

Exogenously-induced, Natural Killer Cell-mediated Neuronal Killing: A Novel Pathogenetic Mechanism

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Summary

Many human neurodegenerative diseases are characterized by the idiopathic death of cells narrowly restricted to a subset of neurons in a specific functional neuroanatomic system. Few in vivo models exist for the analysis of these types of degeneration. This report documents the death of sympathetic neurons resident in the superior cervical ganglia of rats after exposure to an exogenous chemical agent, the drug guanethidine, as being mediated by natural killer (NK) cells. This is the first in vivo model of a disorder of the nervous system in which NK cells appear to be the principal effector cell, and thus could serve a central role in dissecting the normal and pathological function of NK cells. In addition, this pathogenetic mechanism appears to represent a novel type of autoimmune reaction that could have a direct bearing on a number of human illnesses.

NK cells are a subpopulation of CD3⁻, LGL whose function to date has been defined by their ability to mediate the in vitro destruction of certain neoplastic or virally infected cells in a rapid, non-MHC restricted fashion (1-3). NK cells can be phenotypically identified by their characteristic expression of cell surface markers which in the rat include 3.2.3 (NKR-P1), CD8, and asialo-GM₁ (4-7). Although there is data to support a role for NK cells in the protection of the individual from infections and neoplasms (8-13), their role as a primary effector cell causing in vivo, autoimmune tissue destruction remains controversial (14). Recently, a mAb, 3.2.3, was developed (6, 7) that recognizes all rat NK cells, and which selectively depletes recipient rats of circulating NK cells without affecting circulating lymphocytes or monocytes (6). This reagent, which binds to NKR-P1, a cell membrane-signaling molecule for NK cell activation (15, 16), makes it possible to specifically define the in vivo role of NK cells in various disease models.

Guanethidine is an adrenergic neuron-blocking agent whose pharmacology and metabolism have been extensively studied (17-19). In 1971, Erkanko and Erkanko (20) reported that this drug induced destruction of sympathetic neurons in the superior cervical ganglia (SCG)¹, of neonatal and adult rats, while sparing nonsympathetic neurons. Subsequently, this model of selective sympathectomy has been extensively characterized (21-26).

Observations by Manning et al. (27, 28) concerning the

mechanism of this sympathectomy determined that it was at least in part mediated by cellular elements of the immune system, and could be blocked by lethal irradiation or cyclophosphamide administration. One curious feature of this immune-mediated destruction in rats was that it occurred more rapidly than typical T cell-mediated reactions. Cellular infiltrate and neuronal death could be seen a few days after initiation of treatment in neonatal rats, and was virtually complete by 7 d (26). Moreover, athymic rats were as susceptible as euthymic rats to this neuronal destruction (24, 25).

Prior studies in our laboratory have shown that the principal infiltrating cell type during guanethidine-induced sympathectomy is a CD8⁺, lymphocyte-like cell (26). This observation, coupled with the aforementioned phenomena associated with this system, lead us to the hypothesis that NK cells might be central to its pathogenesis. This study was designed to evaluate morphologically and in vivo the role of rat NK cells in guanethidine sympathectomy.

Materials and Methods

Animals. Pregnant Fisher 344 rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). The rats were housed separately with free access to water and rat chow. When the pups were delivered, they were left with their mothers throughout the experiment.

Induction of Sympathectomy. Beginning at 1 wk of age, rat pups were given daily subcutaneous injections of 100 μ l of guanethidine monosulfate (Sigma Chemical Co., St. Louis, MO) dissolved in PBS at a dose of 25.0 mg/kg for 5 d. Control animals received saline injections. All pups were killed 24 h after the last injection,

¹ Abbreviation used in this paper: SCG, superior cervical ganglia.

and their SCG and mesenteric ganglia removed for immunohistochemistry and histological evaluation.

