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CD4⁻CD8⁻ T CELLS FROM MICE WITH COLLAGEN ARTHRITIS DISPLAY ABERRANT EXPRESSION OF TYPE *l* K^+ CHANNELS[1]

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Expression of voltage-gated K^+ channels in mAb-defined T cell subsets from normal mice and mice with experimental autoimmune arthritis was studied with the patch-clamp whole-cell recording technique in combination with fluorescence microscopy. $CD4^+CD8^-$ Th cells from DBA/1 LacJ mice with type II collagen arthritis expressed low levels of type *n* K^+ channels, and $CD4^-CD8^+$ T cells (cytotoxic) showed small numbers of type *l* or *n'* K^+ channels, like their phenotypic counterparts in normal mice. $CD4^-CD8^-Thy\text{-}1.2^+$ (double negative or DN) T cells from the diseased mice, however, displayed an abundance of type *l* K^+ channels compared to DN T cells in normal mice, or mice immunized with CFA. Furthermore, the aberrant expression of type *l* K^+ channels correlated with the presence of active disease. DN T cells from mice with SLE, type-1 diabetes mellitus, and experimental allergic encephalomyelitis, also exhibited a high number of type *l* K^+ channels. These results suggest that expression of numerous type *l* K^+ channels may be a useful marker for DN T cells associated with these autoimmune disorders.

Type II collagen autoimmune arthritis has been extensively investigated as a model for human arthritis (1–3). Immunization of rats, mice, and monkeys with type II collagen results in polyarthritis characterized by synovial hyperplasia, cellular infiltration of the subsynovial tissues, destruction of the joint cartilage, and periostitis. These pathologic features resemble those in humans with rheumatoid arthritis. An antibody response to type II collagen mediates joint destruction. T cells appear to play an accessory role in the pathogenesis of arthritis since type II collagen is a T-dependent Ag (4–8). Type II collagen-responsive T cell lines or lymphokine-rich supernatants induce joint swelling and pathology consistent with arthritis when introduced directly into joints (4–8). Injection of anti-CD4 antibodies retards the development of disease, further substantiating a role for T cells in the pathogenesis of collagen arthritis (9).

Anti-CD4 therapy ameliorates the clinical features of murine SLE (10, 11), type-1 diabetes mellitus (12), chronic EAE[3] (13), and myasthenia gravis (14), suggesting that these disparate autoimmune disorders share common pathogenic mechanisms with collagen arthritis. Anti-CD4 treatment also reduces a population of $CD4^-CD8^-$ (DN) T cells in lupus mice (10), suggesting a link between these cells and $CD4^+CD8^-$ T cells. We have recently demonstrated that DN T cells from mice with SLE, type 1 diabetes mellitus and chronic EAE exhibit abnormally high numbers of a voltage-gated K^+ channel called type *l* (15). Several interesting questions arise from this observation. 1) Do DN T cells from mice with type II collagen arthritis display the identical channel abnormality? 2) Is the augmented expression of type *l* K^+ channels a unique feature of autoimmunity or is it a result of a generalized immune response in vivo? We have addressed these questions in our study.

MATERIALS AND METHODS

Mice

DBA/1 LacJ male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were injected intradermally (using a 26-gauge needle) near the base of their tails with 100 μg of highly purified chick type II collagen emulsified in CFA that contained additional *Mycobacterium butyricum* (Difco, Detroit, MI). Native chick type II collagen was prepared from chick sternal cartilage (16) and was a gift from Dr. Kuniaki Terato (Department of Medicine, Memphis, TN). The collagen/CFA emulsion was prepared by adding 1 volume of modified CFA (30 μg of *M. butyricum*/50 μl of CFA) to 1 volume of collagen solution (100 μg of type II collagen per 50 μl of 0.01 N acetic acid). Seven mice were each immunized with 100 μl of this mixture. As controls, one group of animals ($n = 2$) was not injected and another group ($n = 6$) was injected with modified CFA without collagen. Five mice developed clinically evident arthritis with enlarged, inflamed paws. Three mice had arthritic involvement of one paw, and two had arthritis of two paws. Paw thickness was measured with a Schnelltaster constant-tension caliper from Schlesingers (Dobbs Ferry, NY). Two mice injected with type II collagen and CFA did not develop obvious signs of arthritis. Mice injected with collagen were killed 10 to 12 wk after immunization for patch-clamp studies.

