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Evidence of a Neurogenic Component During IgE-Mediated Inflammation in Murine Skin

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EVIDENCE OF A NEUROGENIC COMPONENT DURING IgE-MEDIATED INFLAMMATION IN MURINE SKIN

by

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ABSTRACT

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The direct stimulation of sensory nerves or the intradermal injection of neuropeptides triggers a cutaneous inflammatory response that involves the degranulation of mast cells. These neurogenic inflammatory reactions are physiologically similar to IgE-mediated hypersensitivity responses, which also depend on mast cells. Although non-antigenic stimuli distinguish neurogenic from IgE-mediated inflammation, the similarity of their effector stages suggested that peripheral nerves may also participate in IgE-mediated responses. To examine this hypothesis, IgE responses were elicited in denervated skin. A murine model was developed where the footpad was denervated by surgically removing a 2 mm segment of the sciatic nerve, proximal to the tibial and common peroneal branch. The other hind leg received either sham surgery or no surgery (untreated). One week after surgery, cutaneous inflammation was induced in denervated footpads with a monoclonal IgE and its antigen, and compared to responses in sham and untreated control footpads. IgE-induced swelling was significantly reduced by approximately one-third in denervated footpads of both passively sensitized and immunized mice. In denervated footpads passively sensitized with IgE, reduced swelling responses were observed over a range of local IgE doses, and were not dependent on the route

of sensitization. In denervated footpads of immunized animals, significantly reduced swelling responses were measured against two different antigens. This reduction in IgE-mediated inflammation detected in denervated footpads was not due to hyporesponsive blood vessels, because denervated footpads responded normally to the vasoactive mediators histamine and serotonin. Hyporesponsiveness to IgE in denervated skin was not the result of the desensitization of mast cells, or to a change in mast cell number. These findings indicate that peripheral nerves contribute significantly to IgE-mediated inflammation, demonstrating *in vivo* the existence of a neurogenic component during IgE responses in the skin.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Inflammation

Inflammation is the response of tissues to injury or irritation, and acts as a protective mechanism to eliminate the source of the injury and remove any damaged tissues. The classical signs of inflammation are redness, swelling, heat, and pain, and are common to all inflammatory responses. Local factors released by damaged tissues initiate a series of events, involving vascular changes and leukocyte activation, to produce these symptoms. Vasoactive factors induce blood vessel dilation and increased blood flow to the affected area, producing local reddening and increased temperature. These factors also cause increased vascular permeability resulting in local plasma extravasation, fluid accumulation, and tissue swelling. Chemotactic factors attract circulating leukocytes to the affected site where they are activated to eliminate the source of inflammation and restore normal tissue conditions. Pain is produced by the stimulation of sensory nerve fibers by particular products of damaged tissues. Additional substances lead to generalized systemic responses, including fever and an elevated number of blood neutrophils (Coleman et al., 1989; Sheldon, 1988; Tizard, 1988).

Immunologically, inflammation can be classified as nonspecific or specific, according to its cause. Nonspecific inflammation represents an innate response to the direct physical damage of tissues, such as a cut, burn, or insect bite. Immunologically specific inflammation occurs when products of an immune reaction lead directly to the response, such as a bacterial infection, an allergic reaction, or graft rejection. Inflammation initiated by the immune system is called hypersensitivity. Historically, hypersensitivity was viewed as immune responses which acted in an excessive or inappropriate form to produce tissue destruction, rather than

immunity. However, these reactions are presently regarded as beneficial in protecting the host against foreign substances. In 1963, the British immunologists, Coombs and Gell, classified hypersensitivity into four basic types: three (Types I, II, and III) within the category of immediate hypersensitivity, and the fourth (Type IV) within the category of delayed hypersensitivity. Immediate hypersensitivity refers to a response which is mediated by antibody, usually develops within hours, and is typified by an infiltration of polymorphonuclear leukocytes, primarily neutrophils, due to their preponderance in the blood. Delayed-type hypersensitivity is mediated by T lymphocytes, occurs within 24 to 48 hours, and is distinguished by an accumulation of mononuclear cells (Coleman et al., 1989; Sheldon, 1988; Tizard, 1988).

Type I hypersensitivity, also called anaphylactic inflammation, is mediated by IgE or some IgG subisotypes attached to the surface of mast cells or basophils. Specific antigen binding to the cell-fixed antibodies stimulates the release of vasoactive mediators from the cells, producing local inflammation within minutes. This class of inflammation includes allergic reactions, which may occur locally, as with hay fever or hives, or systemically, as with anaphylactic shock from a bee sting. Type II hypersensitivity is mediated by cytotoxic antibodies of the IgG or IgM isotypes which coat target cells, and then destroy them by activating complement or by antibody-dependent cellular cytotoxicity mechanisms. This form of inflammation is observed in early graft rejection and in some autoimmune diseases, when the antibody is directed against antigens on an individual's own cells. Type III hypersensitivity occurs when immune complexes form between soluble antigen and IgG or IgM antibodies, and their inefficient removal results in complement activation and tissue destruction. This local accumulation of immune complexes can lead to

an Arthus reaction, a necrotic cutaneous response, or to serum sickness from chronic microbial infections. Type IV, or delayed-type hypersensitivity, is mediated by lymphokines released from a specific population of antigen-activated T lymphocytes, and reaches its peak intensity two to three days after antigen contact. Examples of these responses include tuberculosis, leprosy, and contact sensitivity to poison ivy and heavy metals (Coleman et al., 1989; Paul, 1989; Roitt et al., 1987; Tizard, 1988).

IgE-Mediated Hypersensitivity

The mechanism of an allergic response was first described by Prausnitz and Kustner in 1921, when they found that allergies could be transferred by injecting serum from allergic patients into normal skin, and then challenging these sites with the allergen. However, it was not until 45 years later that Ishizaka and his colleagues demonstrated that this serum "reagin" was a new class of immunoglobulin, termed IgE (Ishizaka and Ishizaka, 1967). IgE is a globular serum protein consisting of paired κ or λ light chains and two ϵ heavy chains. It has a molecular weight close to 190,000 daltons, and is divalent with respect to binding sites for antigen. The characteristics which distinguish IgE from other antibody isotypes are: its low concentration in the serum (20-500 ng/ml), its heat lability (IgE is inactivated by heating at 56° C for two hours), and its ability to bind to mast cells and basophils. Free in the serum, IgE has a half-life of only two or three days. However, when cell-bound, it has a half-life of 11 to 12 days, but can remain bound to mast cells for up to 12 weeks (Paul, 1989; Tizard, 1988).

Certain environmental antigens, such as pollen or dust, have a tendency to stimulate the production of IgE antibodies in allergic individuals (Paul, 1989). Contact with these allergens then induces an anaphylactic response, which consists of two phases: the first is called the sensitization stage , and the second is the atopic

stage. The sensitization stage begins with the first exposure to an antigen, which initiates the production of IgE. Circulating IgE is then distributed throughout the tissues of the body where it binds to receptors specific for the Fc region of the molecule ($Fc_\epsilon R$) on the surface of mast cells and basophils (Coleman et al., 1989). Mast cells are large granular cells found in the loose connective tissues surrounding blood vessels, nerves, and glandular ducts; and are also distributed under epithelial, serosal, and synovial membranes. Basophils are granulytic cells found circulating in the blood, but which migrate into tissues in response to appropriate stimuli (Graziano, 1988). A second exposure to the allergen initiates the atopic stage, or the immediate inflammatory reaction. Antigen binding and cross-linking of surface-bound IgE activates the mast cells and basophils to release their cytoplasmic granules containing vasoactive and chemotactic mediators. In addition, the synthesis and secretion of other biologically active substances is stimulated, primarily the enzymatic break-down products of membrane phospholipids. Collectively, these pre-formed and newly synthesized mediators act to increase vascular permeability, constrict bronchial smooth muscle, induce the emigration of circulating neutrophils and eosinophils, and activate these leukocytes to remove and destroy the antigen (Coleman et al., 1989; Tizard, 1988).

The IgE molecule combines with its Fc receptor on the surface of mast cells through the second and third constant domains of its heavy chain. The binding between these proteins has a very high association constant (10^{10}/M) and a low dissociation constant, making the $Fc_\epsilon R$ a high-affinity receptor. Because IgE binds so firmly, crosslinking of surface-bound IgE by a multi-valent antigen causes the $Fc_\epsilon R$ to accumulate on the same pole of the cell forming a "cap". This formation induces local changes in the membrane structure, and activates membrane-associated

enzymes which initiate a series of biochemical events resulting in degranulation. The most critical of these events is an increase in intracellular Ca^{++} ion concentration, which has two main results. First, microtubules are formed that move the cytoplasmic granules toward the cell membrane. The granule membrane then fuses with the plasma membrane, and the granule contents are exocytosed, releasing pre-formed mediators into the local environment. Second, changes in the plasma membrane activate phospholipase A_2 to release arachidonic acid from membrane phospholipids. Arachidonic acid is then metabolized by lipoxygenase enzymes to produce leukotrienes, and metabolized by cyclooxygenase enzymes to produce prostaglandins and thromboxanes (Ishizaka and Ishizaka, 1984; Paul, 1989).

In addition to a multi-valent antigen, IgE-sensitized mast cells can be activated by anti-IgE antibodies or lectins, proteins which crosslink IgE by binding to carbohydrate residues. Because mast cell activation is mediated through the crosslinking of $Fc_\epsilon R$, antibodies against these receptors can trigger degranulation. Other compounds can directly activate mast cells by causing an influx of Ca^{++} ions, and include compound 48/80, mellitin, and Ca^{++} ionophores. *In vivo*, the cleavage products of complement activation, C3a and C5a, lead to the Ca^{++} ion influx crucial for degranulation (Ishizaka and Ishizaka, 1984; Roitt et al., 1987).

The pre-formed mediators contained in mast cell granules include: histamine, serotonin, chemotactic factors, proteolytic enzymes, and kininogenases. Histamine, an amine formed by the decarboxylation of the amino acid histadine, is bound to the proteoglycan-protein core of the mast cell granule, and is released by exchange with cations once exposed to the extracellular fluid (Pepys and Edwards, 1979). It acts quickly to dilate capillaries and venules, increase vascular permeability, contract the smooth muscle of the lung bronchi, and stimulate glandular secretions. Serotonin,

or 5-hydroxytryptamine, is a derivative of the amino acid tryptophan, and is released primarily from the mast cells of rodents. It also functions rapidly to increase vascular permeability and contract bronchial smooth muscle. Neutrophil chemotactic factor of anaphylaxis attracts neutrophils to the site of inflammation, and activates them to phagocytize and remove damaged cells. Eosinophil chemotactic factor of anaphylaxis attracts and stimulates eosinophils to release antihistamine-like substances, which function to reverse some of the deleterious effects of anaphylaxis. Activated eosinophils contribute significantly to the body's defense against parasitic worms, and represents a beneficial IgE-mediated response. Tryptase directly activates the third component of complement, leading to the production of complement anaphylatoxins (C3a and C5a), which directly cause the degranulation of mast cells. Kininogenases act on local tissue kininogens to generate bradykinen, which stimulates vasodilation, bronchiole contriction, and pain receptors (Schwartz and Austen, 1984; Coleman et al., 1989).

The newly synthesized mediators of mast cells are products of the enzymatic breakdown of membrane phospholipids and include: leukotrienes, prostaglandins, thromboxanes, and platelet activating factor. Leukotrienes C_4, D_4, and E_4 mediate a late phase skin reaction, which is often observed several hours following the immediate response and may last up to 24 hours after contact with the antigen. These leukotrienes induce physiological effects similar to those of the preformed vasoamines, but act slower to produce longer-lasting and generally more severe results. Leukotriene B_4 stimulates neutrophil and eosinophil chemotaxis and motility, and enhances their capability for complement-mediated lysis. The prostaglandins and thromboxanes induce a spectrum of activities, including the modulation of smooth muscle contractility, vascular permeability, and the sensation of pain.

Platelet activating factor stimulates the aggregation and lysis of platelets, active fragments of megakaryocytes which contain granules of serotonin. It also attracts and activates phagocytic leukocytes, and increases the stickiness of the vascular endothelium for these cells (Schwartz and Austen, 1984).

In rodents, there are two subpopulations of mast cells which differ both morphologically and functionally: the connective-tissue mast cell, and the mucosal mast cell. Connective-tissue mast cells are found ubiquitously throughout the body in the connective tissues surrounding blood vessels and nerve fibers. Mucosal mast cells are located primarily in the mucosa of the lung and gastrointestinal tract, where their frequency sharply increases during parasitic infections. Morphologically, connective-tissue mast cells are larger (20 μm diameter), contain many more granules, have a longer life-span (over six months), and express fewer surface $Fc_{\epsilon}R$ (rat peritoneal mast cells express 2 to 3 x 10^5 receptors). The major proteoglycan found in the granules of connective-tissue mast cells is heparin, whereas mucosal mast cells contain chondroitin sulfate instead, giving rise to different staining properties. Mucosal mast cells are also distinguished by their growth characteristics, which are dependent on factors released from T lymphocytes, such as interleukin-3. Functionally, these mast cell populations respond oppositely to anti-allergic drugs, such as cromoglycate and cortisone, which inhibit histamine release from connective-tissue mast cells, but not from mucosal mast cells (Bienenstock, 1988; Tizard, 1988).

The Sensory Nervous System

Most activities of the nervous system are initiated by sensory information coming from sensory receptors throughout the body, including receptors that detect stimuli such as touch, sound, light, and pain. These receptors change sensory stimuli into electrical signals which are carried to the central nervous system through an

afferent neuron. The signal enters the central nervous system through the spinal nerves and is conducted to multiple primary sensory areas in the spinal cord and brain, where it is processed and may be transformed into a motor command. This signal is then carried by an efferent, or motor, neuron to the effector organ to produce the desired reflex response to the stimulus (Kandel and Schwartz, 1985; Guyton, 1987).

The neuron has four morphologically defined areas: the cell body, the dendrites, the axon, and the presynaptic terminals of the axon. The cell body, or soma, is the metabolic center of the neuron and contains most of the organelles characteristic of these cells, including a large nucleus and an extensive endoplasmic reticulum and golgi apparatus. The dendrites are fine branching extensions of the cell body, which function to receive signals from environmental stimuli or from other neurons. The axon is the conducting unit of the neuron, and consists of a single tubular process from the cell body to the presynaptic endings. The diameter of the axon is proportional to its conduction velocity, with larger axons being capable of faster conduction. Myelin, a fatty insulating sheath surrounding the axon, greatly increases the conduction velocity of those neurons which possess it. Near its end, the axon divides into many branches, called presynaptic terminals. The majority of these nerve endings lie on the dendritic surfaces of other neurons. They contain synaptic vesicles, which hold a transmitter substance, and are separated from the dendritic membrane by a synaptic cleft of approximately 20 to 30 nanometers. This junction point from one neuron to the next is called the synapse. It is formed by the presynaptic terminal of one neuron, the receptive surface of the other, and the space between them (synaptic cleft). The synapse is a site where signal transmission from one neuron to the next can be controlled, and thus determines the neural pathway

that a stimulus will follow (Kandel and Schwartz, 1985; Guyton, 1987).

Most afferent, or sensory, neurons are pseudounipolar cells having only a single short process emerging from the cell body, which then splits in a T-shaped fashion into two axons. One axon extends peripherally toward the skin, muscle, and viscera, and the other extends centrally toward the spinal cord. Sensory neurons are located within peripheral nerves and ascend from the receptor to the dorsal horn of the spinal cord, a distance of up to one meter in humans. Their cell bodies lie in clusters called the dorsal root ganglion, which are located between the spinal cord and the nerves emerging from the spinal cord. Efferent neurons emerge from the ventral root of the spinal cord and join the fibers of the dorsal root to form a spinal nerve. Therefore, spinal nerves contain the axons of sensory as well as motor neurons (Kandel and Schwartz, 1985).

There are five basic types of sensory receptors: 1) mechanoreceptors, which detect physical deformations of tissues; 2) thermoreceptors, which detect changes in temperature; 3) nociceptors, or pain receptors, which detect both physical and chemical tissue damage; 4) electromagnetic receptors, which are found on the retina of the eye and detect light; and 5) chemoreceptors, which detect the presence and concentration of particular compounds. Each type of receptor has a specialized structure, and is sensitive to the sensory modality for which it was designed. Excitation of the receptor by its particular stimulus immediately changes the electrical potential across the receptor membrane. If this receptor potential increases above the threshold level, then an electrochemical impulse, called an action potential, will be initiated in the peripheral axon attached to the receptor. The action potential is propagated along the axon and spreads over the presynaptic terminals, causing the synaptic vesicles to fuse with the terminal membrane and

release their transmitter substance into the synaptic cleft. The neurotransmitter diffuses across the cleft and binds to receptors in the postsynaptic membrane. Transmitter binding induces an immediate change in ion permeability, which can lead to either excitation or inhibition of the neuron, depending on the nature of the transmitter and the receptor (Guyton, 1987).

The speed at which the sensory impulse reaches the central nervous system depends on the diameter and myelination of the afferent axon. Sensory signals that must be received rapidly, such as limb positions during exercise, are transmitted through large diameter myelinated fibers, called type A fibers. These fibers are further subdivided into α, ß, γ, and δ fibers, according to their diameter and conduction velocity, with α being the largest and fastest, reaching a diameter of 20 μm and a velocity of 120 meters per second. Other sensory information that does not require such rapid transmission, such as an aching pain or itch, is carried to the central nervous system more slowly by small diameter unmyelinated nerve fibers, called type C fibers. These fibers range from 0.5 to 2.0 μm in diameter, and only reach a maximum velocity of 2.0 meters per second (Guyton, 1987).

Pain is a symptom of most ailments of the body where tissue destruction has occurred, and it serves as a protective mechanism to remove the stimulus causing the pain. The sensory receptors which selectively detect damaging stimuli are called nociceptors, or free nerve endings. The free nerve ending is the morphologically least differentiated sensory receptor, and consists of fine arborizing dendritic structures. Pain is classified into two major types, acute and slow. Acute, or sharp pain, like a needle prick, is usually confined to the skin, and is transmitted through type A-δ fibers. Slow pain, or an aching and throbbing pain, is associated with tissue destruction, and is transmitted through type C fibers. This pain can occur in the

skin, as well as in almost all the internal organs and tissues of the body. The types of stimuli that excite pain receptors include mechanical, thermal, and chemical stimuli. Mechanical nociceptors are excited by strong and abrupt mechanical stimulation, particularly by sharp objects. Thermal nociceptors are stimulated by extreme hot and cold temperatures. Chemical nociceptors respond to substances released by traumatized tissues, including many mast cell mediators released during IgE-mediated inflammation. Histamine, serotonin, bradykinen, prostaglandins, and proteolytic enzymes can stimulate chemosensitive pain receptors (Kandel and Schwartz, 1985; Guyton, 1987).

