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17. The irregular firing patterns were determined by obtaining interspike interval histograms of the DA neurons. These interspike interval histograms revealed increases in the number of short bursting periods and in the number of unusually long interspike intervals. Such firing patterns are rarely seen in control rats. Despite the irregular patterns, these cells met all other criteria for inclusion as DA neurons. Moreover, the A9 and A10 cells exhibiting these irregular firing patterns could usually be antidromically activated by stimulation of the caudate nucleus and nucleus accumbens, respectively. Moreover, their estimated conduction velocities were consistent with those of DA, not those of non-DA, neurons in the substantia nigra zone and ventral tegmental area. For further discussion of these findings and representative examples of interspike interval histograms, see the report by White and Wang (*6*).
18. The proposed process of depolarization inactivation suggests that APD's cause continuous excitation (or disinhibition) of DA cells, driving them above their maximal firing rate, causing excessive depolarization, and, eventually, inactivating the spike-generating mechanism of the cell, that is, raising the resting membrane potential above threshold (*6, 7*).
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21. Repeated treatment with morphine at 1.0 mg/kg significantly ($P < 0.01$) increased the number of A9 and A10 DA cells per track to 120 percent (1.03 ± 0.04) and 126 percent (1.90 ± 0.21) of control, respectively. Repeated morphine treatment at 15.0 mg/kg significantly increased the number of A9 and A10 DA cells per track to 125 and 126 percent of control, respectively. Therefore, this effect of morphine was apparently not dose-related. Similar results were found in rats receiving single injections of morphine. Repeated desipramine treatment also significantly increased the number of A9 (1.20 ± 0.04) and A10 (1.97 ± 0.03) DA cells per track.
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A Serological Test for Leprosy with a Glycolipid Specific for *Mycobacterium leprae*

Abstract. *A phenolic glycolipid from* Mycobacterium leprae *was purified and used as antigen in an enzyme-linked immunosorbent assay. Antibodies directed against the lipid were seen in serums from leprosy patients but not in serums from uninfected controls or patients infected with other mycobacteria, including* Mycobacterium tuberculosis. *The antibody response distinguished between the* Mycobacterium leprae *lipid and the structurally related phenolic glycolipid from* Mycobacterium kansasii. *This assay has considerable potential as a specific serodiagnostic test for leprosy infection.*

Resistance to leprosy is primarily dependent on the cell-mediated immune system, but infection with *Mycobacterium leprae* also stimulates an apparently nonprotective humoral immune response (*1–4*). Antibody levels in leprosy patients have been reported to reflect different clinical forms of the disease (*2, 4*) and to change with alterations in antigen load during chemotherapy (*4, 5*). *Mycobacterium leprae* has not yet been cultivated in vitro, and although preliminary evidence of *M. leprae*–specific antigens has been reported (*6–8*), it has not been possible to obtain these components in a form suitable for use in routine serological tests. Previous studies of humoral immunity in leprosy have therefore made use of mixtures of specific and nonspecific antigens (*4*), homogeneous cross-reactive antigens from other mycobacteria (*9, 10*), or leprosy serums absorbed with other mycobacteria to remove cross-reactive antibodies (*1*).

A phenolic glycolipid with a structure related to that of mycoside A of *Mycobacterium kansasii* (*11*) was found in *M. leprae* preparations by Draper (*12*), and its structure was elucidated by Hunter and Brennan (*13*). The unique trisaccharide component of the glycolipid (2,3-di-*O*-methyl rhamnose, 3-*O*-methyl rhamnose, and 3,6-di-*O*-methyl glucose) suggested to these authors that it may constitute a species-specific antigen, but they were unable to demonstrate serological activity using the purified lipid (*13*). Payne *et al.* (*14*), however, showed that the lipid is active in immunodiffusion experiments when it is incorporated into liposomes, and other workers indicated the possible use of the native phenolic glycolipid in an enzyme-linked immunosorbent assay (ELISA) system (*15, 16*).

We now report the development of an ELISA in which the deacylated form of the phenolic glycolipid is used. The fact that the lipid is found in large amounts in skin lesions of leprosy patients (*17*) and in tissues of experimentally infected nine-banded armadillos (*13*) suggests that it may play an important role during leprosy infection and also that it will be available in sufficient quantities for use in large-scale serological screening.