Antibodies

PE-conjugated-anti-CD4 (L3T4), FITC-conjugated-anti-CD8, FITC-conjugated-anti-Thy-1.2, anti-CD4, and anti-CD8 were purchased from Becton Dickinson (Mountain View, CA). PE-conjugated-goat-anti-rat IgG (affinity purified and absorbed against mouse IgG) were purchased from Caltag (Rupp and Bowman, Tustin, CA).

Separation of T Cells

Single-cell suspensions were prepared from mouse spleens, and T cells were enriched by passage through a nylon wool column. Cells

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[3] Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis, DN, double negative, PE, phycoerythrin, TEA^+, tetraethylammonium chloride; g_K, maximal K^+ conductance; pS, picosiemens.

were then suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT) and 2 mM *l*-glutamine (medium).

Staining

Cells (2×10^6) were incubated with an appropriate dilution of anti-CD4-PE and anti-CD8-FITC for 30 min on ice, washed three times with medium, and resuspended in 1 ml of medium. The stained cells were plated into glass chambers, and the $CD4^+CD8^-$ (appear orange) and $CD4^-CD8^+$ (green) cells were identified by epifluorescence microscopy (17). To identify DN T cells, cells were incubated with anti-CD4 and anti-CD8 for 30 min on ice, washed three times with medium, incubated with PE-labeled-goat anti-rat IgG for 30 min on ice, washed five times with medium, incubated with FITC-labeled-anti-Thy-1.2 for 30 min on ice, washed three times with medium, and then resuspended in 1 ml of medium. The cells were visualized under the microscope and three populations were evident: $CD4^-CD8^-Thy\text{-}1.2^+$ cells appeared green, $CD4^+CD8^-Thy\text{-}1.2^+$ and $CD4^-CD8^+Thy\text{-}1.2^+$ cells appeared yellow, and B cells and macrophages were unstained. These staining protocols do not affect channel expression (15, 17, 18). In most experiments, chambers were coated with polylysine (0.25 mg/ml) to improve cell adherence to the dish. This procedure did not alter channel expression when compared with cells plated into uncoated chambers.

Electrophysiology

After phenotypic identification by epifluorescence microscopy, single T cells were patch-clamped at room temperature (22 to 26°C). Details of the voltage-clamp technique used are described elsewhere (15, 17–20).

Solutions. The cells under investigation were bathed in normal mammalian Ringer solution containing (in mM): 160 NaCl, 4.5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, and 5 Na-HEPES, pH 7.4. The patch pipette contained 134 KF, 11 K_2-EGTA, 1.1 $CaCl_2$, 2 $MgCl_2$, and 10 K-HEPES (pH 7.2). In Ringer solutions containing TEA^+, NaCl was replaced by the appropriate TEA^+ concentrations keeping the osmolarity constant. The bath solution could be changed during recordings by bath perfusion.

Identification of K^+ channel type. We identified K^+ channels on the basis of their inactivation properties, channel closing kinetics, and sensitivity to block by TEA^+ (15, 17–19, 21, 22). Type *n* K^+ channels become inactivated during repetitive pulses of 1 Hz (use dependent), close slowly upon repolarization with a time constant of about 30 ms at −60 mV, and are blocked by TEA^+ (K_d = 8 mM). Type *l* K^+ channels are not use dependent, close more rapidly on repolarization with a time constant of 2 ms at −60 mV, and are much more sensitive to block by TEA^+ (K_d = 0.1 mM). Type *n′* K^+ channels are not use-dependent, close slowly as do type *n* K^+ channels upon repolarization, but are less sensitive to block by TEA^+ (K_d ~100 mM).

Determination of g_K and number of K^+ channels per cell. The largest K^+ currents recorded in each cell at 40 mV along with a reversal potential of −80 mV were used to calculate g_K (20). Each data point in Figures 2 to 4 represents g_K in a single cell. Cells were pooled from two to five animals in each group with about equal numbers of cells from each animal (five animals per group for the diseased study). The number of K^+ channels per cell was calculated by dividing g_K by the single-channel conductances of the appropriate channel type; the single-channel conductances are 18, 27, and 17 pS for *n*, *l*, and *n′*, respectively (15, 17, 18).