Sensory Neuropeptides

More than 40 chemical substances have been identified to function as neurotransmitters in the central and peripheral nervous systems. Biochemically, these transmitters are divided into four different classes: Class I, acetylcholine; Class II, amines; Class III, amino acids; and Class IV, peptides. Acetylcholine is the only known neurotransmitter that is not directly derived from an amino acid, and in most cases it excites the post-synaptic neuron. In the peripheral nervous system, it is secreted by motor neurons that innervate skeletal muscles; and in the central nervous system, it is released by cells throughout the brain, but primarily in the cerebral cortex. Amine transmitters include the catecholamines (norepinephrine, epinephrine, and dopamine) and serotonin. The catecholamines are synthesized from the amino acid tyrosine, and are common neurotransmitters in the brain stem and in the sympathetic nervous system. Serotonin is synthesized from the amino acid tryptophan, and is distributed throughout the brain and spinal cord, where it is thought to control moods and sleep. The amino acid transmitters include glycine, γ-aminobutyric acid, and glutamate. Glycine and γ-aminodbutyric acid are released

at synapses in the spinal cord and brain, and usually act to inhibit post-synaptic neurons. Glutamate is secreted by most sensory neurons, where it usually causes excitation. Over 30 short peptides have been identified in both central and peripheral neurons, where they act as excitatory and inhibitory transmitters. These neuroactive messengers include: hypothalamic-releasing hormones (thyrotropin-releasing hormone, gonadotropin-releasing hormone, somatostatin), pituitary hormones (vasopressin, oxytocin, luteinizing hormone, growth hormone), gastrointestinal peptides (vasoactive intestinal polypeptide, substance P, neurotensin), and others (bradykinen, calcitonin, calcitonin gene-related peptide). A feature common to all peptide neurotransmitters is that they are produced only by neurons or by glandular cells that are developmentally related to nervous tissue (Kandel and Schwartz, 1985; Guyton, 1987).

Class I, II, and III neurotransmitters, often called the classical transmitters, are low-molecular weight compounds which are synthesized by cytoplasmic enzymes, and become distributed throughout the neuron by axoplasmic transport. These substances can be synthesized in all parts of the neuron, including the terminals, giving rise to rapid and sustained release patterns. In contrast, peptide neurotransmitters are derived from large polyprotein precursors, which are produced only in the cell body on ribosomes attached to the endoplasmic reticulum. The polyproteins are packaged by the golgi apparatus into secretory vesicles, where they are processed into neuropeptides by membrane-associated proteases. Usually, the polyprotein precursor is encoded by a single messenger RNA and is post-translationally processed into several copies of the same protein, or into two or more different peptides. The secretory vesicles reach the nerve terminals by axonal transport (Kandel and Schwartz, 1985).

In most cases, a mature neuron will secrete only one type of classical neurotransmitter at all of its terminals. However, peptide and classical transmitters can coexist in the same neuron, furthermore, peptidergic neurons can release several different neuropeptides (Kandel and Schwartz, 1979). Immunohistochemical studies on the localization of neuropeptides in the peripheral nervous system have identified several in the sensory neurons supplying the skin and mucus membranes of the lung and intestines. These sensory neuropeptides include: substance P, vasoactive intestinal polypeptide, somatostatin, neurotensin, neurokinin A, and calcitonin gene-related peptide. In addition, it's been found that several of these peptides coexist in the same sensory neuron (Foreman, 1987).

Many neuropeptides were initially identified as hormones, substances which are released at a considerable distance from their target tissue. However, it is believed that they may also act as chemical messengers in other tissues onto which they are directly released. Neuropeptides have been found in both the central and peripheral processes of C and A-δ sensory neurons, which carry nociceptive signals to the central nervous system (Goetzl et al., 1985). Although these peptides are considered to be neurotransmitters at the central terminals in the dorsal horn of the spinal cord, up to 90 percent of the neuropeptides synthesized in the cell bodies are transported to peripheral processes where they are stored (Payan et al., 1984).

The Sciatic Nerve

The hind limb of rodents is innervated exclusively by branches of the sciatic and femoral nerves. The sciatic nerve is the largest nerve in diameter in the body, and is formed by the convergence of spinal nerve bundles from the fourth and fifth lumbar nerves and the first, second, and third sacral nerves. Anatomically, it passes out of the pelvis and descends along the back of the thigh. Near the lower third of

the thigh, the nerve divides into two large branches, called the tibial and common peroneal nerves. The tibial nerve, the larger division of the sciatic, descends along the back of the leg to the heel, where it divides to feed the tissues of the paw. The common peroneal nerve divides just below the knee into deep and superficial branches which descend to innervate the leg and paw (Warwick and Williams, 1980). The cutaneous branches of the tibial and common peroneal nerves innervate almost the entire plantar surface of the hindpaw. In addition, the lateral half of the dorsal surface is also innervated by sciatic nerve branches. The medial remainder of the dorsal surface and a very thin medial strip of the plantar surface are innervated by the saphenous nerve, a femoral nerve branch (Wall et al., 1988).

When a peripheral nerve is severed, both sensory and motor axons are cut. The neurons then degenerate distal to the site of the lesion, a process called Wallerian degeneration. Although the degenerative process is not complete until one to two months after sectioning, synaptic transmission fails immediately and morphological signs of degeneration begin within hours (Kandel and Schwartz, 1979). In adult mammals, the major changes that occur in the pool of sensory neurons after sciatic nerve section include the following: 1) only a small to moderate percentage (8 to 30 percent) of the dorsal root ganglion cells die, 2) a large proportion of the ganglion cells can survive section of their distal axons, but 3) there is a complete loss of distal processes and normal signal transmission from the periphery (Wall et al., 1988).

Neurogenic Inflammation

The influence of peripheral nerves on cutaneous inflammation was first recognized by Stricker in 1876, when he found that mechanical stimulation of the peripheral segment of a cut nerve produced arteriolar dilatation (Pernow, 1985).

Since then, numerous investigators have shown that cutaneous hyperemia and edema can be induced by the application of chemical irritants to the skin, or by antidromic (conduction in a peripheral direction) electrical stimulation of peripheral nerves (Jansco et al., 1960; Kiernan, 1974; Kowalski and Kaliner, 1988). This inflammatory response, called neurogenic inflammation, is dependent on the integrity of peripheral nerves (Jansco et al., 1967).

Neurogenic inflammation induced with chemical irritants can be completely inhibited by severing peripheral nerves and allowing their distal segments to degenerate (Jansco et al., 1967). The irritants produce an intense inflammatory response and a severe burning pain in normal mammalian skin, and include noxious chemicals such as mustard oil, xylene, and capsaicin, an extract of red pepper. Neurogenic inflammation can also be blocked by repeated application of capsaicin, which induces a selective insensitivity to slow burning pain by degenerating the unmyelinated C sensory nerve fibers (Jansco, 1960). These results demonstrate that this response represents a pure neurogenic form of inflammation, which is dependent on the function of pain sensory nerve endings. In contrast, marked inflammation is observed in both surgically denervated and capsaicin-desensitized skin with other inflammation-inducing compounds, such as dextran, egg white, compound 48/80, histamine, and serotonin. Some of these compounds (dextran, egg white, compound 48/80) act by inducing the release of vasoactive substances, and others (histamine, serotonin) by directly affecting the vasculature. These findings indicate the existence of other inflammation-inducing agents which do not act by a neurogenic route (Jansco, 1960; Jansco et al., 1967).

It is believed that neurogenic inflammation is mediated by neuropeptides released from the peripheral processes of C fiber sensory nerves by an axon reflex

mechanism. The axon reflex, a local neural mechanism, is initiated when a noxious stimulus elicits an afferent impulse in a sensory neuron. As the impulse travels orthodromically (in a central direction) along the axon, it initiates antidromic conduction down afferent nerve collaterals. When the impulse reaches the peripheral nerve processes, it causes the release of sensory neuropeptides stored there. These neuropeptides then act on the local tissues to produce the classical symptoms of inflammation (Payan et al., 1984, 1986; Pernow, 1985; Barnes, 1986). It appears that substance P may be a particularly important mediator of neurogenic inflammation, because it has been recovered from blister fluid in rat skin when the sciatic nerve was electrically stimulated, and pretreatment of the skin with substance P-antagonists inhibits neurogenic inflammation (Goetzl et al., 1985; Kowalski and Kaliner, 1988; Payan, 1989).

Mast Cell/Nerve Interactions

Although a few sensory neuropeptides released from peripheral terminals exert inhibitory effects on inflammation, most elicit inflammation by directly acting on blood vessels and smooth muscle, and indirectly by stimulating the release of mediators from mast cells (Payan et al., 1986; Foreman, 1987; Matsuda et al., 1989; Mousli et al., 1989). Substance P, neurokinin A, calcitonin gene-related peptide, and possibly other neuropeptides directly stimulate smooth muscle fibers, leading to pronounced bronchoconstriction in the lung and peristalsis in the gastrointestinal tract (Pernow, 1985; Barnes, 1986; Perdue and Davison, 1986). Substance P and vasoactive intestinal polypeptide can directly act on the microvasculature to induce dilatation and increase vascular permeability when injected intradermally into human and rodent skin at physiologically relevant concentrations. In fact, substance P has potent vasodilatory activities estimated to be 400 times greater than that of

histamine, and can induce profound hypotension after interarteriolar administration (Payan, 1989). The inflammation-inducing activity of most neuropeptides, however, is largely a function of their mast cell stimulating effects (Payan and Goetzl, 1987). Kiernan (1972) was the first to demonstrate that electrical stimulation of sensory nerves can induce mast cell degranulation. Several sensory neuropeptides, including substance P, vasoactive intestinal polypeptide, somatostatin, and neurotensin, cause the noncytotoxic degranulation of mast cells during *in vitro* incubations, as well as the generation of unstored mediators (Shanahan et al., 1987; Mousli et al., 1989). In addition, subcutaneous injections of substance P or neurotensin can lead to the *in vivo* degranulation of mast cells in humans and mice (Payan et al., 1986; Matsuda et al., 1989). Mast cells from different sites within the body respond heterogenously to neuropeptides. Most neuropeptides exert stimulatory effects on peritoneal or cutaneous mast cells, types of connective-tissue mast cells. In contrast, substance P is the only known neuropeptide that can stimulate intestinal mucosal mast cells (Shanahan et al., 1985).

Microanatomical studies have provided evidence of close associations between mast cells and nerve fibers containing sensory neuropeptides (Olsson, 1968; Dimitriadou et al., 1987; Stead et al., 1987). Stead and coworkers (1987) found that 67 percent of intestinal mucosal mast cells in rats were in direct membrane contact with peptidergic nerve membranes, and that more than 87 percent of the mast cells were within half a cell diameter from a nerve. In fact, mast cells interdigitating with and wrapping around nerve terminals was a frequent observation. Similar mast cell/nerve associations have been identified in a variety of other tissues, including the skin, lung, heart, and brain (Bienenstock et al., 1987). This preferrential localization of mast cells with the peripheral endings of sensory nerve fibers provides

a structural framework for direct communication, presumably through soluble mediators (Payan et al., 1984).

Nerve growth factor, a protein essential for the survival, growth, and function of peripheral nerves, also has significant direct effects on mast cells (Pearce and Thompson, 1985; Tomioka et al., 1988). It is thought that every cell type has the capability to produce nerve growth factor, since it is constitutively found in the serum of all mammals tested for it (Pearce and Thompson, 1985). Not only is nerve growth factor synthesized by regenerating and growing nerves, but is also produced in response to inflammation and tissue injury (Stanisz et al., 1987). *In vitro*, nerve growth factor can cause over a two-fold enhancement of histamine release from antigen-stimulated rat peritoneal mast cells, as well as a significant increase in the kinetics of mediator release (Tomaika et al., 1988). The administration of nerve growth factor to neonatal rats induces *in vivo* mast cell hyperplasia in several organs examined, including the skin (Stanisz et al., 1987).

Modulation of Immediate Hypersensitivity by Neuropeptides

The ability of sensory neuropeptides to elicit inflammation and the evidence of mast cell/nerve interactions, support the hypothesis that sensory nerves may be involved in immediate hypersensitivity. Local tissue concentrations of neuropeptides during immediate hypersensitivity reactions are elevated, and reach levels sufficient to directly affect the vasculature and to activate mast cells (Payan and Goetzl, 1987). The *in vivo* reactions elicited by sensory neuropeptides in the skin, the lung, and the intestines are physiologically similar, both qualitatively and temporally, to immediate hypersensitivity responses mediated by IgE (Goetzl et al., 1985). Although non-antigenic stimuli distinguish neurogenic inflammation from IgE/antigen-induced immediate hypersensitivity, the similarity of their effector stages suggest that a

neurogenic component may be evoked during IgE-mediated responses.

In support of this hypothesis, *in vivo* studies in the lung have shown that peripheral nerves significantly enhance IgE-mediated inflammation (Leff et al., 1986; Sestini et al., 1989). Leff and colleagues (1986) demonstrated that stimulation of the vagus nerve significantly augments the secretion of histamine from pulmonary mast cells during antigen challenge in the dog. Although they had no direct evidence of neuropeptide involvement, they believed that the neurotransmitters released from the vagus nerve intensified the response to antigen by increasing the degranulation of mast cells. Sestini and coworkers (1989) used the clearance rate of an aerosolized radiolabeled solute from the lungs as a measure of lung epithelial permeability to examine IgE-mediated inflammation in sensitized adult rats which were neonatally treated with capsaicin. Control animals not treated with capsaicin responded to antigen challenge with severe respiratory distress and an increased lung solute clearance rate. However, capsaicin-treated animals displayed only mild respiratory symptoms and a markedly reduced lung solute clearance rate. This report indicates that sensory nerves play an important role in IgE-mediated reactions in the lung, where they contribute significantly to respiratory symptoms and lung epithelial permeability.

II. SPECIFIC OBJECTIVES

The purpose of this study was to test the hypothesis that peripheral nerves participate in cutaneous inflammation induced by IgE, and consisted of the following specific objectives:

1. The development of a murine model where the footpad was denervated by surgically removing a segment of the sciatic nerve.

2. The use of this local denervation model to examine the swelling response of denervated skin to vasoactive mediators released from mast cells during IgE-mediated inflammation.

3. The use of this model to determine the effects of local denervation on the cutaneous swelling response induced by a monoclonal IgE and its antigen.

III. MATERIALS AND METHODS

Mice

Female B6CBAF1/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and female 6CBF/CUM mice were obtained from Cumberland View Farms (Cumberland, TN). Both strains are hybrids of C57BL/6 females and CBA males. For each experiment, the mice were matched by age and strain, and were at least 8 weeks old.

Animals were cared for in the Old Dominion University Animal Facility, where they were housed five per cage and received food and water *ad libitum*. All experimental procedures involving animals were approved by the Old Dominion University Institutional Animal Care and Use Committee. For those experiments which required animal sacrifice, cervical dislocation was used, a method consistent with the recommendations from the Panel on Euthanasia of the American Veterinary Medical Association.

Anesthetics

Mice were anesthesized for surgery, sensitization, and challenge with an intraperitoneal injection of a 2.5% solution of 2,2,2-tribromoethanol (TBE: Aldrich Chemical Co., Milwaukee, WI) at a dose of 0.15 ml per 10 g of weight. The solution was prepared by dissolving 1.0 g TBE into 1.0 ml tertiary amyl alcohol (99%, Aldrich) and adding 39 ml warm distilled water. Ether (Sigma Chemical Co., St. Louis, MO) was used to immobilize mice for measurements of footpad thickness.

Surgical Procedures

Sciatic denervation was performed by making an incision over the pelvic joint, removing the muscle fascia between the *vastus lateralis* of the *quadraceps* and the long head of the *biceps femoris*, and carefully separating these muscles to locate the

sciatic nerve. A 2-mm segment of the nerve was removed at a location proximal to its division into the tibial and common peroneal nerves (see Appendix A). The incision was closed with sutures, and a period of at least seven days was allowed for the degeneration of the distal segment of the sciatic nerve prior to experimentation. Verification of denervation was confirmed by the partial dysfunction (a limp and loss of grasping function) of the hind limb receiving this surgery.

Sham surgery served as the surgery control and was performed in exactly the same manner as the denervation procedure, except that once the sciatic nerve was located it was not severed. Hind limbs receiving sham surgery retained full and normal function. Untreated control footpads were those from mice receiving no surgery.

Each experiment included equal numbers of denervated, sham-surgery control, and untreated control footpads. In some experiments, animals received a single surgery on the left leg (either denervation or sham surgery), and only the swelling of the left footpad was analyzed in all three experimental groups (denervated, sham, and untreated). In those experiments where animals received double surgery, sciatic denervation was performed on one leg and sham surgery on the other. Each experiment included equal numbers of animals denervated on the right and left sides, therefore each experimental treatment contained equal numbers of left and right footpad responses.

Histology

Sixteen weeks following surgery, denervated and sham footpads were prepared for histological analysis using the methods of Fong and Mosmann (1989). The mice were euthanized and their hind paws were severed from the leg at the hair line. The paws were fixed in 10% phosphate-buffered formalin for seven days, and then

decalcified in Perenyi's solution (8% hydroformic acid and 8% hydrochloric acid, in a 1:1 volume ratio) for three days. After decalcification, the tissues were embedded with paraffin (Tissue Tek III vacuum infiltration processor, Naperville, IL) and cut into 10 μm serial sections. Mounted sections were stained with hematoxylin (Weigert's) and eosin following the methods of Humason (1979), or with toluidine blue (Sigma) using the methods of Preece (1972). Mounted sections were photographed at 50× magnification under a Nikon Optiphot microscope.

Mast cells, visualized by toluidine blue staining, were counted along 1.0 linear mm of footpad dermis in every sixth section (every 60 μm) on a gridded microscopic field at 40× magnification, as previously described (Weinreich and Undem, 1987).

Antibody

A mouse monoclonal IgE anti-dinitrophenol ascites fluid, H1-DNP-ϵ-26, was used to passively sensitize footpads (Liu et al., 1980).

Antigen

Bovine serum albumin (BSA: Sigma) haptenated with dinitrophenol was used to challenge sensitized footpads. This antigen (DNP-BSA) was prepared with 2,4-dinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, NY) as described by Mishell and Shiigi (1980). In some experiments, BSA was used as the antigen.

Induction of Footpad Inflammation

Cutaneous inflammation was elicited in footpads with histamine (diphosphate salt, Sigma), serotonin (5-hydroxytryptamine, creatine sulfate complex, Sigma), or IgEαDNP and DNP-BSA. All dilutions of vasoamines, IgE, and antigen were made in phosphate-buffered saline (PBS, pH=7.2-7.4).

A. Vasoamines

Footpads were subcutaneously injected with a single dose of histamine (0.01 to 50 μg/footpad) or serotonin (0.001 to 10 μg/footpad), delivered in a 6 μl volume using a Hamilton syringe.

B. Local IgE

Passive cutaneous anaphylaxis (PCA) was induced by sensitizing footpads with a subcutaneous dose of IgE (0.0016 to 25 μg/footpad), delivered in a 20 μl volume with a Hamilton syringe. Two hours after sensitization, when the footpads returned to their pre-injection thickness, the animals were challenged with intravenous DNP-BSA (1.0 mg /animal), administered retroorbitally in a 0.5 ml volume.

C. Systemic IgE

PCA was also induced by systemically sensitizing mice with intravenous IgE (100 μg/animal), delivered retroorbitally in a 0.25 ml volume. Eighteen hours after sensitization, the footpads were locally challenged with subcutaneous DNP-BSA (60 μg/footpad), injected in a 6 μl volume with a Hamilton syringe. This local dose of antigen was determined by subcutaneously injecting the footpads of nonsensitized animals with DNP-BSA (30 to 120 μg/footpad, in a 6 μl volume), and selecting a dose which did not cause substantial inflammation on its own.

Measure of Footpad Inflammation

Footpad swelling was used as a measure of inflammation, and was determined from footpad thickness measurements taken with an engineer's micrometer (Walter Stern, METR.E, ASP, West Germany). Footpad thickness was measured at 0.5, 2.0, and 4.0 h after inflammation was induced, and swelling at each time was calculated by subtracting footpad measurements taken immediately before challenge with vasoamines or antigen. Measurements of footpad thickness were taken in a "blinded" manner, whereby the surgical procedure performed on each hind limb was unknown.

In each experiment, footpad swelling was reported as the mean of the treatment group ± the standard deviation in units of 0.001 cm.