Antibody Treatment. The antibodies employed in an attempt to prevent the neuronal necrosis are listed in Table 1. The majority of the antibodies used were murine IgGs directed specifically to rat antigens. All murine antibodies were obtained from Bioproducts for Science (Indianapolis, IN), except for the 3.2.3 antibody which was supplied (by J. Hiserodt) as ascites. The antisialo-GM₁ was a rabbit antiserum (Wako Chemicals, Dallas, TX). All of these antibodies were diluted in PBS to a protein concentration of 1.0 mg/ml, and specific pups were given 0.5 ml of the antibody by intraperitoneal injection on the day before the first and at the time of the third guanethidine injection. As a control for the antibody injections, 0.5-ml injections of normal mouse serum diluted 1:5 with PBS were given at similar times.

Tissue Processing. At the time of killing, the SCGs were removed from each rat pup. One was fixed in buffered 3% glutaraldehyde and embedded in plastic resin for histological and ultrastructural studies (26). The other SCG was snap frozen in OCT (Miles Laboratories Inc., Elkhart, IN) for immunohistochemical analysis. Immunohistochemistry was performed on 5.0- μ m thick sections by a previously detailed method (29). Both 1- μ m-thick plastic sections and immunohistochemically stained sections from each rat were assessed for the presence and extent of inflammation and neuronal death (Table 2). In addition, the spleen and other ganglia were removed from selected pups for immunohistochemical analysis to detect the effectiveness of the antibodies in depleting the NK cells or for the presence of inflammation at other ganglionic sites.

Results and Discussion

Guanethidine produced the expected, widespread neuronal destruction in the SCG within 5 d (Fig. 1 A). As shown in Table 2, normal mouse serum (Fig. 2 A), as well as antibodies against T cell epitopes (including CD5 and CD4), macrophage/monocytic molecules, or MHC class I and II antigens, all failed to protect the rat pups from the guanethi-

dine effect. Complete or near complete protection of pups from guanethidine-induced neuronal death was provided by either a rabbit antiserum against asialo-GM₁, or a combination of 3.2.3 and antisialo-GM₁. Normal mouse serum had no effect. The antibodies 3.2.3 and OX-8 alone protected to an extent greater than any of the other mAbs. In 4 of 12 pups given 3.2.3 treatment, this mAb completely prevented both neuronal killing and cellular inflammation of the ganglia (Fig. 1 B). The efficacy of both antisialo-GM₁ and 3.2.3 antibodies in depleting the NK cells of the rats has been previously documented (6), the latter by inducing degranulation via the NKR-P1 membrane-signaling molecule (15, 16). In this study, these antibodies, alone or together, extensively reduced or rendered undetectable the splenic population of NK (3.2.3 positive) cells in treated pups.

Immunohistochemical analysis of the damaged ganglia showed a marked infiltrate by 3.2.3 positive NK cells in all the groups that exhibited neuronal destruction and inflammation (Fig. 1 C). In addition to NK cells, other CD4⁺, CD8⁺, and macrophage/monocytic (CD11b/c) cells were present. In the ganglia of animals treated with 3.2.3 and/or antisialo GM₁, 3.2.3 positive cells were very sparse or undetectable. Moreover, the other attendant cellular components of the inflammatory infiltrate were likewise reduced or eliminated.

The data obtained by these experiments strongly suggest that the guanethidine-induced death of neurons in the SCG is mediated by cells morphologically and phenotypically consistent with the NK lineage. Moreover, this destruction can be substantially inhibited by antibodies known to interfere with NK cell function. One of the striking features of guanethidine-induced sympathectomy is that it is predominantly restricted to SCG sympathetic ganglion cells, whereas sympathetic ganglion cells in the prevertebral superior mesenteric and celiac ganglia exhibit far less cell death (21, 23, 26). There are species and strain variations in sensitivity to the

Table 1. *Antibodies Used*

Antibody designation	Specificity	NK cells	CD8 ⁺ T cell	CD4 ⁺ T cell	Monocyte/Macrophage	B cells
3.2.3	rat NK cells	+++	-	-	-	-
antiAsialo-GM ₁ *		+++	+	-	++	-
OX-8	rat CD8	++	+++	-	-	-
W3/25	rat CD4	-	-	+++	+	-
OX-42	rat CR3 (CD11b/c)	+	-	-	+++	-
OX-19	rat CD5	-	++	++	-	-
OX-52	peripheral T cells	+	++	++	-	-
OX-18	rat MHC class I	++	++	++	++	++
OX-6	rat MHC class II	-	±	+	+++	++

* This is a rabbit antiserum produced by Wako Chemicals USA, Inc. (Dallas TX, code #986-10001.). 100 μ l of this antiserum injected into an adult BALB/c mouse 72 h before testing completely inhibits YAC-1 target cell killing by the NK cells obtained from that mouse.