RESULTS

DN T cells from mice with collagen arthritis possess abnormally large numbers of type l K^+ channels. Figure 1 shows K^+ outward currents in DN T cells from normal DBA/1 LacJ mice and mice with collagen arthritis. In the experiments shown in Figure 1, *upper panel*, we measured the decline in the size of the K^+ current elicited by a repetitive (1/s) depolarizing voltage-step from −80 mV to +40 mV for a duration of 200 ms. This property of K^+ channels which is termed use dependence is characteristic of type *n* K^+ channels, but not of types *l* or *n′*. Another feature of type *n* K^+ channels is their rate of closing during membrane hyperpolarization, after an initial period of depolarization. This property, called deactivation, can be assessed by analysis of tail currents as

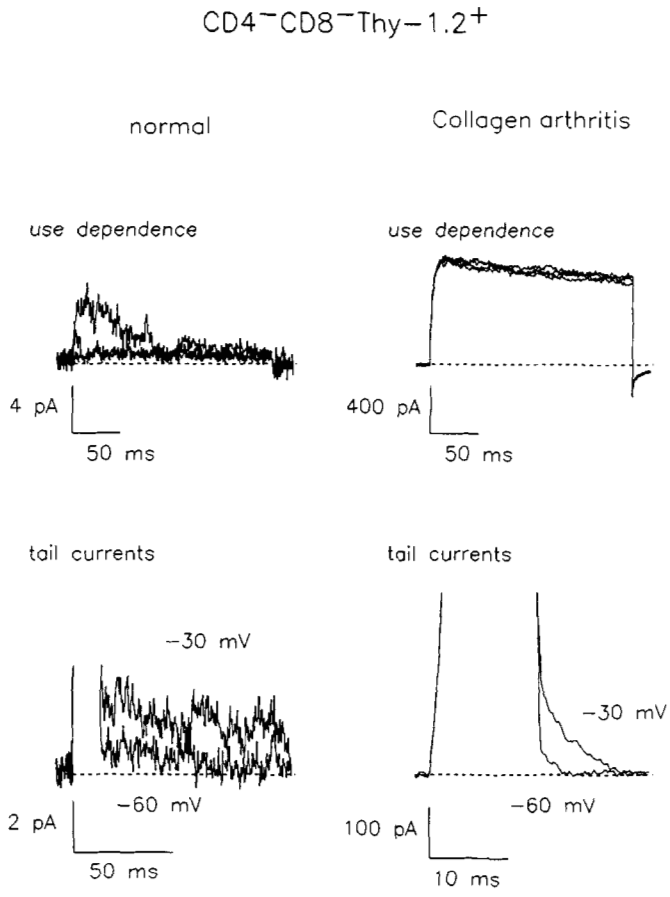

Figure 1. Whole cell K^+ currents in $CD4^-CD8^-Thy\text{-}1.2^+$ T cells in normal mice (*left*) and mice with collagen arthritis (*right*). *Top*, K^+ currents were elicited with 200 ms depolarizing pulses to 40 mV from a holding potential of −80 mV. Pulse interval was 1 s to assay cumulative (use dependent) inactivation. Shown are representative current traces recorded in one cell from a normal mouse (*left*) and in one cell from a mouse with collagen arthritis (*right*) in response to the first four pulses. Note the different current scale. *Bottom*, Tail currents were elicited by voltage steps to −60 and −30 mV after a 15 ms prepulse to 40 mV. Note the different current and time scale.

shown in the *bottom panel* of Figure 1. In these studies, the membrane potential was held at −80 mV, then stepped to +40 mV for a 15 ms duration to open all the channels, and then stepped back to either −30 or −60 mV. Type *n* and *n′* channels close slowly (time constants for closure are 75 and 40 ms at −30 and −60 mV, respectively), whereas type *l* K^+ channels close rapidly (time constants for closure are 6 and 2 ms at −30 and −60 mV, respectively).