Statistics

Inflammation was induced with local IgE (0.20 μg/footpad) in the footpads of mice receiving different combinations of single or double surgeries. Footpad swelling measurements taken at 0.5 h after antigen challenge in these animals were evaluated in a 1-factor analysis of variance (ANOVA), a 1-factor randomized block ANOVA, and a Tukey's honestly significant difference (HSD) test using the methods of Sokal and Rohlf (1981). The results of these analyses permitted the use of the Student's t-test to calculate the statistical difference between denervated and sham footpad responses in each experiment.

Active IgE Sensitization

Mice were immunized with DNP-BSA to produce IgE anti-DNP-BSA, according to the methods of Kato and Yamamoto (1982). Each animal was given an intraperitoneal injection of 1.0 mg DNP-BSA in 0.2 ml of a 1:1 emulsion with incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI). Three weeks following immunization, mice received surgery, and one week later were challenged locally with a footpad injection of DNP-BSA or BSA (60 μg in 6.0 μl/footpad). Footpad thickness measurements were taken (blinded) at 0.5, 2.0, 4.0, 24, and 48 h after antigen challenge. At each time point, the swelling responses of denervated and sham footpads of unimmunized mice locally challenged with antigen were subtracted from those of immunized animals.

Serum IgE activity in immunized mice was verified with passive cutaneous anaphylaxis in rat skin. Marked sites on the back of an adult male Sprague-Dawley rat (weighing approximately 400 g) were subcutaneously injected with 50 μl volumes

of dilutions of immune mouse serum, preimmune mouse serum, and IgEαDNP. Immune mouse serum was collected (retroorbitally) immediately after the 48 h footpad thickness reading, and pooled; preimmune mouse serum was collected prior to immunization, and pooled. Two hours after sensitization, the rat was challenged intravenously with 2.0 ml phosphate-buffered saline containing 2.0 mg DNP-BSA and 1% Evan's Blue Dye (Sigma). Forty-five minutes later, the animal was euthanized, the skin from the back was removed, and the x and y diameters of the blued areas were measured with a metric ruler.

IV. RESULTS

Histology of Denervated and Sham Footpads

A histological analysis was conducted 16 weeks after surgery to detect any changes in the appearance of footpad tissues as a result of sciatic denervation. Because blood vessels and mast cells are involved in IgE-mediated inflammation, these tissues were examined in denervated and sham footpad sections. Hematoxylin and eosin stains were used to inspect nervous and vascular tissues. Toluidine blue, a dye specific for connective-tissue mast cells, was used to visualize mast cells.

A comparison of the nervous tissues in denervated and sham footpad sections revealed the presence of many more darkly stained nuclei within the nerve fiber bundles of denervated sections (Figure 1). This cellular proliferation is indicative of nervous degeneration and represents that of Schwann cells. In normal nervous tissues, Schwann cells lay down the myelin shealth surrounding some axons. However, when there is damage to a peripheral nerve, these cells proliferate and dedifferentiate to form phagocytes that absorb the degenerating neuron (Kandel and Schwartz, 1985). The nerve fiber bundles in denervated footpads exibited a looser organization and a loss in axon density. These characteristics confirmed nervous degeneration in denervated footpads. There were no detectable changes in the morphology of blood vessels as a result of sciatic denervation.

Denervated and sham sections stained with toluidine blue displayed a similar number and distribution of mast cells (Figure 2). Denervated footpad sections contained 115 ± 16 toluidine blue-stained mast cells per linear millimeter of footpad dermis, and sham sections contained 120 ± 17 (Table 1). The length of dermis counted in both surgical groups included sites of blood vessel and nerve fiber clusters within the footpad, locations which contained high concentrations of connective-

tissue mast cells. In both denervated and sham sections, there were few mast cells outside these sites.

Establishing Conditions of Inflammation Induction

Preliminary experiments were conducted in normal mice to standardize the conditions of inducing and measuring footpad inflammation in mice receiving surgery. The kinetics of the inflammatory responses induced in normal footpads with both vasoamines and IgEαDNP were such that swelling peaked around 0.5 h after challenge, gradually decreased thereafter, and disappeared by 4.0 h. Therefore, in each experiment, footpad thickness was measured at 0.5, 2.0, and 4.0 h after challenge, representing the peak, midpoint, and end of the swelling response (respectively). For each mediator of inflammation, the swelling responses of normal footpads to a range of local doses were measured at 0.5 h after challenge, and then used to select a single dose to test cutaneous inflammation in denervated footpads. Unless otherwise stated, the swelling responses reported in this text represent those measured at 0.5 h after challenge.

A. Vasoamines

The swelling responses of normal footpads to subcutaneous doses of histamine or serotonin are shown in Figure 3. Histamine-induced swelling reached a plateau of 62 ± 7 at a dose of 6.25 μg per footpad, and remained near this level at all higher doses tested (Fig. 3a). Because 6.25 μg represented the lowest dose to cause maximum swelling, a local dose of 6.00 μg was chosen to examine histamine responses in denervated footpads.

Murine footpads responded more strongly to serotonin, and produced a swelling response of 102 ± 15 at a dose of 10 μg per footpad (Fig. 3b). Greater doses were not examined, because the swelling induced by 10 μg already approached

twice the normal footpad thickness. Therefore, this dose was selected to test serotonin-induced inflammation in denervated footpads.

All vasoamine doses were delivered in a 6 μl volume, because there were difficulties associated with smaller and larger volumes. With all subcutaneous footpad injections, a volume of one or two microliters escaped from the skin when the needle was removed immediately following injection. Volumes less than 6 μl were avoided, because a substantial proportion of the mediator could be lost when removing the needle. Due to the small size of the footpad, volumes greater than 6 μl were not used because they could cause considerable volume-induced swelling at the 0.5 h reading.

B. Local IgE

The swelling responses of normal footpads sensitized with subcutaneous doses of IgE are shown in Figure 4. Maximal swelling was reached at a dose of 1.00 μg IgE per footpad, which produced a response of 86 $\pm$ 8 units. Because it was not known how denervation would affect IgE-mediated inflammation, a submaximal dose of 0.20 μg per footpad was selected to be tested in denervated footpads. This local dose produced a swelling response of 66 $\pm$ 10 in normal footpads, a level which would permit a possible increase or decrease in swelling when induced in denervated footpads.

To determine the effects of IgE dosage on the swelling response in denervated skin, swelling in denervated footpads locally sensitized with a lower dose of 0.02 μg IgE or a higher dose of 2.00 μg IgE was also examined. In this way, the three doses of local IgE selected would test the effects of local denervation on IgE-mediated inflammation which produced mild, intermediate, and maximal swelling responses in normal murine skin.

In these experiments, footpad thickness was not measured earlier than 0.5 h after antigen challenge, because a preliminary experiment revealed that the swelling responses in denervated and untreated control footpads at 0.25 h were not significantly different than those measured at 0.5 h (see Appendix B). Also, each experiment included at least 15 animals, making a 0.25 h reading unfeasible.

C. Systemic IgE

PCA in denervated skin was also examined by systemically sensitizing mice with IgEαDNP, and later challenging the footpads with a local injection of DNP-BSA. However, because this antigen may itself cause inflammation, the footpads of nonsensitized animals were injected subcutaneously with various doses of DNP-BSA to identify a local dose which would not induce substantial nonspecific swelling (Fig. 5). A line of regression was plotted from the results of this experiment, and it was estimated that a local dose of 60 μg DNP-BSA would produce only 10 units swelling at 0.5 h after injection. When systemically sensitized mice were locally challenged with this dose of antigen, a swelling response of 90 units was produced in the footpads. Therefore, 60 μg was selected as the antigen dose to locally challenge systemically sensitized animals.

Responses of Denervated Footpads to Vasoamines

Innervation to cutaneous blood vessels was lost when the sciatic nerve was severed, raising a concern that these vessels may not function normally during an immediate hypersensitivity reaction. Therefore, the responses of denervated footpads to the mast cell vasomediators histamine and serotonin were examined.

One week after surgery, a footpad injection of histamine produced similar swelling responses in all three experimental treatments: 47 ± 8 in denervated footpads, 50 ± 6 in sham footpads, and 56 ± 6 units in untreated footpads (Fig. 6).

Swelling measured at 2.0 or at 4.0 h also did not differ among the footpad groups.

The swelling responses induced with serotonin one week after surgery were not significantly different among the three footpad treatments (Fig. 7a). Denervated footpads swelled 88 ± 9, sham footpads 90 ± 8, and untreated footpads 92 ± 7 units after local serotonin injection. There were similar swelling responses in all footpad groups at 2.0 and at 4.0 h as well.

Footpad responses to serotonin were repeated at 16 weeks after sciatic denervation. No significant differences were observed between denervated and sham swelling responses at any post-injection point (Fig. 7b). However, swelling in untreated footpads (90 ± 5) was significantly greater ($P<0.05$) than that measured in sham footpads (72 ± 8), although it was not statistically different from the response in denervated footpads (74 ± 12). It is not clear why denervated and sham footpads did not respond as strongly to subcutaneous serotonin at 16 weeks after surgery. Nevertheless, there were no differences between denervated and sham swelling responses induced with serotonin at either one or 16 weeks.

Responses of Denervated Footpads to Passive IgE Sensitization

Once it was established that denervated footpads responded normally to common vasomediators released from immunologically activated mast cells, the denervation model was then used to examine the involvement of peripheral nerves in IgE-induced responses. Denervated footpads were passively sensitized with IgE by two different routes: local subcutaneous injection, and systemic intravenous injection.

A. Local IgE Sensitization

1. Dose Responses of Denervated Footpads

One week after surgery, PCA was induced in denervated footpads with three doses of local IgE (0.02, 0.20, or 2.00 μg/footpad). In mice sensitized with the lowest dosage, swelling in denervated footpads (31 ± 8) was reduced by one-third ($P<0.01$) of that observed in sham controls (48 ± 8) (Fig. 8). In contrast, the swelling responses measured in sham and untreated control footpads (50 ± 6) were similar. There were no significant differences in swelling among the three footpad treatments at the 2.0 or the 4.0 h readings.

An analogous pattern was observed with PCA responses induced with the other two IgE doses. Denervated footpads sensitized with 0.20 μg IgE swelled 66 ± 15 units, a response which was significantly ($P<0.01$) less than that of sham (103 ± 12) and untreated (118 ± 8) controls (Fig. 9). There was no significant difference between sham and untreated footpad responses at 0.5 h, nor among the swelling responses in all three footpad groups at 2.0 or 4.0 h. This experiment was repeated two times, and similar results were reproduced with each trial.

A local dose of 2.00 μg IgE produced a swelling response of 74 ± 7 in sham footpads and 51 ± 4 in denervated footpads, a reduction ($P<0.001$) of 31 percent (Fig. 10). This was the only significant difference in swelling detected among the footpad treatments in the experiment. When this experiment was repeated, a significant difference was observed between denervated and sham footpads, however, the responses of denervated and untreated footpads were not statistically different (see Appendix C). This was the only experiment in IgE-sensitized animals where there was not a significant difference between the swelling responses measured in denervated and untreated control footpads.

2. Time Course of Sciatic Denervation

PCA was induced with local IgE (0.20 μg/footpad) at 3 and 16 weeks after surgery, to determine if the response in denervated footpads was influenced by the length of time which elapsed after sciatic nerve sectioning. Three weeks following surgery, sham footpads swelled 98 ± 7 and denervated footpads 75 ± 7 (Fig. 11a), a significant ($P<0.01$) reduction similar to that observed at one week (Fig. 9). Sixteen weeks after surgery, swelling in denervated footpads (59 ± 16) was reduced by almost 40 percent ($P<0.001$) compared to that measured in sham footpads (97 ± 7) (Fig. 11b). These results were also similar to those found at one week, indicating that the hyporesponsiveness to IgE detected in denervated footpads was maintained for 16 weeks after severing the sciatic nerve.

3. Statistics

A statistical analysis was conducted to dismiss any artificial influences of the methods of surgery on footpad swelling responses, and to ensure that the denervation model was suitable to investigate the hypothesis of this project. Swelling responses induced with local IgE (0.20 μg/footpad) in the footpads of mice receiving different combinations of single and double surgery were used in this evaluation (Table 2).

The swelling measurements from groups B (sham surgery on the left leg, no surgery on the right), D (sham surgeries on both legs), and E (sham surgery on the left, denervation on the right) were included in a 1-factor analysis of variance (ANOVA). The sham responses in the left footpads were compared among the three groups to detect any differences in this response when the animal received single or double surgery. This analysis determined that sham surgery did not significantly affect footpad swelling, and that the swelling responses in sham footpads were not affected by contra-lateral sham or denervation surgery (Table 3a).

The swelling measurements from all six groups (A through F, Table 2) were evaluated in a 1-factor randomized block ANOVA to determine if there were any differences among the three experimental treatments. The results of this analysis demonstrated that there were highly significantly ($P<0.001$) differences in footpad swelling responses among the surgery treatments, and that these results did not differ among experimental trials (*i.e.* blocks) (Table 3b). The responses in all six groups were then evaluated in a Tukey's honestly significant difference (HSD) test to determine which of these treatments was different. This analysis concluded that denervation significantly reduced the swelling response when compared to either sham or untreated controls (Table 3b). In addition, these analyses established that denervation and sham treatments, when applied to the same individual animal, retained this highly significant difference.

Because the statistical analyses determined that there was no significant difference between sham and untreated footpad responses, the Student's t-test was then used to calculate the statistical difference between denervated and sham responses in each experiment. The analyses also concluded that there were no effects of either surgery on the swelling responses in contra-lateral footpads, indicating that the data collected from animals receiving one or two surgeries were both relevant. Within each treatment, there was a significant difference between left and right footpad measurements, the left being slightly smaller, however this difference represented only one percent of the mean swelling response. Furthermore, this difference between left and right measurements was compensated for by: 1) performing surgery on the left leg and analyzing only the left footpad response in those experiments where animals received a single surgery, or 2) including equal numbers of animals denervated on the left and right sides in those

experiments where animals received double surgeries.

B. Systemic IgE Sensitization

The footpads of mice were sensitized with intravenous IgE to determine whether the route of sensitization influenced PCA in denervated skin. In systemically sensitized mice, swelling in denervated footpads (66 ± 8) was significantly ($P<0.001$) less than that in sham (88 ± 10) and untreated (89 ± 1) controls (Fig. 12). As with locally sensitized footpads, there were no differences in swelling among the footpad treatments at the 2.0 and 4.0 h readings. This experiment was repeated with similar results. In fact, the results obtained in systemically sensitized animals closely parallel those observed in footpads locally sensitized with 0.20 μg IgE (Fig. 9).

Responses of Denervated Footpads to Active IgE Sensitization

Mice were immunized with DNP-BSA to examine the swelling response of denervated footpads in actively sensitized animals. The protocol for immunization has been shown to promote the production of the IgE isotype (Kato and Yamamoto, 1982). IgE activity to DNP-BSA was verified in the serum of immunized animals in a PCA reaction in the skin of a rat, where immune mouse serum produced a titer of 25 (see Appendix D). One week after surgery, the footpads of immunized mice were challenged with a local injection of DNP-BSA or BSA.

In animals challenged with DNP-BSA, the swelling response in sham footpads was 75 ± 8, whereas a significantly ($P<0.01$) lower response of 52 ± 7 was measured in denervated footpads (Fig. 13a). Swelling measurements taken after 0.5 h were highly variable among individual animals, and none were statistically different between denervated and sham treatments. Footpad swelling in both surgery groups decreased slightly at 2.0 h, and then increased to reach peak levels at 4.0 h after

challenge (denervated, 67 ± 15; sham, 94 ± 30). Swelling measured at 24 and 48 h indicate that a slight delayed-type hypersensitivity response to DNP-BSA may have occurred.

Immunized mice challenged locally with BSA had similar swelling responses at 0.5 h to those challenged with DNP-BSA, suggesting that most of the response to DNP-BSA at this time may have been to the BSA molecule. Sham footpads swelled 74 ± 5, whereas a significantly ($P<0.01$) weaker response of 56 ± 6 was seen in denervated footpads (Fig. 13b). There were no significant differences between denervated and sham swelling responses at any point after 0.5 h. Footpad swelling measured at 4.0 h (denervated, 34 ± 29; sham, 42 ± 32) was not as great as that observed with DNP-BSA challenge. No swelling was measured at 24 and 48 h after BSA challenge, indicating that there was no detectable delayed response to this antigen.

V. DISCUSSION

In the mouse, the majority of the footpad skin is innervated by sciatic nerve branches. However, a small portion of the medial footpad is innervated by the saphenous nerve, a femoral nerve branch (Wall et al., 1988). In the denervation model developed for this project, only the sciatic nerve was severed, because a preliminary experiment in which both the sciatic and femoral nerves were cut resulted in total loss of functon of the hind limb and self-mutilation of the paw. Because the femoral nerve remained intact, the results of this study do not reflect conditions of complete denervation.

The method of surgical denervation (Appendix A) used in this model offered advantages over other denervation techniques. The surgical procedure itself was simple, mild, and specifically abolished most nerve function to the footpad. Surgery was performed near the hip, a site proximal to the footpad, so there was no direct damage to the tissues of the footpad. There was no surgery-induced swelling in the footpad at the time inflammation was induced. When compared with chemical denervation, this method eliminated the severe trauma, shock, and possibly other nonspecific effects induced by exposing tissues to a noxious chemical.

The results reported here demonstrate that IgE-mediated inflammation was significantly reduced in surgically denervated skin. Denervated footpads passively sensitized over a range of local IgE doses showed between a 23 to 39 percent reduction in swelling when compared to surgery control footpads (Figs. 8, 9, 10). These findings imply that a portion of IgE-induced swelling in the footpad was contributed by the sciatic nerve, and because denervated footpads were missing this input, the response in these footpads did not reach its full potential. Significantly lower swelling responses were observed in denervated footpads only at the 0.5 h

reading, indicating that the modulation by denervation occurred early in the reaction (within the first half hour). Measurements taken at 2.0 and 4.0 h were not significantly different among the experimental treatments, suggesting that the decline of the response was not affected by denervation.

In this study, there was negligible regeneration of the sciatic nerve, perhaps because a relatively large segment (2 mm) of the nerve was excised. The histological analysis showed that nerve fiber bundles in denervated footpads were in a degenerative condition at 16 weeks after surgery (Fig. 1b). IgE-induced swelling responses in denervated footpads were similar at one, three, and 16 weeks after severing the sciatic nerve (Figs. 9, 11), demonstrating that hyporesponsiveness to IgE was maintained for 16 weeks.

Denervated footpads of mice systemically sensitized with IgE displayed a comparable reduction in swelling to those footpads sensitized locally, demonstrating that the weakened response to IgE in denervated skin was not dependent on the method in which PCA was induced. Previous studies have shown that nervous stimulation can directly cause an increase in vascular permeability. Therefore, in those animals intravenously challenged with antigen, a functional sciatic nerve in control footpads could lead to a greater amount of antigen crossing the vascular endothelium and gaining access to sensitized mast cells. However, this possibility as a source of decreased swelling in denervated footpads was dismissed because denervated footpads systemically sensitized with IgE and challenged with subcutaneous antigen showed a 25 percent reduction in swelling when compared to sham footpads (Fig. 12).

Swelling responses were significantly reduced in denervated footpads of immunized animals shown to have serum IgE-like activity (Appendix D), revealing

the existence of this defect in IgE-mediated inflammation in denervated skin of actively sensitized mice. In addition, this weaker response in denervated footpads was observed against two different antigens (Fig. 13). Like passively sensitized footpads, only the swelling at one-half hour after challenge was significantly lower, confirming the early involvement of the sciatic nerve in immunized animals. The footpad swelling responses measured in immunized mice did not follow the progression observed in animals passively sensitized with IgE, but instead suggested the production and participation of other antibody isotypes. Strong swelling responses and severe reddening at four hours after challenge suggested the occurrence of an Arthus reaction, possibly mediated by IgG antibodies produced during immunization. In addition, swelling measured at 24 and 48 h after DNP-BSA challenge suggested the occurrence of a slight delayed-type hypersensitivity response to this antigen. However, because swelling was highly variable after one-half hour, additional studies must be conducted to determine whether other immediate or delayed reactions are influenced by local denervation.

