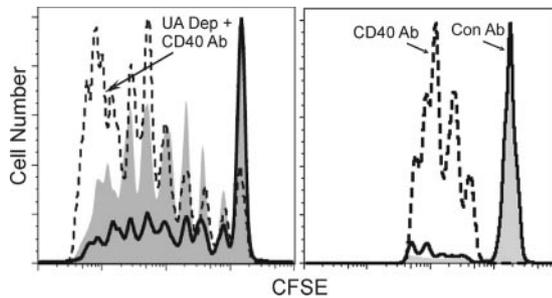
FIGURE 5. APCs in uric acid-depleted mice are fully responsive to CD40 Ab-mediated activation. *A*, Similar to Fig. 2*C*, except that the 25 μg of FGK45 Ab (dashed line) was delivered i.p. into uric acid-depleted RIP-mOVA on the same day of OT-1 infusion. *B*, Similar to *A*, except that a uric acid-depleted mouse infused with a control IgG was used to replace the untreated control.

adjuvants in general and danger signals in particular help to stimulate immune responses.

The danger hypothesis postulated that endogenous factors released from injured cells provided the adjuvant activity that allowed immune responses to develop to transplanted cells and tumors, and also to autologous cells in autoimmunity. The present findings provide support for these concepts and that uric acid plays a role as a danger signal. Previous work had shown that cell lysates could function as an adjuvant when admixed with an exogenous Ag and that depletion of uric acid from cell lysates reduced this adjuvant effect. Although this was an artificial system, these results suggested that uric acid would similarly promote immune responses to cells and their constituent Ags. The present findings confirm this prediction for transplanted cells and for a form of autoimmunity. Our findings are consistent with a recent report showing that allopurinol and uricase delayed the rejection of an immunogenic tumor (19), although the precise mechanism for this delay was not defined. Our results show that uric acid depletion leads to a reduction in T cell responses that is probably occurring because of reduced activation of APCs.

Some further comment is warranted on the findings in the RIP-mOVA model of autoimmunity. Although this is not formally a model of spontaneous autoimmune disease, it does measure the activation of autoreactive transgenic T cells and has been useful to analyzing how cross-presentation contributes to this process. When OT1 T cells are transferred into B6 BM → RIPmOVA chimeric mice on the H-2^{bm1} background, they are stimulated by H-2K^b BM-derived-APCs, but the autoreactive effector cells do not cause diabetes because they cannot recognize the host H-2K^{bm1} islet cells. In this situation, the activated autoreactive T cells undergo deletion over a period of many weeks/months (20), a phenomenon termed "cross tolerance." Why this occurs and whether it is related in any way to endogenous adjuvants (e.g., not fully activating APCs) or other mechanisms, such as clonal exhaustion, is not clear. However, it is clear that the initial activation of the T cells in the OT1 → RIP-mOVA model leads to bonafide autoimmunity; therefore, the BM-derived APCs and the signals that are activating them are initially leading to full activation of the T cells and not tolerance.

It is clear from the published data that in the RIP-mOVA model nonsecreted cellular Ags are released and acquired by APCs in the pancreas. Our data indicate that there is release of not only integral Ags but also endogenous adjuvants. An inter-

esting unresolved question is what is causing the release of these cellular constituents in this model. Presumably, this occurs as a consequence of the death of cells that normally occurs in the pancreas (21) and/or perhaps from some damage caused by small numbers of OT1 effectors present in the transferred cells.

The depletion of uric acid reduces, but does not eliminate, the generation of T cell responses to cellular Ags either in the RIP-mOVA-transgenic model or the transfer of gp120-expressing cells. The uric acid-independent component of this response is either adjuvant independent or, we feel more likely, reflects the activity of other endogenous adjuvants. Previously, we have found biochemical evidence for other endogenous adjuvants (16), and recently, high mobility group box 1 has been reported to have such activity (22, 23). It will be important to define these other endogenous adjuvants and determine their role in activating T cells to tumors, transplants, and autoimmunity.

Disclosures

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