DN T cells from normal DBA/1 LacJ mice express small numbers of type *n*, *n′*, or *l* K^+ channels, and thus display small K^+ currents upon depolarization. Characteristic recordings from a normal DN T cell with type *n* K^+ channels are shown in the *left panel* of Figure 1; the small K^+ currents are use dependent (*top*), display slow deactivation kinetics (*bottom*), and are half-blocked by 10 mM TEA^+ (not shown). In contrast, DN T cells from diseased mice have large K^+ currents (*right panel* of Fig. 1) that are not use dependent (*top*), exhibit rapid deactivation kinetics (*bottom*), and are half-blocked by 0.1 mM TEA^+ (not shown), indicating that these cells express an abundance of type *l* K^+ channels.

Figure 2*a* shows the g_K of splenic DN T cells from normal and diseased mice; Fig. 2b represents the fraction of cells with large numbers of type *l* K^+ channels. The

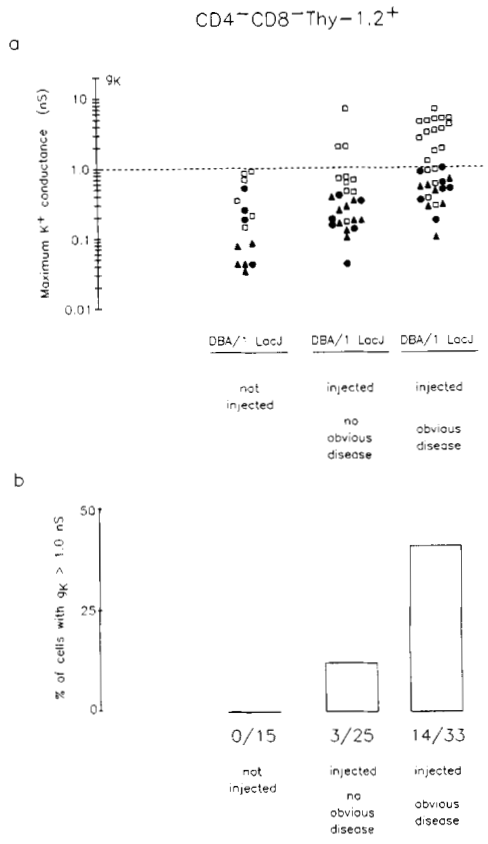

Figure 2. Maximal K^+ conductance (g_K) of $CD4^-CD8^-$Thy-1.2^+ T cells: *a*, ▲, □, ● represent *n*, *l*, *n'* K^+ channel types. Each data point represents g_K in one single cell. *b*, Percentage of cells with type *l* K^+ channels and g_K > 1000 pS.

upper limit for g_K in DN T cells from normal DBA/1 LacJ mice is 1000 pS, with an average of 299 + 78 pS (mean + SEM; n = 15). This value is similar to the g_K of DN T cells from C3H-HeJ, BALB/c, and C57BL of 405 + 53 pS (1). These data indicate that DN T cells from normal DBA/1 LacJ mice express roughly 10 to 20 K^+ channels/cell of types *n*, *l*, or *n'* as with other normal strains of mice. In marked contrast, 14 of 33 DN T cells from diseased DBA/1 LacJ mice immunized with type II collagen, exhibited a g_K of more than 1000 pS (Fig. 2 *a* and *b*), and the channels in these cells were exclusively type *l*, averaging 180 channels/cell. Interestingly, DN T cells exhibiting large numbers of type *l* K^+ channels were substantially fewer (3/25) in DBA/1 LacJ mice immunized with type II collagen, but which developed no obvious clinical signs of arthritis compared with symptomatic mice (Fig. 2 *a* and *b*). These data suggest that increased levels of type *l* K^+ channel expression in DN T cells parallel the development of collagen arthritis.

Other T cell subsets from diseased mice retain their normal pattern of expression. Figure 3 shows the g_K values in helper ($CD4^+CD8^-$) and cytotoxic ($CD4^-CD8^+$) T cells from normal and diseased mice. $CD4^+CD8^-$ T cells from both normal and diseased mice exhibited small numbers of K^+ channels, (averaging 10 to 20 channels/cell), which were predominantly type *n*. $CD4^-CD8^+$ T cells from mice with collagen arthritis displayed small numbers of types *n'* or *l* K^+ channels as with their phenotypic counterparts from normal mice. Thus, the augmentation of the number of type *l* K^+ channels appears to be a feature confined to DN T cells.