Collectively, the results of this project have shown that swelling induced by IgE was reduced by approximately one-third in murine footpads receiving sciatic denervation. An interpretation of these findings is that the sciatic nerve enhances IgE-mediated inflammation in footpads. However, it can be argued that the swelling reductions observed in denervated footpads may represent an artifact of the murine model used in this study, rather than the involvement of the sciatic nerve during IgE-mediated hypersensitivity. Therefore, alternative explanations for the swelling reductions measured in denervated footpads, such as the loss of normal blood vessel function or the desensitization of mast cells, were eliminated in order to substantiate the participation of peripheral nerves during IgE-induced cutaneous inflammation.

Removing a segment of the sciatic nerve caused the partial dysfunction of the hind limb, including a limp and a loss of the grasping function of the hind paw. Perhaps other less obvious tissue defects were also induced by sciatic denervation, possibly affecting those tissues critically important to IgE-mediated inflammation, such as blood vessels. However, the reducton in swelling measured in denervated footpads could not be attributed to hyporesponsive vasculature. Functionally, denervated footpads responded normally to histamine and serotonin (Figs. 6, 7), indicating that the loss of innervation did not affect the ability of cutaneous blood vessels to respond to these vasomediators of immediate hypersensitivity. The histological analysis revealed that sections from denervated footpads contained degenerated nerve fiber bundles surrounded by tissues that appeared to be normal (Fig. 1b). Morphologically, the blood vessels in denervated skin did not appear to change. These results indicate that, for the purposes of examining IgE-mediated inflammation, the integrity of cutaneous blood vessels was not adversely affected by sciatic denervation.

Another alternative interpretation of the decreased swelling responses found in denervated footpads is that the mast cells in these tissues may be partially desensitized to antigen. The desensitization of murine mast cells has been reported in anaphylactic reactions, where exposure of sensitized tissues to sub- or supraoptimal doses of antigen, or to optimal levels of antigen in the absence of extracellular Ca^{++}, renders the cells unresponsive to the same antigen upon rechallenge (Kagey-Sobotka et al., 1982; Fox et al., 1982; Mendoza and Orner, 1983; Ishizaka et al., 1985). Although this phenomenon of desensitization is poorly understood, it is not due to the removal of IgE antibody from the cell surface, or to a decrease in the number of $Fc_\epsilon R$ expressed by the cell. In addition, comparable

numbers of antigen molecules have been shown to bind to sensitized and desensitized mast cells. Instead, it is believed that this process of desensitization is caused by the activation of membrane-associated enzymes (Mendoza and Orner, 1983; Ishizaka et al., 1985). Interestingly, the same enzymes involved in mast cell activation are also involved in desensitization. Desensitization is short-lived, and mast cells remain in this unresponsive state for only a few days following sub- or supraoptimal stimuli. Although a prior exposure to antigen is required to induce desensitization in anaphylactic reactions, it seems possible that stimulation resulting from an explosive release of products from sciatic nerve fibers immediately following nerve sectioning could induce desensitization shortly after surgery. However, PCA induced in denervated footpads at one, three, and 16 weeks after surgery were similar at all three post-surgery times (Figs. 9, 11), demonstrating that mast cell desensitization was not the source of IgE hyporesponsiveness in denervated skin.

Alternatively, IgE hyporesponsiveness in denervated footpads could not be attributed to a decrease in mast cell numbers. In this study, the number of dermal mast cells in the footpads did not change significantly at 16 weeks following sciatic denervation (Table 1), a time when the hyporesponsiveness to IgE was most pronounced (Fig. 11). In contrast to these findings, there have been reports of mast cell proliferation in certain lesions of peripheral nerves, both at the site of the lesion and along the entire distal degenerating nerve (Olsson, 1968; Bienenstock et al., 1987). Olsson (1968) found that the number of mast cells along and within the distal segment of severed sciatic nerves in the rat significantly increased two weeks following damage. He also observed over an 800 percent increase in mast cell number 16 weeks after cutting the nerve. In addition, Gamble and Goldby (1961) reported a significant increase in the proportion of degranulating mast cells in rat

sciatic nerves cut one to eight days previously, although the total number of cells had not changed. In view of this evidence, it has been speculated that mast cell hyperplasia and the mediators released from these cells may be involved in normal nerve repair processes. Investigators have proposed that mast cell mediators act to recruit and stimulate other inflammatory cells (*i.e.* macrophages) to repair the nerve (Olsson et al., 1968; Perry et al., 1987), and to directly degrade the myelin of damaged nerves (Johnson et al., 1987). Adopting this view, it then seems possible that those mast cells involved in nerve repair may not be available for antigen-induced activation, perhaps creating the reduction in inflammation observed in denervated footpads. Unfortunately, due to an inadequacy in the tissue-fixing techniques used in this project, all mast cells in both denervated and innervated footpad sections were degranulated, making it impossible to determine the proportion of degranulating cells in denervated footpads.

Nevertheless, mast cell recruitment away from antigen-induced reactions does not appear to be responsible for the swelling reductions observed in denervated footpads in this study. First, there was no change in the number of mast cells 16 weeks after denervation. Second, there were no differences in denervated responses between one and 16 weeks after damage to the sciatic nerve. Third, Olsson found no change in the occurrence of degranulated mast cells after severing the sciatic nerve in the rat. And fourth, Kiernan (1974) found that neither the abundance of dermal mast cells nor the proportion of degranulating cells was changed one week after complete surgical denervation of the rat ear. In addition, the report conducted by Gamble and Goldby claiming increased mast cell degranulation showed no tables, figures, or photographs of their evidence.

From the literature published on the involvement of mast cells in various inflammatory processes and the evidence for mast cell/nerve interactions, it has been suggested that a bidirectional communication system exists between local sensory nerves and mast cells during inflammation. Microanatomical studies have revealed that most tissue mast cells are closely associated with neuropeptide-containing nerve endings (Olsson, 1968; Dimitriadou et al.,1987; Stead et al., 1987). Previous reports have demonstrated that peripheral nerve stimulation can directly cause neurogenic inflammation (Jancso, 1960; Jancso et al., 1967; Kiernan, 1974; Kowalski and Kaliner, 1988). The neuropeptides released from peripheral terminals of sensory nerve fibers mediate this inflammatory response by directly acting on the vasculature to induce vasodilatation and increase vascular permeability, and indirectly by stimulating the degranulation of mast cells (Pernow, 1985; Payan et al., 1986; Matsuda et al., 1989; Mousli et al., 1989). In addition to the evidence of sensory nerve products stimulating the degranulation of mast cells, an opposite interaction where mast cell products stimulate peripheral nerves has also been demonstrated. Immunologically activated mast cells, or the direct application of several mast cell mediators, can initiate impulses through afferent sensory nerves in the dog lung and the guinea pig peripheral nervous system (Widdicombe, 1983; Weinreich and Undem, 1987).

Recent literature has supported the involvement of sensory neuropeptides in antigen-induced inflammation. Substance P has been found in the nasal secretions of allergic individuals after local antigen challenge (Payan and Goetzl, 1987). Leff and coworkers (1986) found that vagal stimulation augments the secretion of histamine from antigen-challenged mast cells in the dog lung. Sestini and colleagues (1989) demonstrated that antigen-induced inflammation is markedly reduced in

chemically denervated rat lungs. The findings of this study have shown that IgE-mediated inflammation is significantly reduced in surgically denervated murine skin. Collectively, these *in vivo* studies strongly support the proposed interactions between mast cells and peripheral nerves during antigen-induced inflammation.

This proposed interaction between mast cells and nerves during inflammation is thought to be provided by an axon reflex mechanism (Barnes, 1986; Bienenstock et al., 1987; Sestini et al., 1989). At present, there is no data to directly support this mechanism. The suggested sequence of events underlying the axon reflex in cutaneous inflammation induced by antigen is as follows: Contact with antigen, and the subsequent formation of IgE/antigen complexes on the surface of sensitized mast cells, causes their degranulation. Preformed and newly synthesized mediators are released into the local environment to produce the classical manifestations of inflammation. Particular mast cell-derived mediators may amplify this initial inflammatory response by directly stimulating C-fiber afferent endings and eliciting an afferent impulse in these sensory neurons. As the impulse travels along the axon, it initiates antidromic conduction down afferent nerve collaterals, leading to the release of sensory neuropeptides stored in the peripheral nerve terminals. These neuropeptides then act immediately on the local vasculature to increase dilatation and permeability, and stimulate the degranulation of additional mast cells. Perhaps this neuronal network spreads the response to other areas where antigen has not made contact with sensitized mast cells. Therefore, these proposed interactions between sensory neurons and tissue mast cells could achieve the optimal expression of IgE-mediated hypersensitivity reactions (Payan et al., 1984; Pernow, 1985; Barnes, 1986).

In conclusion, the results of this project demonstrate that peripheral nerves enhance IgE-mediated inflammation in murine skin. Using a murine model where the footpad was surgically denervated, this study showed that IgE-induced swelling was reduced by about one-third in denervated footpads of both passively sensitized and immunized mice. These weaker swelling responses in denervated footpads were observed over a range of local IgE doses, were not dependent on the route of passive sensitization, and were detected against two antigens. This defect in denervated footpads was not due to hyporesponsive blood vessels, the desensitization of mast cells, or a change in mast cell number. Instead, these findings suggest that a portion of IgE-mediated hypersensitivity in skin is dependent upon local nerves, and strongly support the existence of a neurogenic component during IgE responses. This neurogenic component represents an as yet undefined interaction between IgE-sensitized mast cells and local nerves. This study is the first to report the influence of peripheral nerves on cutaneous IgE responses.

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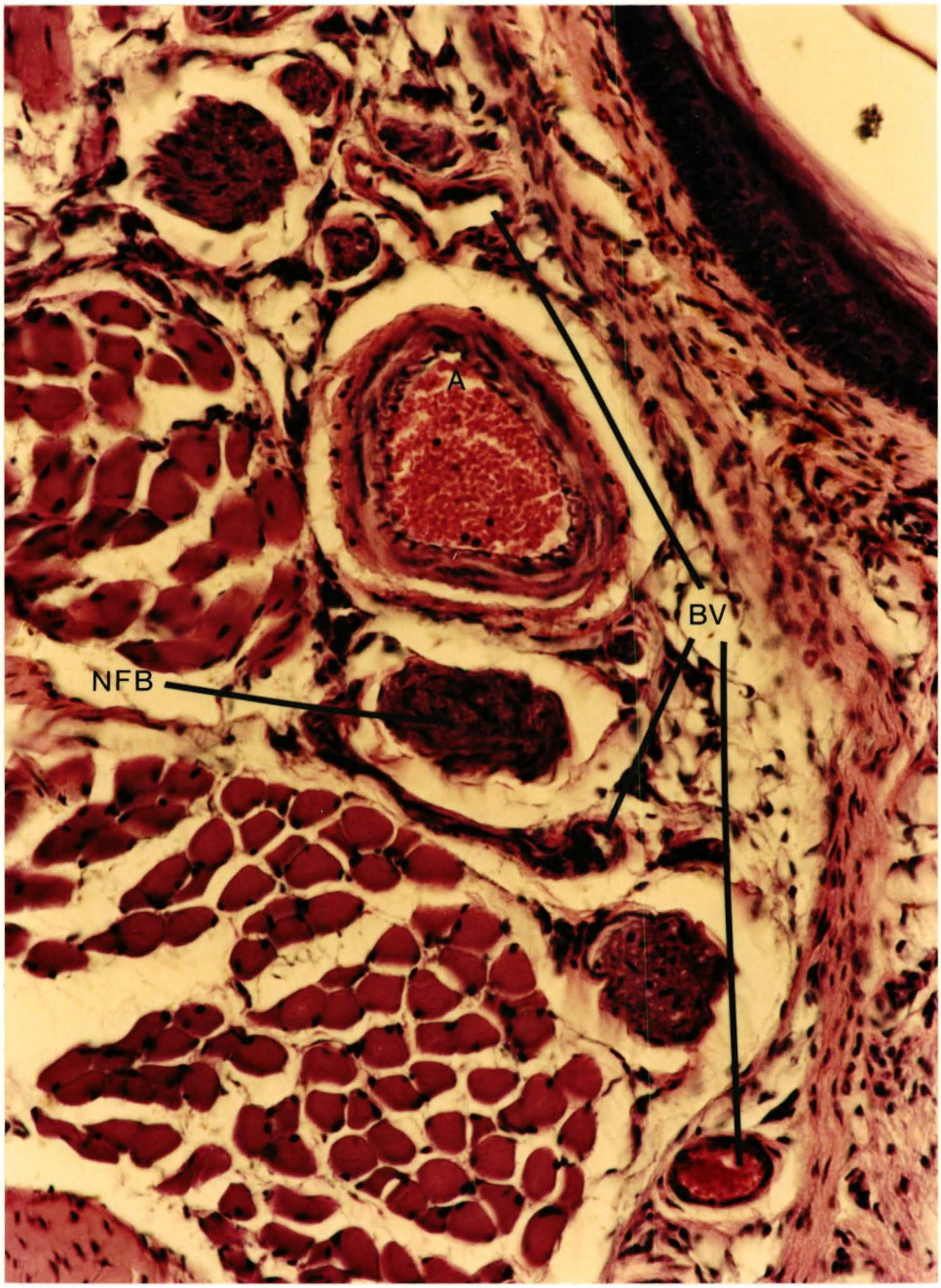
Figure 1. Photomicrographs of sham (a) and denervated (b) footpad sections processed for histological analysis 16 weeks after surgery and stained with hematoxylin and eosin. Mounted sections were photographed at 50× magnification. Key: A, artery; BV, blood vessel; NFB, nerve fiber bundle; dNFB, degenerated nerve fiber bundle.

(a)



(b)

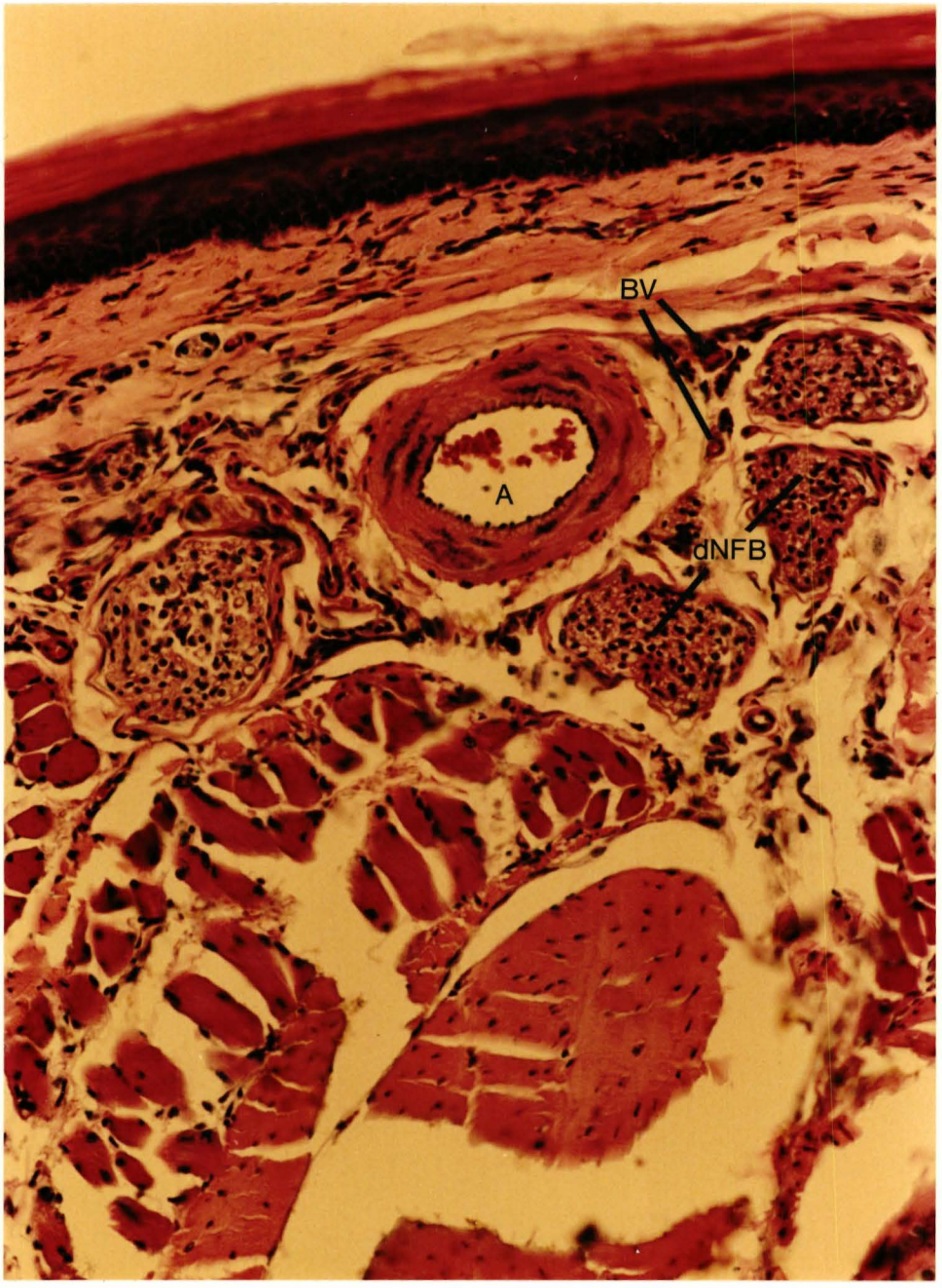
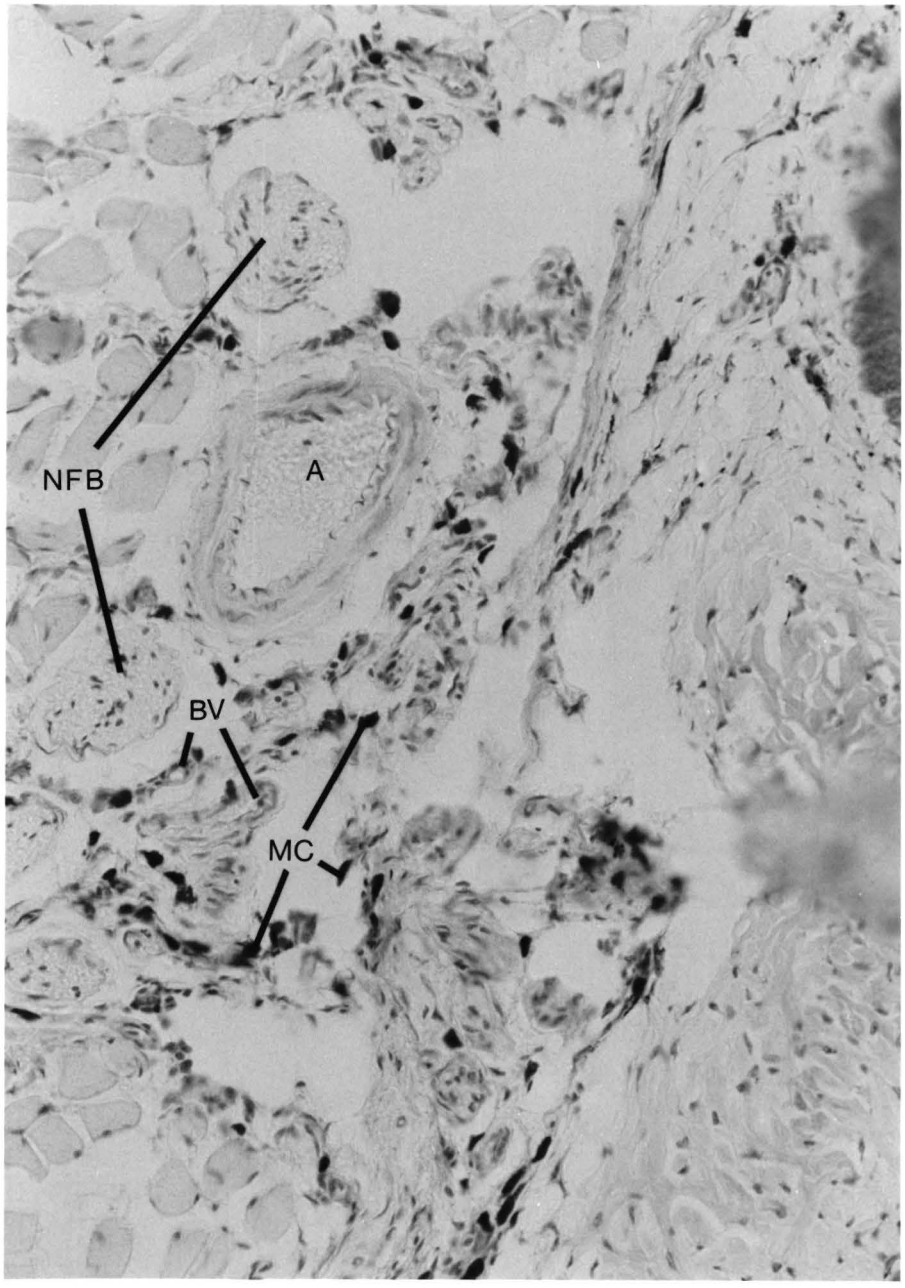


Figure 2. Photomicrographs of sham (a) and denervated (b) footpad sections processed for histological analysis 16 weeks after surgery and stained with toluidine blue. Mounted sections were photographed at 50× magnification. Key: A, artery; BV, blood vessel; NFB, nerve fiber bundle; dNFB, degenerated nerve fiber bundle; MC, mast cell.