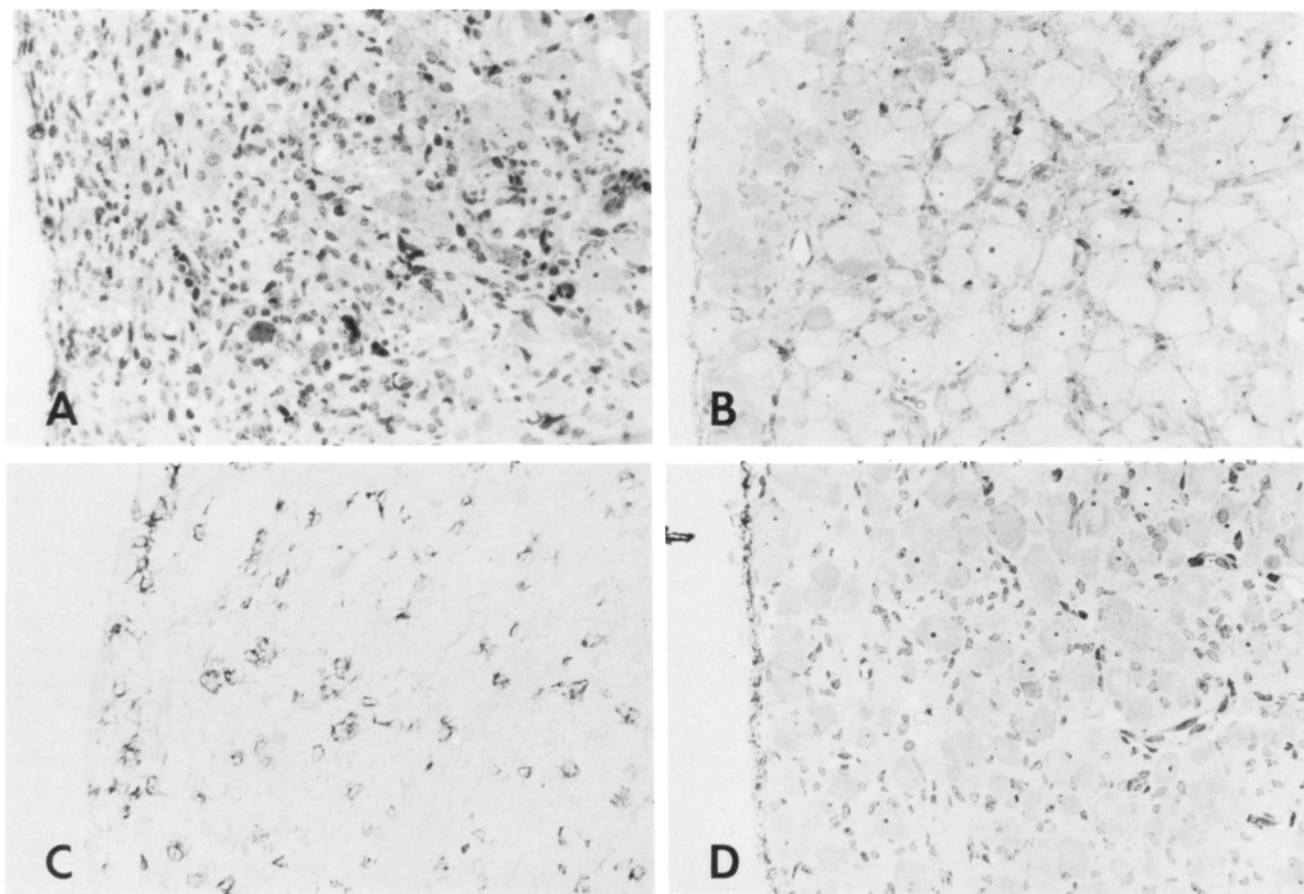


Figure 1. Photomicrographs of SCG from rat pups. (A) SCG from a rat receiving guanethidine and normal mouse serum as a control (graded 3 for both neuron loss and inflammation). (B) SCG from a rat pup given guanethidine and 3.2.3. Note the extent of protection and lack of inflammation in the ganglion of the pup in which NK cells have been depleted. (C) Ganglion from a pup during the acute phase of neuronal destruction which has been immunohistochemically stained with the anti-NKR-P1 antibody 3.2.3. Note the number of NK cells identified in the inflamed ganglion. (D) SCG from a normal, untreated pup, age matched with the treated groups (histologically normal ganglion). A, B, and D were obtained from toluidine blue-stained, 1- μ m-thick plastic sections. $\times 250$.

drug suggesting a strong effect of the subject's genetic composition on the process (23, 26). Thus, the guanethidine model of sympathetic neuron killing is a useful system for studying neuroanatomically specific cell death. This study also provides evidence for the existence of a unique mechanism of autoimmunity: an exogenously/chemically induced alteration in a specific subset of bodily cells that targets them for NK cell-mediated killing.

Immunohistochemical and ultrastructural studies previously performed in our laboratories support a potential role for NK cells in this process (26). As evidence for this hypothesis, immunohistochemical analyses of SCG from rat pups undergoing guanethidine-induced sympathectomy revealed that a large proportion of the CD8⁺ cells in the ganglion 5–7 d after the initiation of guanethidine—during the stage of neuronal destruction—was also positive with the selective NK cell marker 3.2.3 (Fig. 1) as well as for the expression of asialo-GM₁, also characteristic of NK cells. However, immunofluorescent double-labeling of an affected ganglion simul-