Abundant type l K^+ channel expression in DN T cells is not feature of generalized immune response in vivo. To discern whether the enhanced number of type *l* K^+ channels in DN T cells is unique to autoimmune disorders, or whether it reflects a generalized immune response in vivo, we examined DN T cells from mice immunized with CFA (Fig. 4). The DN T cells that were studied were of a comparable size (0.37 to 2.7 picofarads) to DN T cells seen in mice with autoimmune disorders. For the CFA experiments, some mice received one CFA immunization (26 days before patch-clamp experiments); others were given two booster injections of CFA either 62 and 26 days before or 26 and 2 days before patch-clamp experiments. DN T cells from CFA immunized mice possessed small numbers (g_K = 511 + 46 pS; mean + SEM; n = 32) of types *n*, *l*, or *n'* channels, regardless of whether they received one immunization or booster doses. Collectively, these data suggest that expression of a high number of type *l* K^+ channels by DN T cells is not associated with a generalized immune response and appears to correlate with the symptoms of autoimmune diseases.

DISCUSSION

Studies with the patch-clamp recording technique have revealed the presence of three distinct types of voltage-gated K^+ channels in murine T cells (15, 17–19). These channels, termed *n*, *n'*, and *l*, are distinguishable on the basis of biophysical and pharmacologic criteria. Th and cytotoxic T cells characteristically exhibit different patterns of K^+ channel expression. $CD4^+CD8^-$ (Th) T cells display about 10 to 20 type *n* K^+ channels whereas $CD4^-CD8^+$ (cytotoxic) T cells exhibit 10 to 20 type *l* or *n'* K^+ channels (Fig. 3) (1, 18). Mitogen-activated T cells express about 20 times more voltage-gated K^+ channels than unstimulated cells, which are exclusively type *n* (22). Quiescent DN T cells possess small numbers of one of these types of channels (1, 18). We recently found that abundant type *l* K^+ channel expression is a marker for DN T cells associated with murine SLE, type-1 diabetes mellitus, and chronic EAE; other T cell subsets from mice with these autoimmune diseases retain their normal pattern of K^+ channel expression (15). We have extended our observations to mice with type II collagen arthritis, an autoimmune disorder that shares many immunologic features with SLE, type 1 diabetes and chronic EAE.

A large fraction of DN T cells from mice with active type II collagen arthritis display elevated numbers of type *l* K^+ channels compared to phenotypically similar cells from normal mice (Figs. 1 and 2). Helper ($CD4^+CD8^-$) and cytotoxic ($CD4^-CD8^+$) T cells from these arthritic mice exhibit a normal pattern of K^+ channels. The augmented numbers of type *l* K^+ channels in DN T cells does not appear to reflect an in vivo immune response because mice immunized with CFA (Fig. 4), or mice injected with type II collagen but without evidence of active disease, exhibited small numbers of types *n*, *n'*, or *l* K^+ channels. In addition, mice at the terminal stages of acute experimental allergic encephalomyelitis displayed a normal K^+ channel phenotype (1), suggesting that abundant display of type *l* K^+ channels by DN T cells is not related to the animals being sick and in pain. The most interesting explanation of the phenomenon is that it is related to the pathogenesis of chronic autoimmune diseases and may reflect an activation pathway distinct from that triggered