(a)



(b)

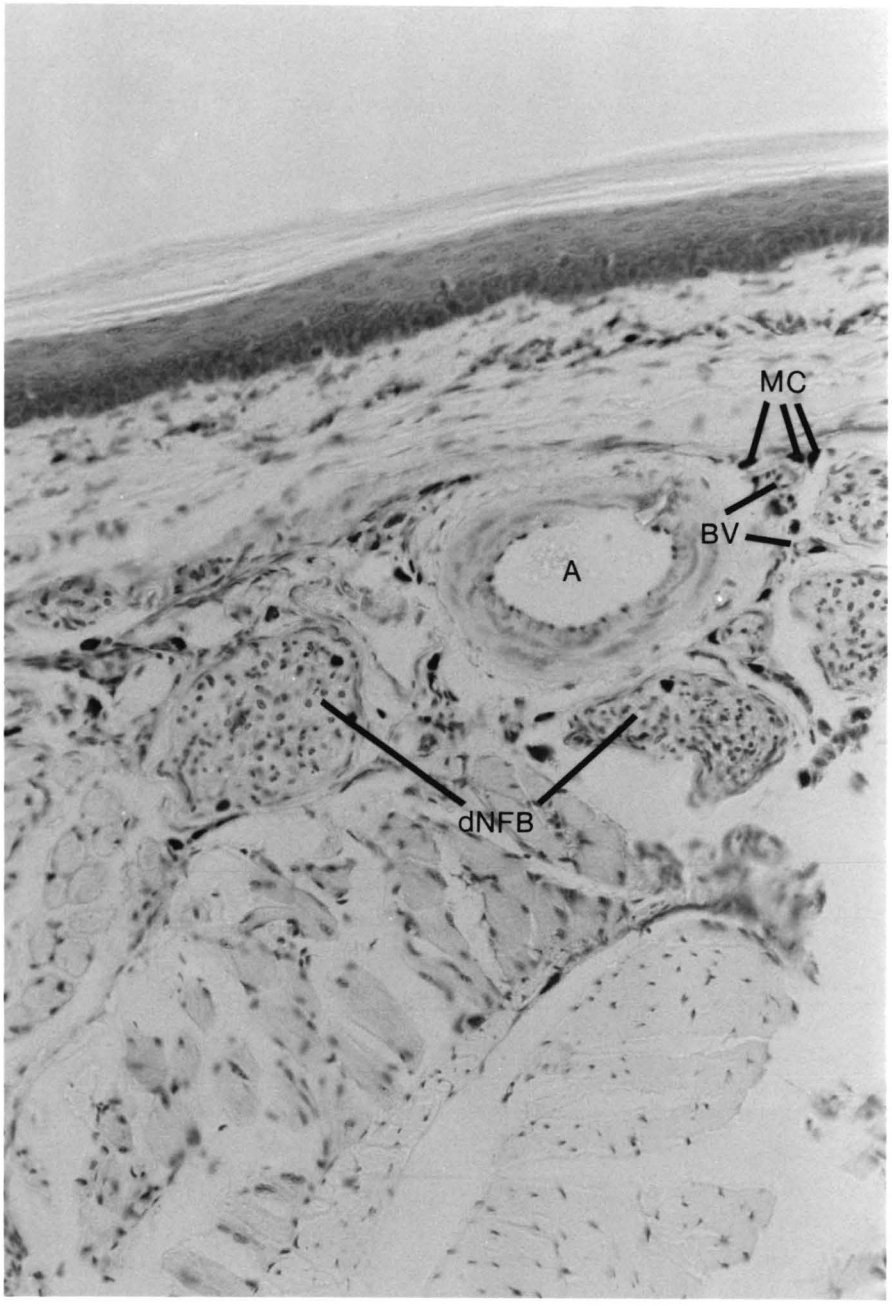


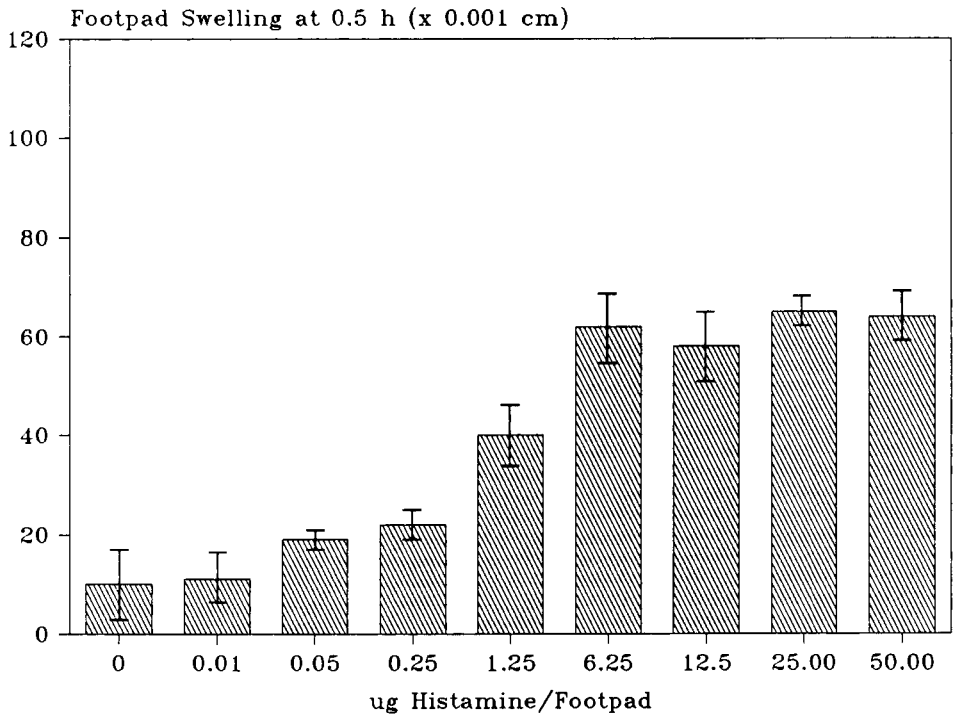
Table 1. Toluidine blue stained-mast cells were counted along 1.0 linear mm of footpad dermis in every sixth section on a gridded microscopic field at 40× magnification.

Table 1. Number of toluidine blue-stained mast cells per linear mm of dermis in sham and denervated footpad sections.

Footpad Section	Sham	Denervated
1	132	134
2	110	126
3	121	146
4	148	129
5	129	138
6	157	141
7	130	116
8	123	99
9	145	118
10	135	113
11	127	115
12	107	101
13	113	98
14	112	94
15	98	102
16	110	95
17	104	114
18	109	116
19	94	112
20	103	93
Mean ± SD	120 ± 17	115 ± 16

Figure 3. Swelling responses of normal footpads to local doses of histamine (a) or serotonin (b). The footpads of mice receiving no surgery were injected subcutaneously with a single dose of histamine or serotonin, and footpad swelling was measured at 0.5 h after injection. All dilutions of vasoamines were made in PBS (pH=7.2-7.4), and the 0 dose represents a local injection of PBS. Each bar represents the mean ± standard deviation of three footpads.

(a)


Footpad Swelling at 0.5 h (x 0.001 cm)
120
100
80
60
40
20
0
0
0.01
0.05
0.25
1.25
6.25
12.5
25.00
50.00
ug Histamine/Footpad

(b)

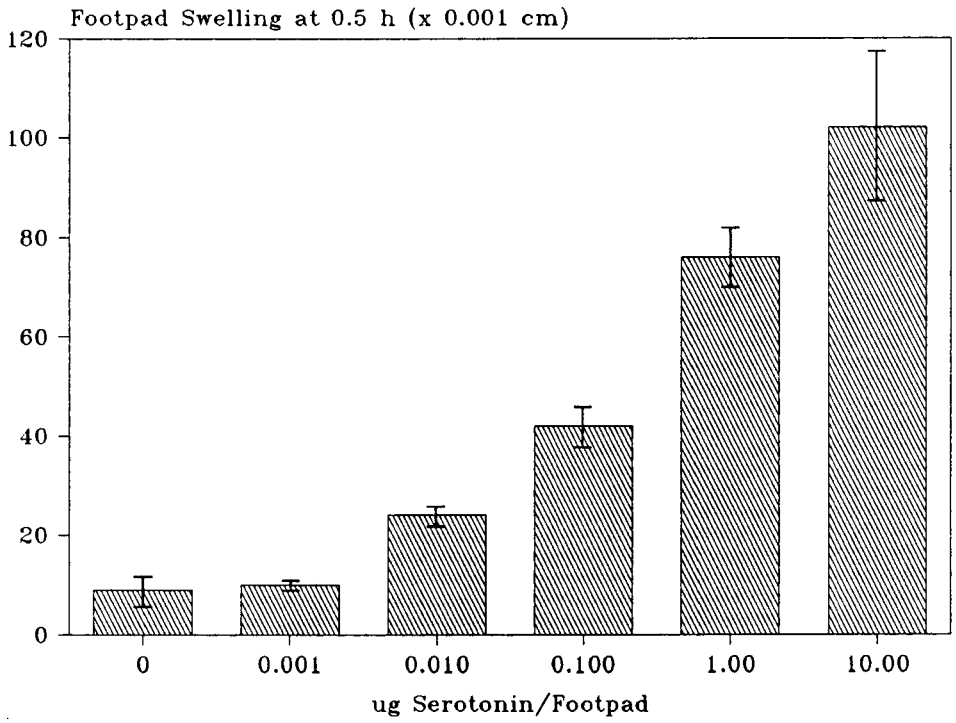

Footpad Swelling at 0.5 h (x 0.001 cm)
120
100
80
60
40
20
0
0
0.001
0.010
0.100
1.00
10.00
ug Serotonin/Footpad

Figure 4. PCA in normal footpads locally sensitized with IgE. The footpads of mice receiving no surgery were sensitized with a subcutaneous injection of IgEαDNP, and later challenged with intravenous DNP-BSA. Footpad swelling was measured at 0.5 h after challenge. All dilutions of IgE were made in PBS (pH=7.2-7.4), and the 0 dose represents a local injection of PBS. Each bar represents the mean ± standard deviation of three footpads.

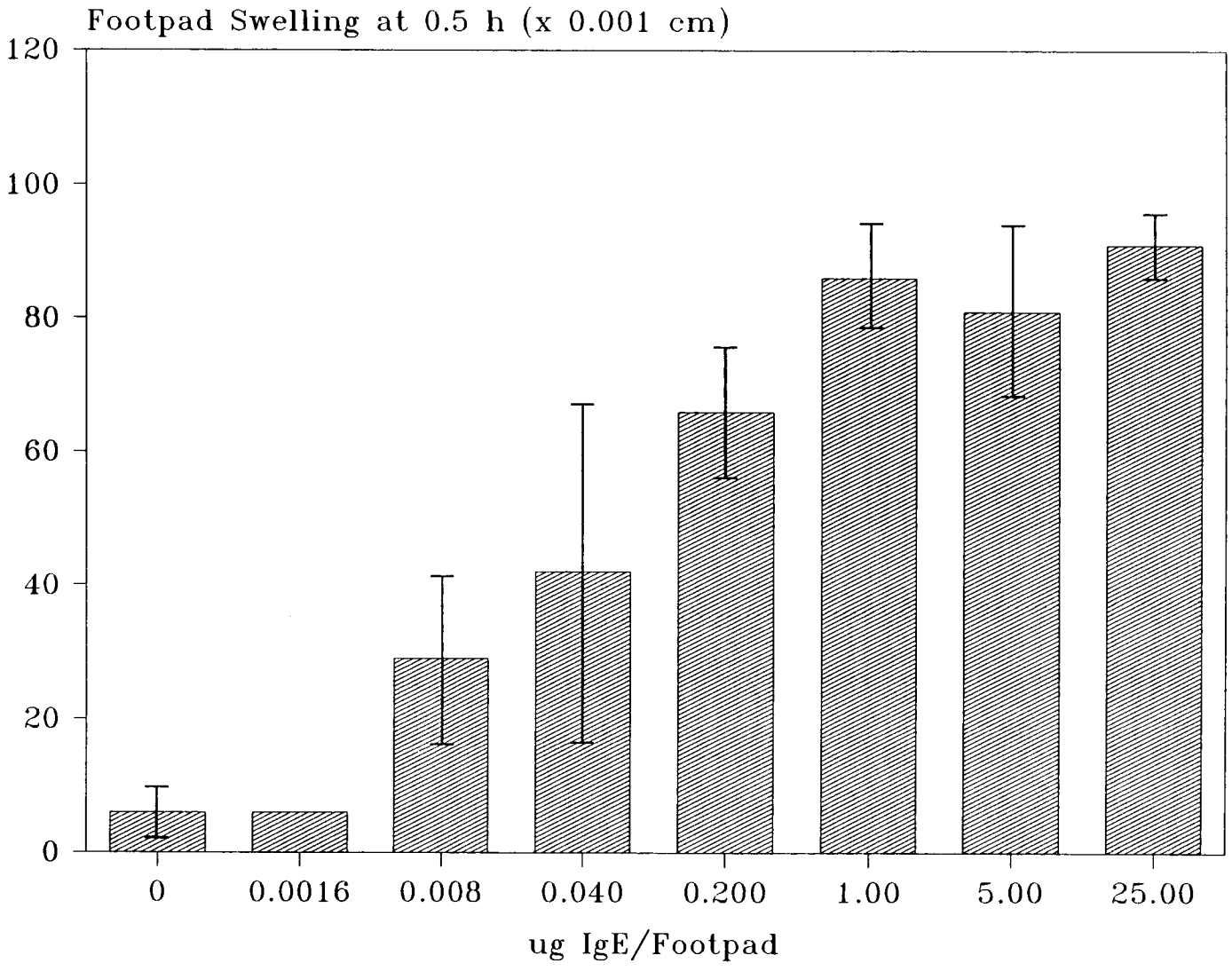

Footpad Swelling at 0.5 h (x 0.001 cm)
120
100
80
60
40
20
0
0
0.0016
0.008
0.040
0.200
1.00
5.00
25.00
ug IgE/Footpad

Figure 5. Nonspecific swelling responses of normal footpads to local doses of DNP-BSA. The footpads of nonsensitized mice receiving no surgery were injected subcutaneously with a dose of DNP-BSA, diluted in PBS (pH=7.2-7.4). Footpad swelling was measured at 0.5 h after injection. Each bar represents the mean ± standard deviation of three footpads. The line of regression includes all four doses.

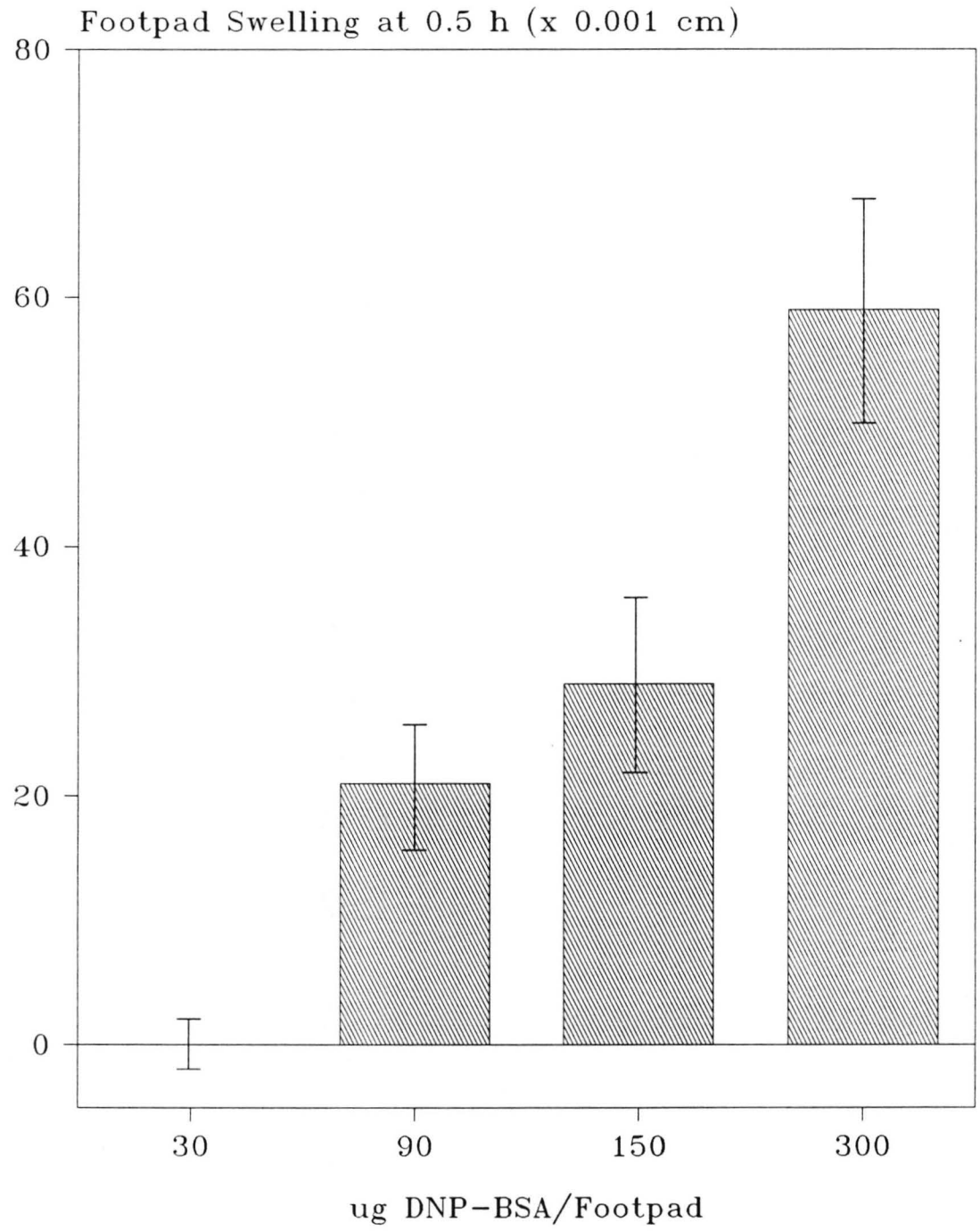

Footpad Swelling at 0.5 h (x 0.001 cm)
80
60
40
20
0
30
90
150
300
ug DNP-BSA/Footpad

Figure 6. Swelling response of denervated footpads to local histamine. One week after surgery, footpads were injected subcutaneously with 6.0 μg histamine. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after injection. Each bar represents the mean ± standard deviation of six footpads.