taneously with 3.2.3 and anti-CD3 (expressed on all mature T cells [30]) mAbs revealed that the majority of the infiltrating CD8⁺ cells were 3.2.3 positive, but CD3 negative, thus confirming their NK cell phenotype (31–33). Normal rat SCGs (Fig. 1 D) contain neither T cells nor 3.2.3 positive NK cells. Ultrastructural examination of ganglia in which neurons were degenerating demonstrated the presence of large lymphocytoid cells with granular cytoplasmic inclusions which were not a normal cellular constituent of the ganglia (26). These LGL cells in the rat are typical of cells with natural killer function (4–7, 13). Moreover, in the SCG they could occasionally be found within the neuronal Schwann cell sheath in intimate apposition to neurons (Fig. 2). The present study is consistent with a cytotoxic role of NK cells in this neuroanatomically restricted system of neuronal killing by using antibodies directed to various hematogenous cell types to selectively inactivate specific cellular participants.

Since NK cells do not possess the antigen-specific receptor complex characteristic of T and B lymphocytes, the recogni-

Table 2. *Experimental Protocol and Results*

Antibodies injected*	Number of rat pups	Neuronal death [‡]	P value [†]	Inflammatory infiltrate	P value [†]
<u>Controls:</u>					
No antibody and no guanethidine [§]	8	0.03 ± 0.13	<0.001	0.2 ± 0.1	<0.001
Normal mouse serum + guanethidine	9	2.5 ± 0.5	-	2.5 ± 0.6	-
<u>Test groups (all with guanethidine):</u>					
3.2.3	12	0.7 ± 0.7	<0.001	1.0 ± 1.0	<0.005
antiasialo GM ₁	8	0.5 ± 0.4	<0.001	0.4 ± 0.4	<0.001
3.2.3 plus antiasialo GM ₁	6	0.4 ± 0.3	<0.001	0.5 ± 0.3	<0.001
OX-8	5	0.8 ± 0.6	<0.001	1.0 ± 0.6	<0.001
anti T-cell (OX-19, OX-52 plus W3/25)	5	2.9 ± 0.3	NS	3.0 ± 0.2	NS
OX-18	2	1.9 ± 0.3	NS	2.0 ± 0.4	NS
OX-6	3	2.1 ± 0.7	NS	2.3 ± 0.4	NS
OX-42	2	1.8 ± 0.5	NS	2.1 ± 0.3	NS

* mAbs were obtained as ascites, and the protein concentration adjusted to 1 mg total protein/ml. The antiserum against asialo-GM₁ was reconstituted according to manufacturer's instructions. All antibodies were given by intraperitoneal injection of 100 µg of the antibody indicated in a total volume of 0.5 ml on days -1 and +2. The first guanethidine injection was given on day 7 of life, and continued daily. The pups were killed 24 h after the 5th injection.