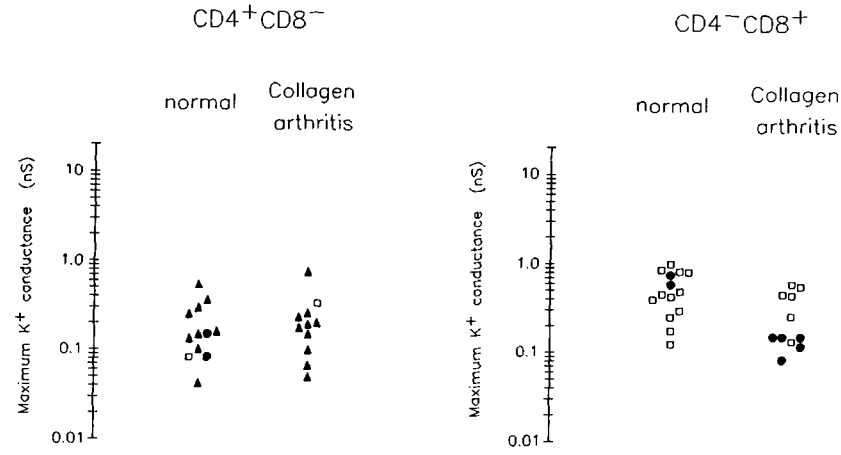

Figure 3. g_K of $CD4^+CD8^-$ and $CD4^-CD8^+$ T cell subsets in normal mice and mice with collagen arthritis. ▲, □, ● represent *n*, *l*, *n'* K^+ channel types.

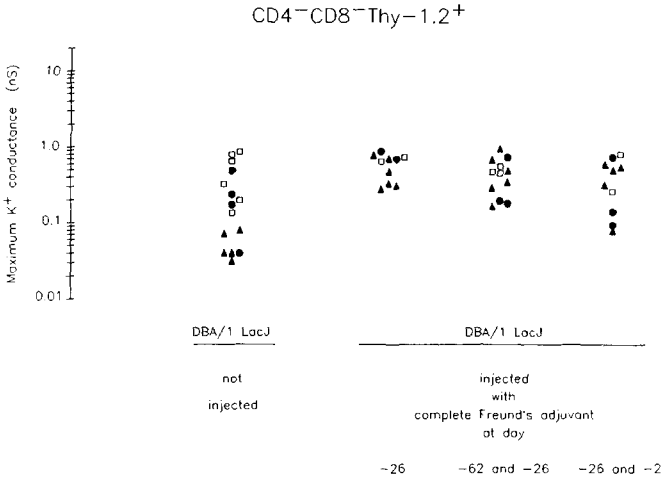

Figure 4. g_K of $CD4^-CD8^-$Thy-1.2^+ T cells in uninjected DBA/1 LacJ mice and DBA/1 LacJ mice injected either once with CFA at day −26, or injected twice with CFA at day −62 and −26 or at day −26 and −2. Day 0 refers to the day, the electrophysiologic experiments were performed. ▲, □, ● represent *n*, *l*, *n'* K^+ channel types.

by mitogens or Ag (e.g., CFA). However, it is not clear whether the alteration is causally related to autoimmunity or whether it is a result of the disease process.

The DN T cell population accounts for <5% of T cells in normal peripheral lymphoid organs. Flow cytometric studies indicated that DN T cells in both prediseased and diseased DBA/1 LacJ mice were also less than 5% of T cells in lymphnodes or spleen (G. M. Peterman and D. C. Hanson, unpublished studies). Furthermore, the numbers of $CD3^+$ α/β and/or γ/δ DN T cells in diseased mice were similar to that in normals.

Recent reports show that human γ/δ TCR^+ DN T cells respond to mycobacterial Ag and accumulate in leprotic skin lesions, cutaneous leishmaniasis, and joints affected by rheumatoid arthritis (23, 24). These DN T cells may contribute to inflammatory processes by inducing the aggregation of monocytes (23). Human and mouse DN T cells have been reported to act as helper cells, inducing autoreactive B cells to secrete pathogenic anti-DNA antibodies (25–27). DN T cells have also been reported to abolish oral tolerance (28). Collectively, these observations suggest that DN T cells may have a significant role in immune responses and may be involved in the pathogenic mechanisms that result in tissue damage found in autoimmune diseases.

Several voltage-gated K^+ channels genes have been recently cloned (29–33). Interestingly, the NGK2 cDNA, structurally related to the *Shaw* gene in *Drosophila* (34) when expressed in *Xenopus* oocytes, has biophysical properties that resemble those of type *l* K^+ channels (32). Future studies may help in identifying the type *l* K^+ channel gene and defining the mechanisms responsible for augmented type *l* K^+ channel expression in DN T cells associated with autoimmunity.

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