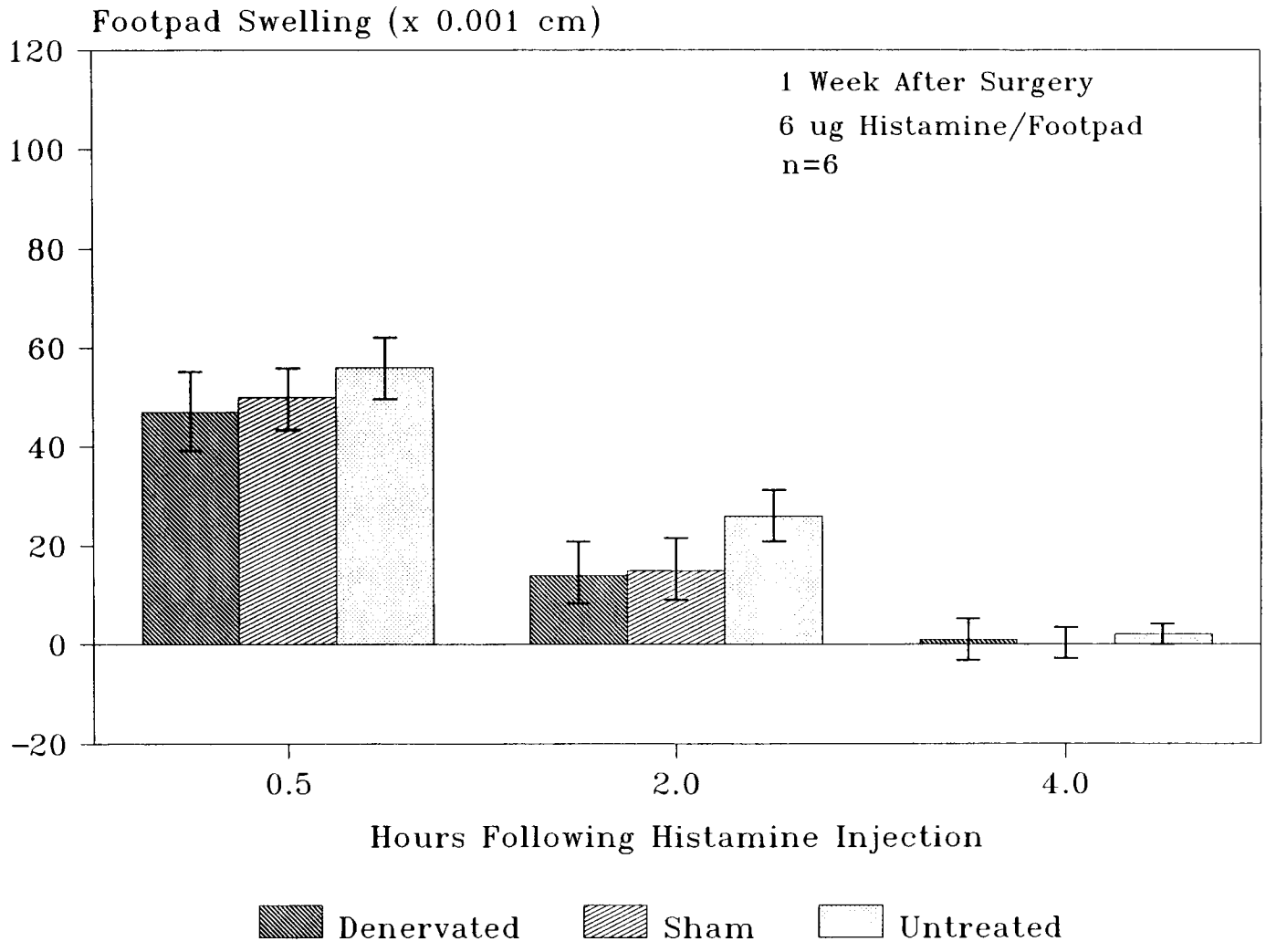
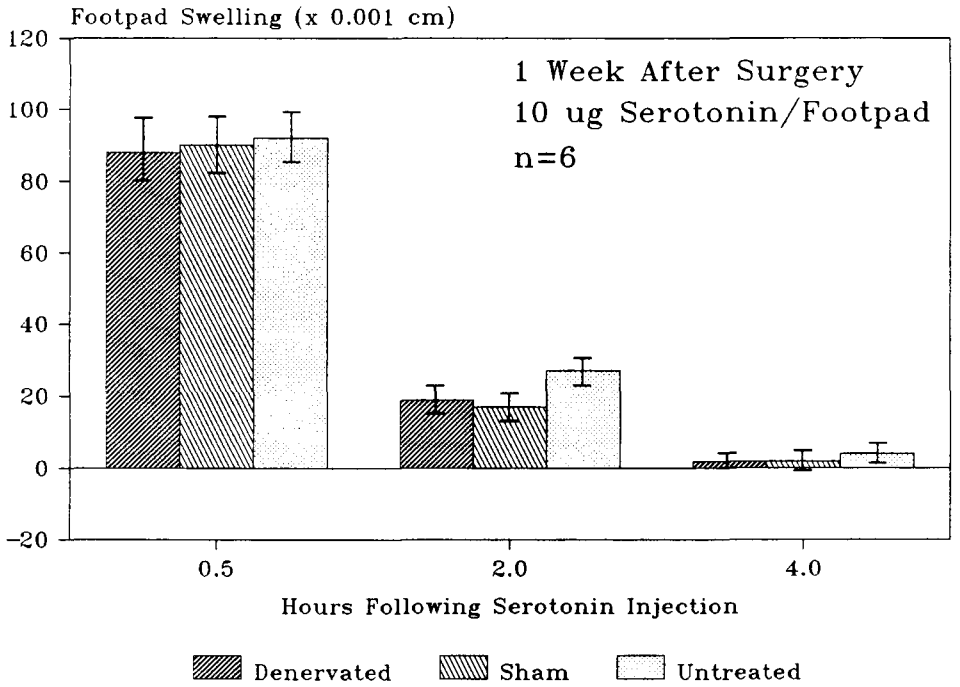

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
1 Week After Surgery
6 ug Histamine/Footpad
n=6
0.5
2.0
4.0
Hours Following Histamine Injection
Denervated
Sham
Untreated

Figure 7. Swelling responses of denervated footpads to local serotonin. One week (a) or sixteen weeks (b) after surgery, footpads were injected subcutaneously with 10 μg serotonin. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after injection. In (a), each bar represents the mean ± standard deviation of six footpads, in (b), of five footpads.

(a)


Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
1 Week After Surgery
10 ug Serotonin/Footpad
n=6
0.5
2.0
4.0
Hours Following Serotonin Injection
Denervated
Sham
Untreated

(b)

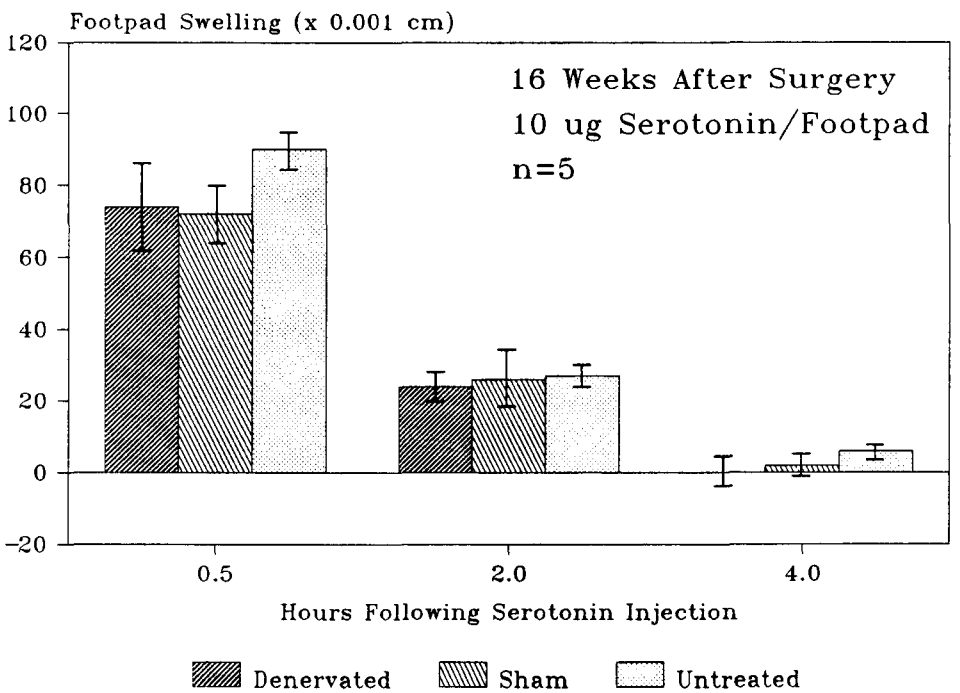

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
16 Weeks After Surgery
10 ug Serotonin/Footpad
n=5
0.5
2.0
4.0
Hours Following Serotonin Injection
Denervated
Sham
Untreated

Figure 8. PCA in denervated footpads locally sensitized with 0.02 μg IgE. One week after surgery, footpads were passively sensitized with a subcutaneous injection of 0.02 μg IgEαDNP. Two hours after sensitization, the animals were challenged with an intravenous injection of DNP-BSA. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after challenge. Each point represents the mean ± standard deviation of seven footpads. The statistical difference between denervated and sham swelling responses at 0.5 h was $P<0.01$.

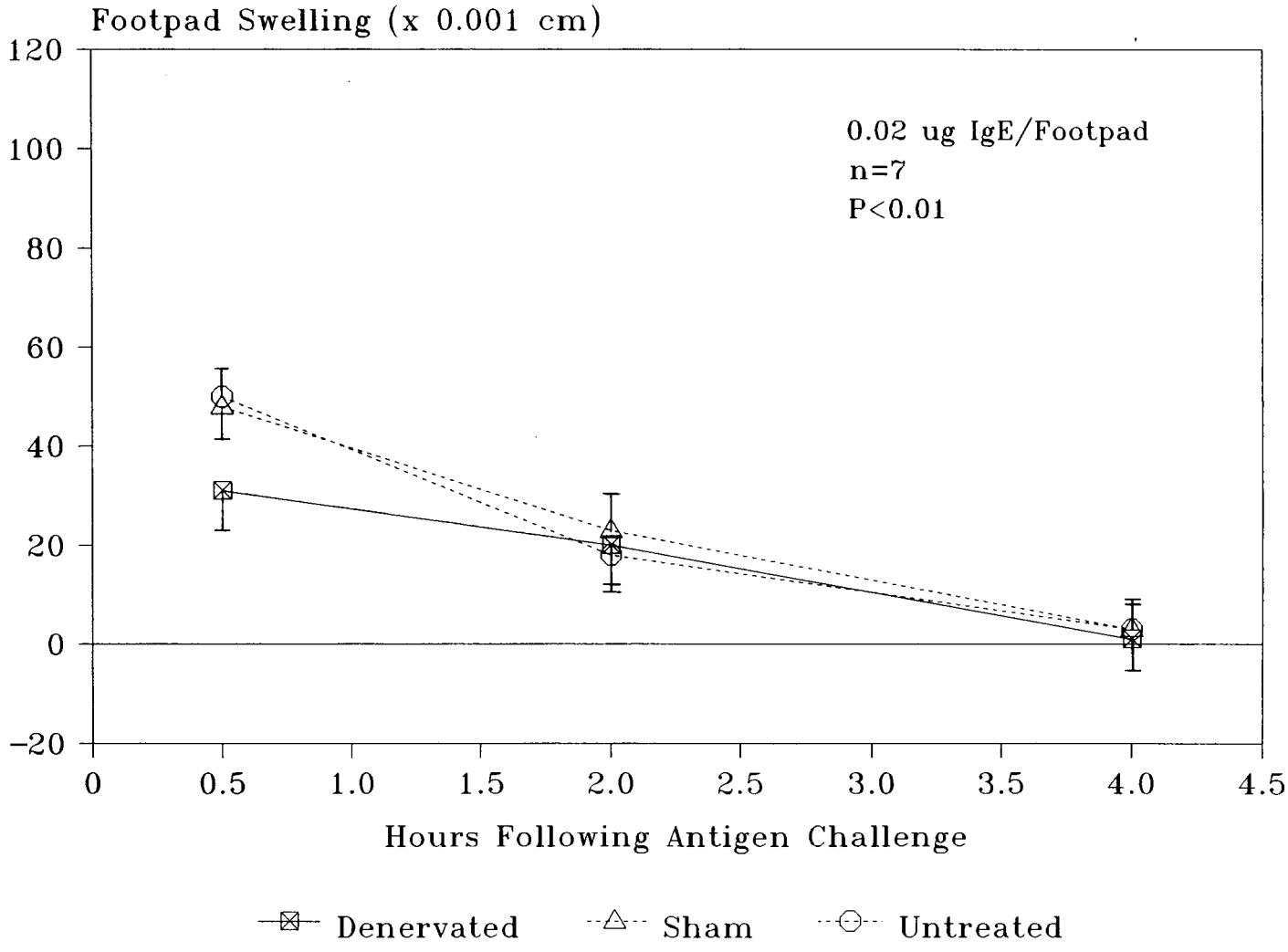

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
0.02 ug IgE/Footpad
n=7
P<0.01
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Hours Following Antigen Challenge
Denervated
Sham
Untreated

Figure 9. PCA in denervated footpads locally sensitized with 0.20 μg IgE. One week after surgery, footpads were passively sensitized with a subcutaneous injection of 0.20 μg IgEαDNP. Two hours after sensitization, the animals were challenged with an intravenous injection of DNP-BSA. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after challenge. Each point represents the mean ± standard deviation of five footpads. The statistical difference between denervated and sham swelling responses at 0.5 h was $P<0.01$. This experiment was repeated twice, and similar results were reproduced with each trial.

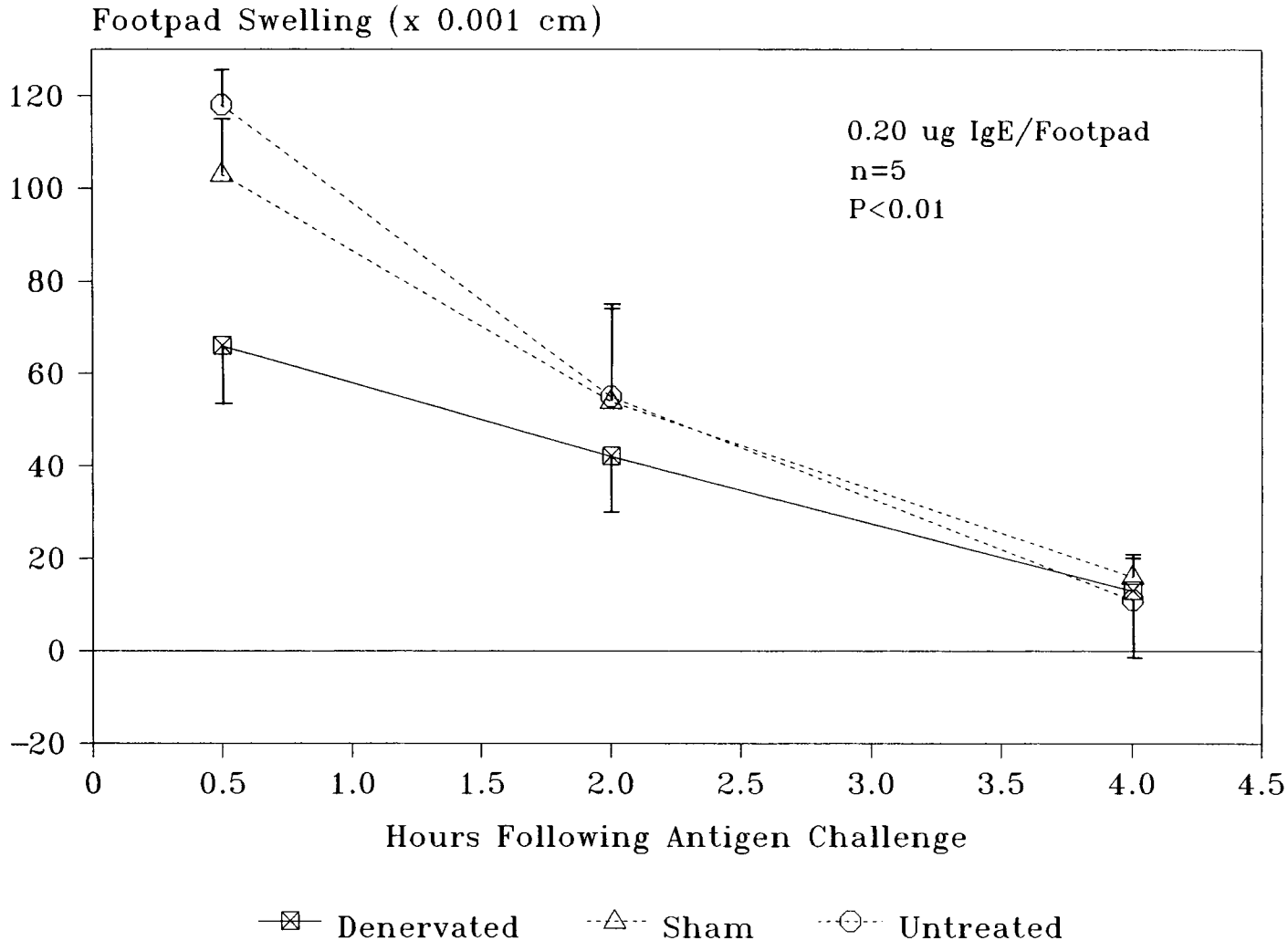

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
0.20 ug IgE/Footpad
n=5
P<0.01
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Hours Following Antigen Challenge
Denervated
Sham
Untreated

Figure 10. PCA in denervated footpads locally sensitized with 2.00 μg IgE. One week after surgery, footpads were passively sensitized with a subcutaneous injection of 2.00 μg IgEαDNP. Two hours after sensitization, the animals were challenged with an intravenous injection of DNP-BSA. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after challenge. Each point represents the mean ± standard deviation of seven footpads. The statistical difference between denervated and sham swelling responses at 0.5 h was $P<0.001$.