‡ 1-µm-thick plastic sections of all experimental ganglia (including controls from normal rat pups) were scored blindly by two investigators using a semiquantitative scale. The scale ranged from 0 to 3, with 0 indicating no cell death; 1, scattered (<10%) dead neurons; 2, clusters of dead neurons; and 3, widespread or total neuronal destruction. The mean ± SD was derived from the aggregate values from both observers for all pups in that group.

|| As with neuronal death, the presence of infiltrating T cells, NK cells, and monocytes was judged on a semiquantitative scale of 0-3 ranging from no detectable cells to confluent inflammation.

† P values were computed for two independent samples of unequal size and unknown population variance using a two-tailed Student's *t* test. The P values represent the significance of the statistical difference of the values in the column to the left, between pups receiving guanethidine plus normal mouse serum, and the group for which the value is given. NS, *P* > 0.05.

tion system and the "antigen" or substance being identified as foreign by NK cells in the ganglia after guanethidine treatment, remains unknown (3, 31, 34). Nevertheless, their recognition of targets does not appear to follow the classical rules of induction and memory pertaining to other lymphocyte classes (1-3). At this time, it cannot be determined if the NK-mediated destruction is by direct neuronal cytotoxicity, or whether the NK cells produce killing via another more indirect immune mediated process requiring the presence of additional inflammatory cell types. However, a central role for the NK cell in this process seems to be supported. The precise molecular events occurring in the ganglion cells which render them susceptible to this cytotoxic mechanism have yet to be identified.

Among human neurological disorders, many of the most enigmatic are those in the group of so-called "neurodegenerative diseases." Conditions such as Parkinson's disease, amyotrophic lateral sclerosis (ALS), striato-nigral degeneration, the cerebellar degenerations, Hallevorden-Spatz disease and others, have no currently known etiology. They do, however, have in common the specific, highly selective degeneration of neurons in particular neuroanatomic systems.

At present, we have no evidence that such an NK cell-

mediated system of neuronal destruction is involved in any of the human neurodegenerative conditions. Moreover, the above mentioned diseases, save perhaps ALS (35, 36), are not currently believed to have an immunologically mediated pathogenesis. Nevertheless, four observations must be considered while evaluating the possibility that some human neurodegenerative disorders might occur via an immunologically mediated mechanism: (a) In rat ganglia examined 2 mo after guanethidine-induced cell destruction, there is no evidence of an antecedent inflammation event. The ganglia are devoid of infiltrating lymphoid cells. Therefore, histological examination of the destroyed neuronal group at time points significantly beyond the acute phase of injury may give no clue as to the mechanism causing the neuronal destruction. (b) In the guanethidine model of NK killing of neurons, the exposure of all the target neurons to the offending substance is simultaneous, high in concentration, and regularly repeated over a short interval. In human disease, if such a mechanism is acting, the exposure of neurons to the offending agent might be selective within a set of neurons, of low dosage, and irregularly administered over a protracted period of time. (c) Guanethidine and its close analog guanacaine are both known to produce SCG inflammatory neuronal killing in subhuman