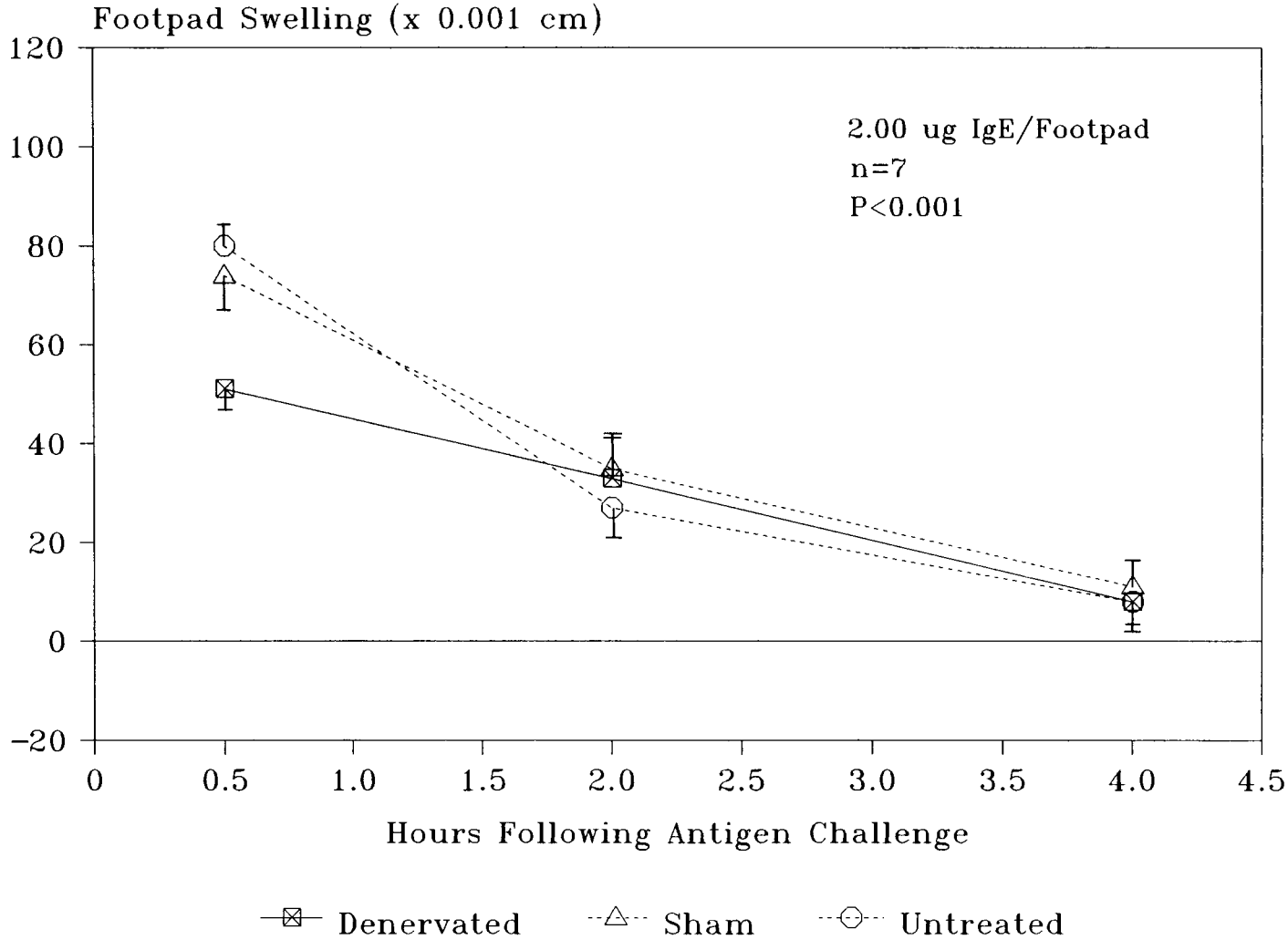
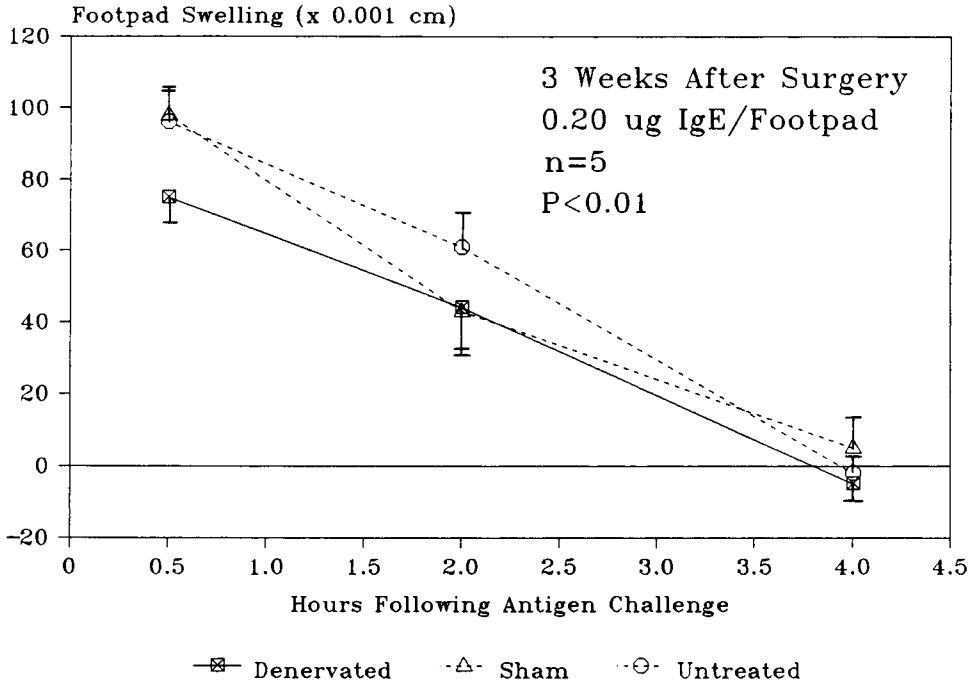

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
2.00 ug IgE/Footpad
n=7
P<0.001
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Hours Following Antigen Challenge
Denervated
Sham
Untreated

Figure 11. PCA in denervated footpads locally sensitized with 0.20 μg IgE at three (a) and 16 (b) weeks after sciatic denervation. Three or 16 weeks after surgery, footpads were passively sensitized with a subcutaneous injection of 0.20 μg IgEαDNP. Two hours after sensitization, the animals were challenged with an intravenous injection of DNP-BSA. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after challenge. Each point represents the mean ± standard deviation of five footpads. The statistical difference between denervated and sham swelling responses at 0.5 h at three weeks was $P<0.01$, and at 16 weeks was $P<0.001$.

(a)


Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
3 Weeks After Surgery
0.20 ug IgE/Footpad
n=5
P<0.01
Hours Following Antigen Challenge
Denervated
Sham
Untreated

(b)

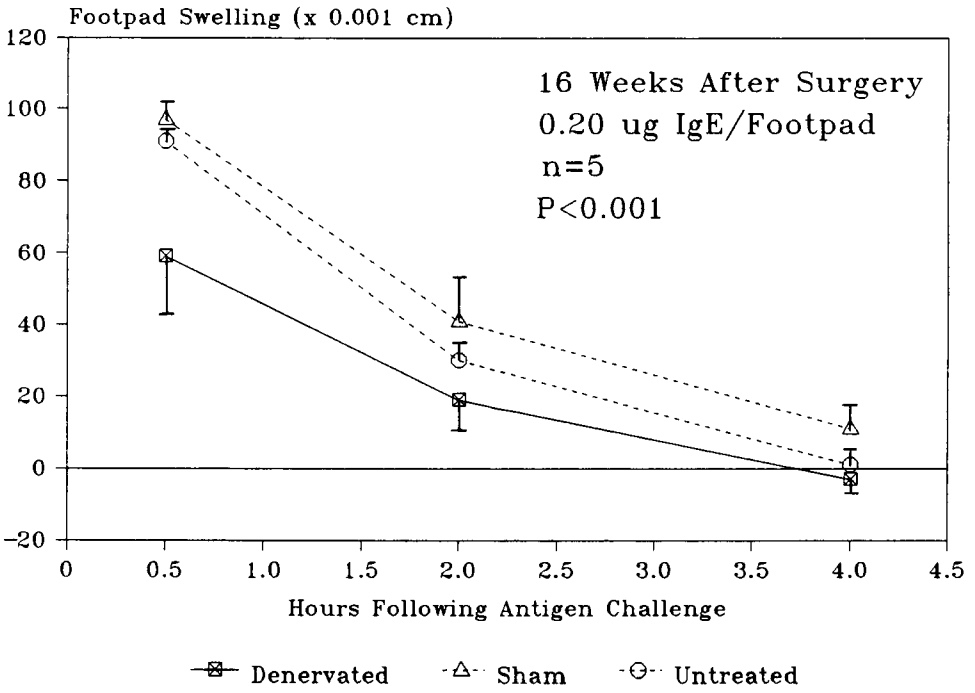

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
16 Weeks After Surgery
0.20 ug IgE/Footpad
n=5
P<0.001
Hours Following Antigen Challenge
Denervated
Sham
Untreated

Table 2. Mice received different combinations of single or double surgery (sciatic denervation, sham surgery, or no surgery) on their hind legs. One week after surgery, footpads were passively sensitized with a subcutaneous injection of 0.20 μg IgEαDNP. Two hours after sensitization, the animals were challenged with an intravenous injection of DNP-BSA. Footpad swelling was measured at 0.5 h after challenge. These swelling responses were used in the statistical analyses shown in Table 3. (ND, not done.)

Table 2. PCA induced with 0.20 μg local IgE in the footpads of mice receiving combinations of single or double surgery.

Group	Surgery		Footpad Swelling at 0.5 h (× 0.001 cm)					
			Expt. 1		Expt. 2		Expt. 3	
	Left Leg	Right Leg	Left	Right	Left	Right	Left	Right
A. Untreated	ND	ND	128	131	83	95	89	86
			115	120	100	92	85	88
			117	112	100	95	72	75
			105	110	95	96	88	87
			125	121	100	108	103	101
B. Single Surgery	Sham	ND	121	111	101	95	ND	
			90	92	102	110		
			110	106	107	107		
			103	107	85	82		
			92	87	97	90		
C. Single Surgery	Denervated	ND	63	89	72	74	ND	
			86	105	84	109		
			65	82	76	102		
			41	89	78	69		
			77	76	64	99		
D. Double Surgery	Sham	Sham	87	87	ND		ND	
			82	85				
			78	81				
			81	82				
			85	81				
E. Double Surgery	Sham	Denervated	81	75	ND		ND	
			83	75				
			90	92				
			86	70				
			111	86				
F. Double Surgery	Denervated	Sham	67	83	ND		ND	
			70	79				
			68	89				
			73	92				

Table 3. Footpad swelling responses listed in Table 2 were evaluated in three statistical analyses. Swelling responses from groups B, D, and E were included in a 1-factor ANOVA (a), and responses from all groups (A through F) were evaluated in a 1-factor randomized block ANOVA and a Tukey's HSD test (b).

Table 3. Statistical analyses of PCA in denervated and control footpads locally sensitized with 0.20 μg IgE.

a. Sham surgery did not affect footpad swelling. Furthermore, swelling responses in sham footpads were not affected by contra-lateral sham or denervation surgery.

1-Factor ANOVA

Source	df	SS	F	P	
treatments	2	0.00094	0.13	0.88	not sig.
error	17	0.06251			
Total	29	0.36340			

b. Denervation significantly reduced the swelling response compared to sham or untreated controls. These results did not differ among experimental trials (*i.e.* blocks).

1-Factor Randomized Block ANOVA

Source	df	SS	F	P	
treatments	2	0.2183	20.88	<0.001	highly sig.
trials	1	0.0092	1.76	0.196	not sig.
error	26	0.1359			
Total	29	0.3634			

Results of **Tukey's HSD test**: Denervated Sham-surgery Untreated

Figure 12. PCA in denervated footpads systemically sensitized with IgE. One week after surgery, mice were passively sensitized with an intravenous injection of IgEαDNP. Eighteen hours later, footpads were challenged with a subcutaneous injection of DNP-BSA. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after challenge. Each point represents the mean ± standard deviation of six footpads. The statistical difference between denervated and sham swelling responses at 0.5 h was $P < 0.001$. This experiment was repeated with similar results.

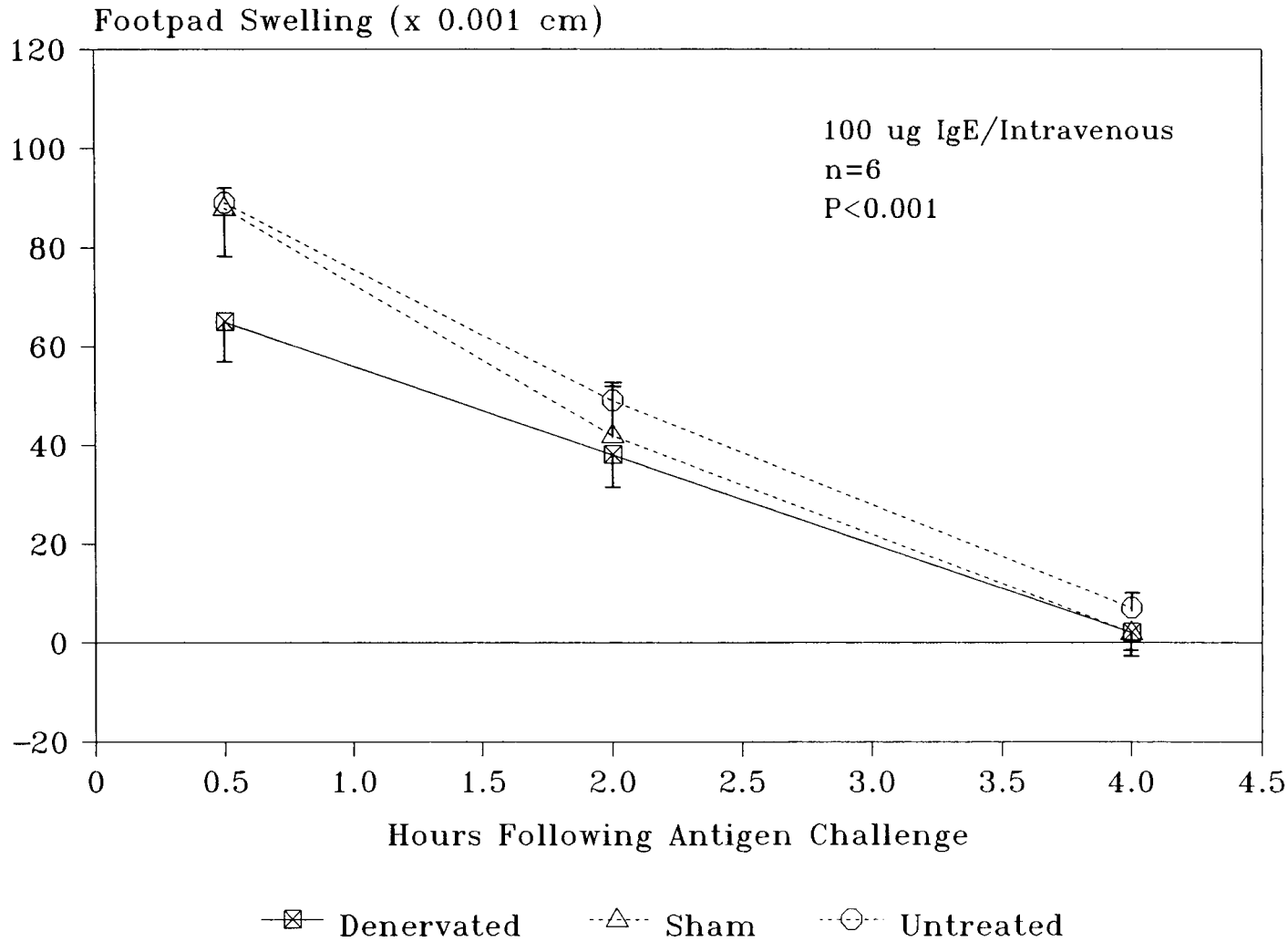
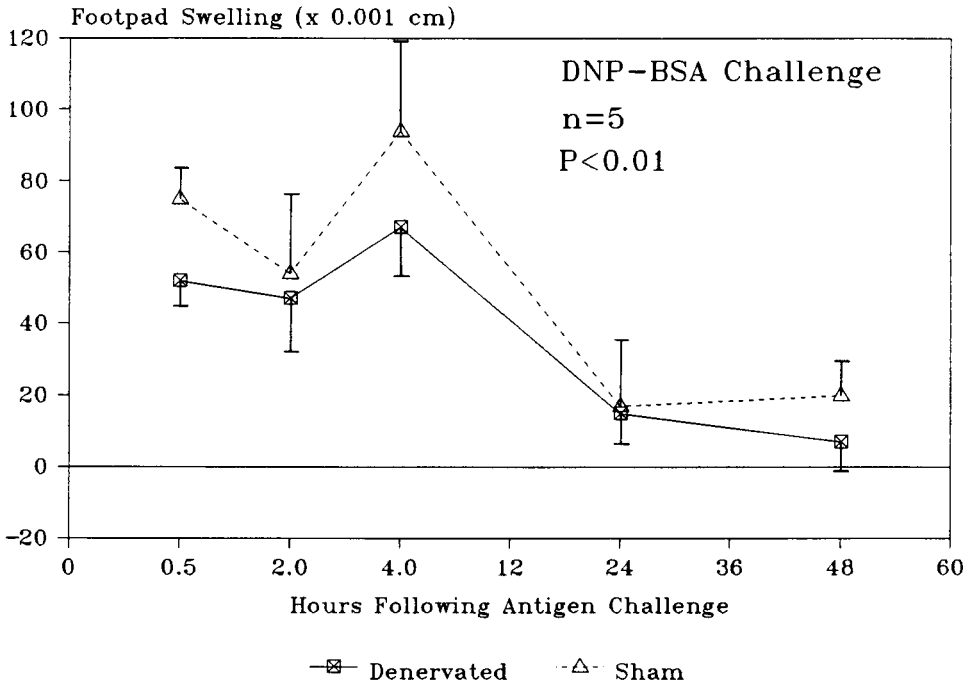
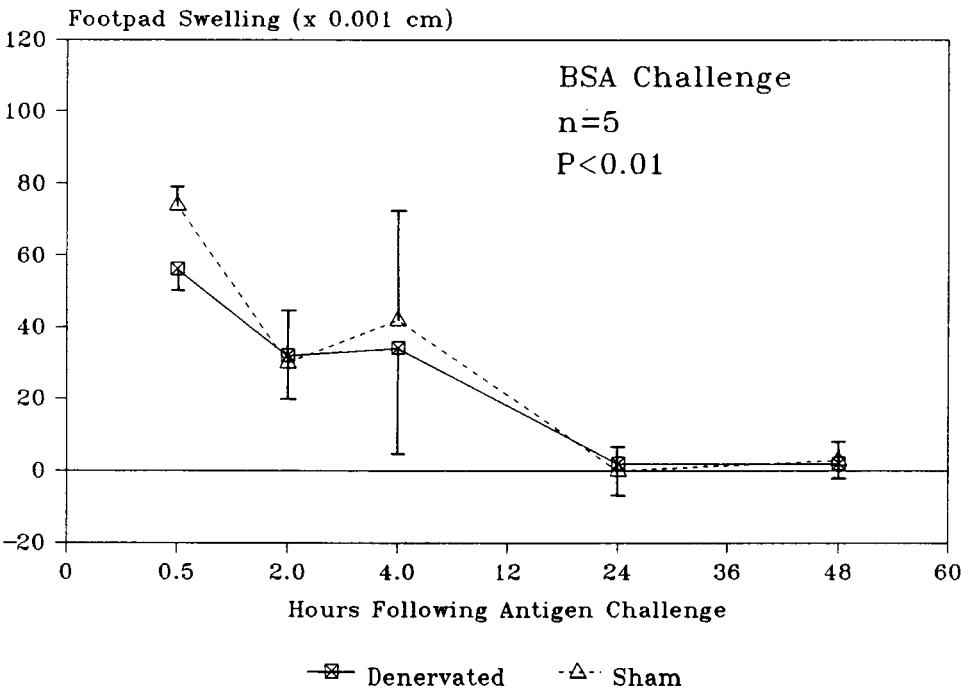

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
100 ug IgE/Intravenous
n=6
P<0.001
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Hours Following Antigen Challenge
Denervated
Sham
Untreated

Figure 13. PCA in denervated footpads of actively sensitized mice. Mice were immunized with DNP-BSA to produce IgEαDNP-BSA. Three weeks after immunization, the animals received surgery, and one week later footpads were challenged with a subcutaneous injection of DNP-BSA (a) or BSA (b). Footpad swelling was measured at 0.5, 2.0, 4.0, 24, and 48 h after local challenge. At each point, the swelling responses of footpads of unimmunized mice locally challenged with antigen were subtracted from those of immunized animals. Serum IgE-like activity to DNP-BSA was verified at the time of antigen challenge with PCA in rat skin (see Appendix C). Each point represents the mean ± standard deviation of five footpads. For both antigens, the statistical difference between denervated and sham swelling responses at 0.5 h was $P<0.01$.

(a)

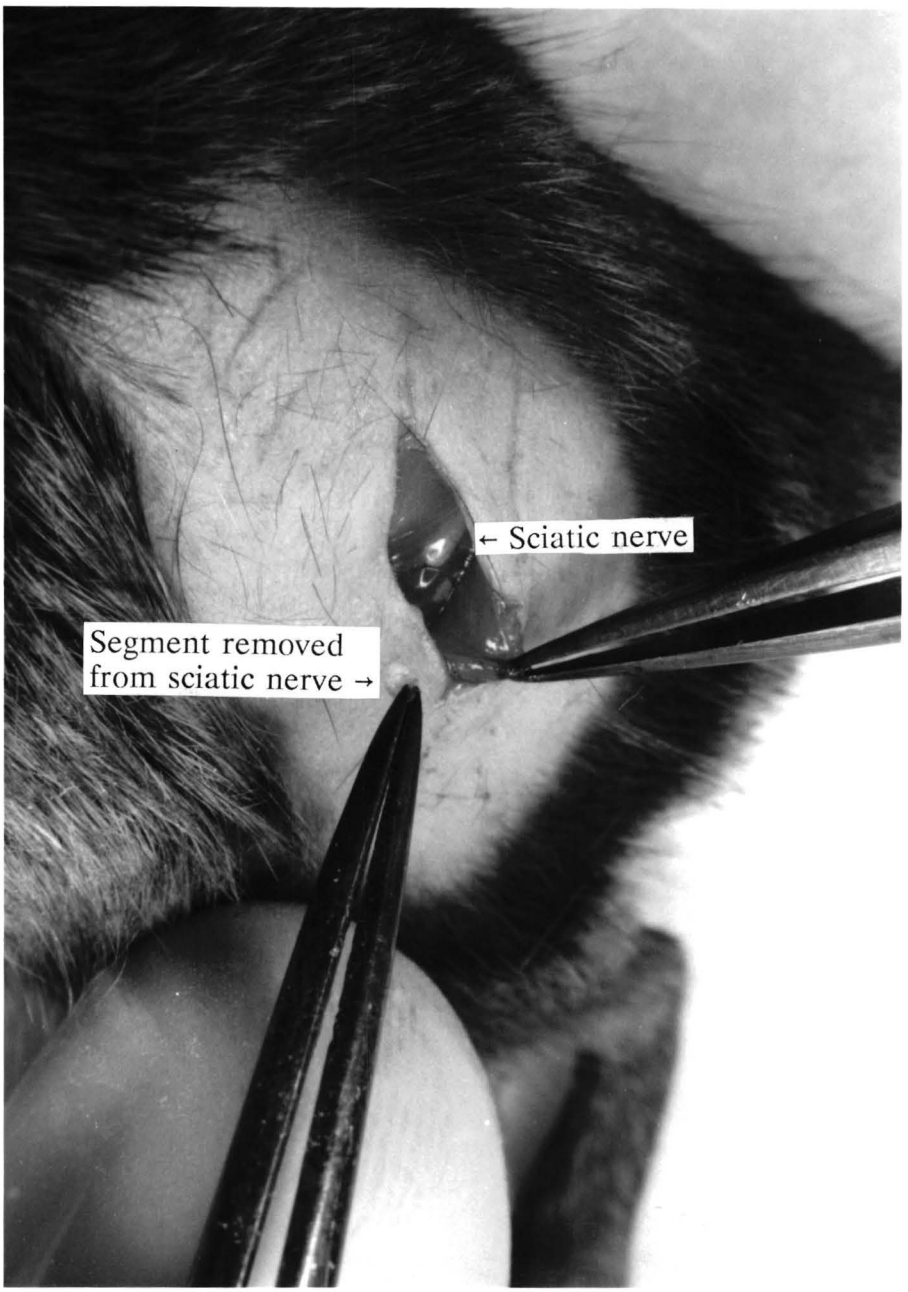

Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
DNP-BSA Challenge
n=5
P<0.01
0
0.5
2.0
4.0
12
24
36
48
60
Hours Following Antigen Challenge
Denervated
Sham

(b)


Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
BSA Challenge
n=5
P<0.01
0
0.5
2.0
4.0
12
24
36
48
60
Hours Following Antigen Challenge
Denervated
Sham

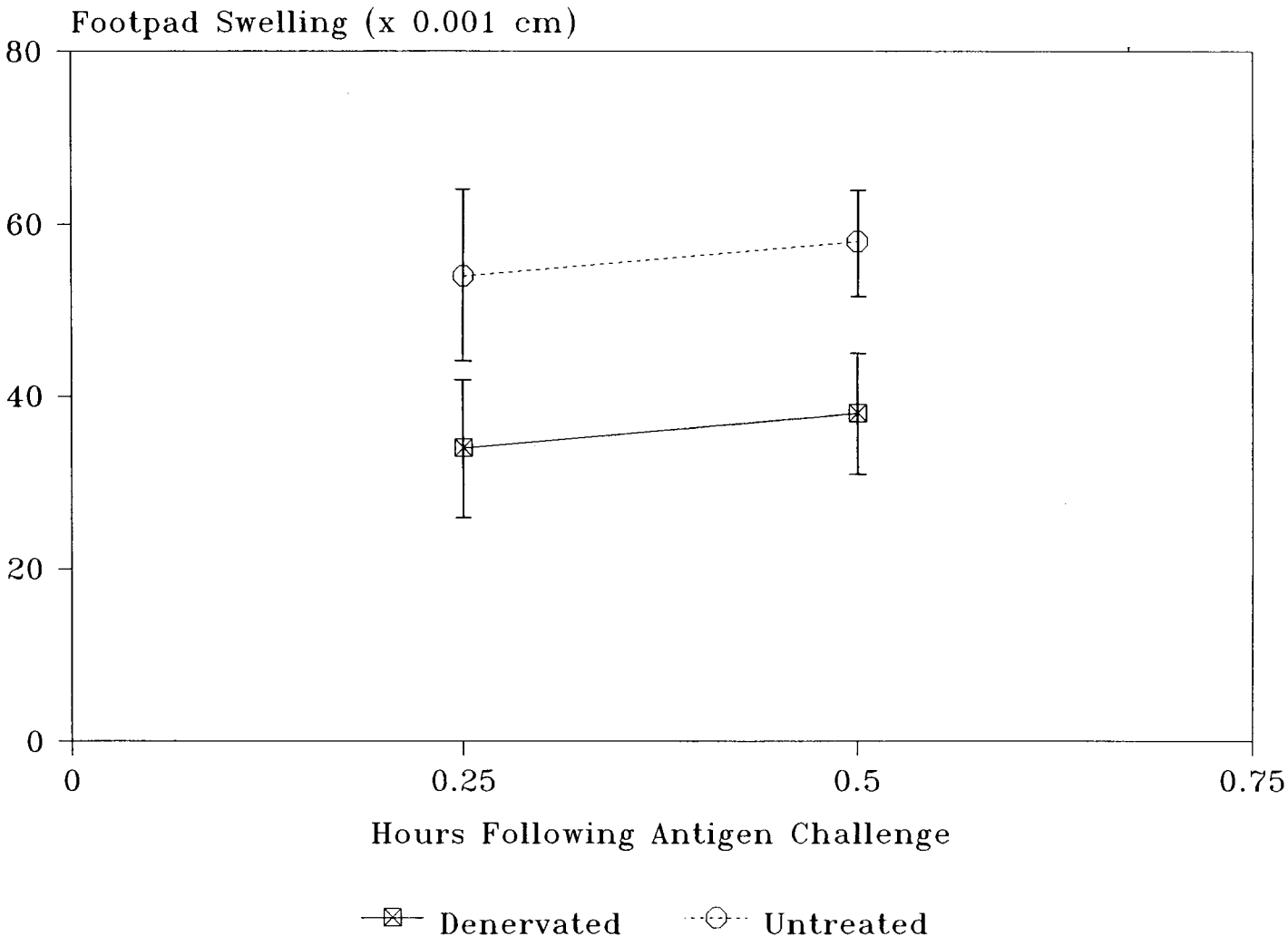
APPENDIX A

Photograph of denervation surgery in the hind leg of a mouse. The procedure for sciatic denervation involved making an incision over the pelvic joint, removing the muscle fascia between the *vastus lateralis* of the *quadraceps* and the long head of the *biceps femoris*, and separating these muscles to locate the sciatic nerve. A 2-mm segment of the nerve was then excised at a location proximal to its division into the tibial and common peroneal nerves.


← Sciatic nerve
Segment removed
from sciatic nerve →

APPENDIX B

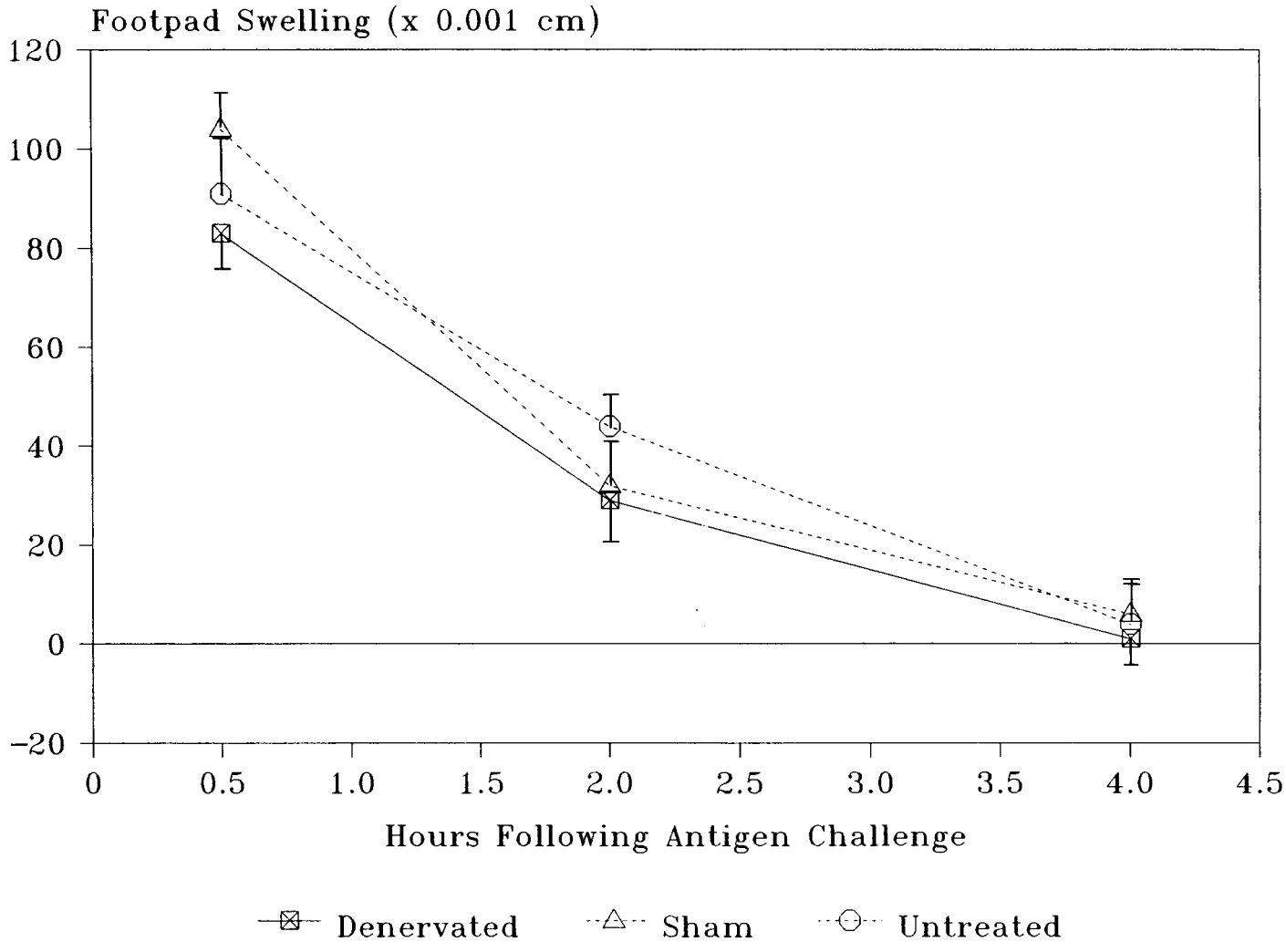
PCA measured at 0.25 and 0.5 h in denervated footpads of DBA-LAC/J mice (Jackson Laboratories, Bar Harbor, ME). One week after surgery, footpads were passively sensitized with a subcutaneous injection of 0.20 μg IgEαDNP. Two hours after sensitization, the animals were challenged with an intravenous injection of DNP-BSA. Footpad swelling was measured at 0.25 and 0.5 h after challenge. Each point represents the mean ± standard deviation of five footpads. The statistical difference between denervated and untreated swelling responses at both times was $P < 0.01$. For either footpad treatment, there was no significant difference between swelling responses measured at 0.25 and 0.5 h.


Footpad Swelling (x 0.001 cm)
80
60
40
20
0
0
0.25
0.5
0.75
Hours Following Antigen Challenge
Denervated
Untreated

APPENDIX C

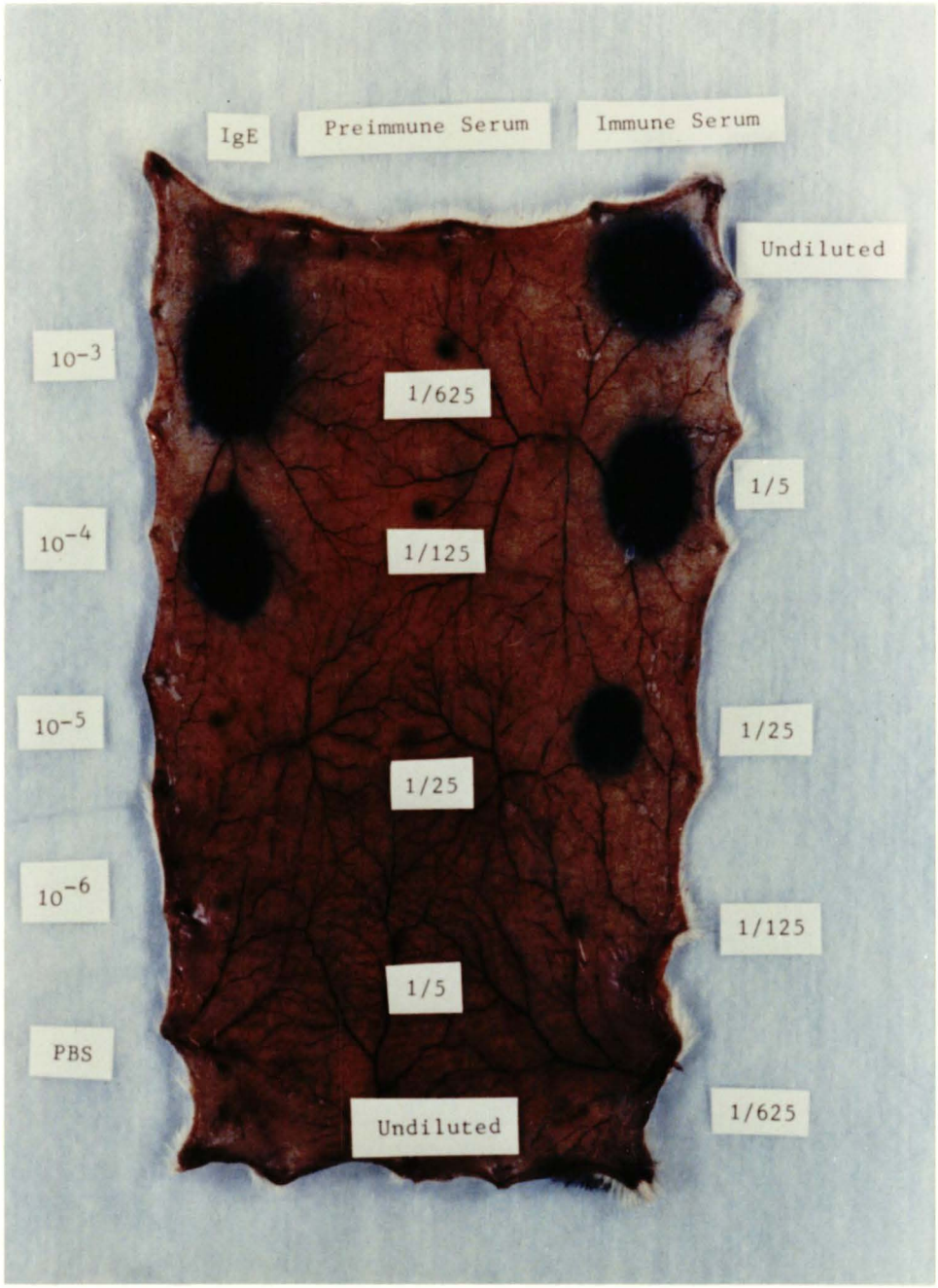
PCA in denervated footpads locally sensitized with 2.00 μg IgE. One week after surgery, footpads were passively sensitized with a subcutaneous injection of 2.00 μg IgEαDNP. Two hours after sensitization, the animals were challenged with an intravenous injection of DNP-BSA. Footpad swelling was measured at 0.5, 2.0, and 4.0 h after challenge. Each point represents the mean ± standard deviation of five footpads.

This was the only experiment in IgE-sensitized animals where there was not a significant difference between denervated and untreated control footpads at 0.5 h. Nevertheless, there was a significant difference between denervated and sham swelling responses ($P<0.02$).


Footpad Swelling (x 0.001 cm)
120
100
80
60
40
20
0
-20
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Hours Following Antigen Challenge
Denervated
Sham
Untreated

APPENDIX D

Photograph of PCA in rat skin locally sensitized with preimmune and immune sera from mice immunized with DNP-BSA. Marked sites on the back of a rat were subcutaneously injected with dilutions (shown in parentheses) of IgEαDNP, preimmune mouse serum (PMS), and immune mouse serum (IMS). Two hours after sensitization, the rat was challenged intravenously with DNP-BSA and Evan's Blue Dye. Forty-five minutes later, the animal was euthanized and the skin from the back was removed. The x and y diameters of the blued areas were as follows: IgE (0.001) = 16 × 24 mm, IgE (0.0001) = 13 × 18 mm, IMS (undiluted) = 17 × 18 mm, IMS (1/5) = 13 × 19 mm, and IMS (1/25) = 9 × 14 mm.


IgE
Preimmune Serum
Immune Serum
Undiluted
10^{-3}
1/625
1/5
10^{-4}
1/125
10^{-5}
1/25
1/25
10^{-6}
1/125
1/5
PBS
Undiluted
1/625