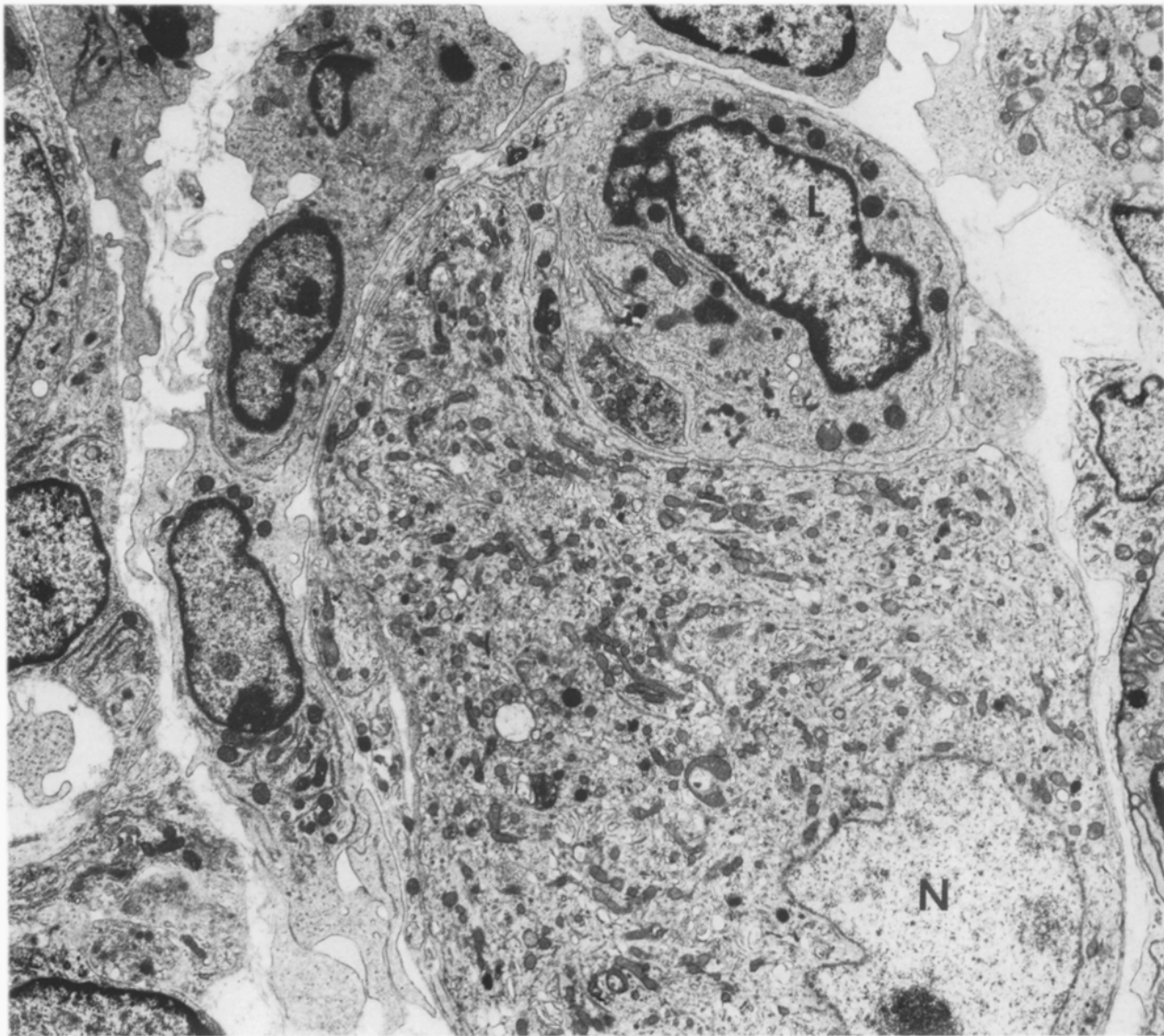


Figure 2. Ultrastructural appearance of cells in the SCG 7 d after the initiation of guanethidine treatment. A large granulated lymphocyte (*L*) has breached the satellite cell sheath and is intimately apposed to the cell body of a principal sympathetic neuron (*N*). The neuronal cytoplasm exhibits a diminished content of Nissl substance and an accumulation of a variety of subcellular organelles. $\times 6,600$.

primates (37), further supporting the possibility that this pathogenetic pathway might be active in some human diseases. (*d*) Persistent orthostatic hypotension (a condition referable to autonomic/sympathetic neuron dysfunction) has been reported in human patients given high doses of guanacine (38, 39), although, the pathology and pathogenetic mechanism have not been described.

The present report raises the possibility that one or more of the idiopathic human neurodegenerative diseases may develop via an exogenously induced, autoimmune, NK cell-mediated pathogenetic process heretofore not known to exist.

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