The *M. leprae* phenolic glycolipid was purified from supernatant material derived from homogenization of infected armadillo liver (*13*). Extraction by the Bligh-Dyer monophasic method (*18*) was followed by elution from a silica gel–Celite column with methanol (2 percent, by volume) in chloroform (*13*) and preparative thin-layer chromatography (TLC) on silica gel G plates developed with a mixture of chloroform and methanol (15:1, by volume). The phenolic glycolipid from *M. kansasii* was purified in the same way from bacteria grown on Middlebrook's 7H11 medium. The identities of the glycolipids were established by combined gas-liquid chromatography and mass spectroscopy of the alditol acetate derivatives generated by trifluoroacetic acid hydrolysis, followed by borohydride reduction and acetylation with acetic anhydride (*19*). Comparison of mass spectrograms of derivatives from the two glycolipids with those of standard partially methylated alditol acetates confirmed the carbohydrate composition of the *M. leprae* lipid as 2,3-di-*O*-methyl rhamnose, 3-*O*-methyl rhamnose, and 3,6-di-*O*-methyl glucose and that of the *M. kansasii* lipid as 2,4-di-*O*-methyl rhamnose, 2-*O*-methyl rhamnose, and 2-*O*-methyl fucose.

The phenolic glycolipid is a strongly hydrophobic compound and therefore difficult to use in routine immunological tests, which are generally carried out under aqueous conditions. To facilitate its use in such tests, we removed the major part of the lipid portion of the molecule by hydrolysis of the two long-chain fatty acids from the phthiocerol core in methanolic NaOH (10 percent, weight to volume) at 100°C for 16 hours. The deacylated phenolic glycolipid was recovered by preparative TLC. The more polar deacylated forms of the phenolic glycolipids from *M. leprae* and *M. kansasii* migrated more slowly than the native molecule in the organic solvent used for TLC and as a single spot, indicating complete deacylation.

The concentrations of deacylated and native phenolic glycolipid solutions were determined by assaying the carbohydrate moiety, with rhamnose used as standard (*20*). The deacylated phenolic glycolipid was prepared in aqueous suspensions by vortex-mixing the lipid (50 μg) with distilled water (1 ml) and incubating at 55°C for 15 minutes. Lipid preparations were stable for at least 1 week when stored at 0° to 4°C. For the ELISA, deacylated phenolic glycolipid was diluted with water (1:10) and added to 96-well polystyrene microtiter plates (Linbro) at 0.1 ml per well. Half of the wells on each plate were coated with the

Table 1. Results of serological testing with the phenolic glycolipid ELISA. All serums were tested at a dilution of 1:20, as described in the text. Values of ΔA_{492} are given as means ± standard deviation.

Group	Number of serums	ΔA_{492}	Number with $\Delta A_{492} \geq 0.1$
Normal	10	0 ± 0.02	0
Vaccinated with BCG	10	0 ± 0.05	0
Tuberculosis	10	0.01 ± 0.04	0
Other mycobacteria	10	0 ± 0.03	0
Leprosy*			
TT	12	0.12 ± 0.12	5
BT	12	0.32 ± 0.39	7
BB	12	0.67 ± 0.46	9
BL	12	0.73 ± 0.36	11
LL	12	0.82 ± 0.34	12

*Classification by Ridley-Jopling criteria (*21*).

lipid antigen; the rest contained distilled water only. After overnight incubation at 37°C, plates were washed four times with phosphate-buffered saline (PBS) and then incubated for 2 hours at 37°C with bovine serum albumin (5 percent, weight to volume) in PBS (0.1 ml per well). The bovine serum albumin was then replaced with serum samples (0.1 ml) diluted in PBS containing fetal calf serum (5 percent, by volume), and plates were incubated for two more hours at 37°C. The plates were washed four times with PBS, and peroxidase-linked goat antiserum to human immunoglobulin G + M + A (Cappel Laboratories, West Chester, Pennsylvania) was added at a dilution of 1:1000 in PBS containing fetal calf serum (1 percent, by volume). After a further 1-hour incubation at 37°C, the plates were washed four times with PBS, and *o*-phenylenediamine was added at a concentration of 0.1 mg/ml in PBS containing 0.05 percent Tween 20 and 0.003 percent H_2O_2 (0.1 ml per well). Reactions were stopped by adding 8*N* H_2SO_4 (25 μl per well) after 20 minutes incubation at room temperature, and absorbances were read at 492 nm with a spectrophotometer (Titertek Multiscan). The antibody reactivity to phenolic glycolipid for each serum sample was calculated by subtracting the mean absorbance of duplicate samples in uncoated wells from the mean of duplicates in antigen-coated wells (ΔA_{492}). Each plate contained a negative control (pooled normal human serums) and a positive control (pooled serums from leprosy patients). The ΔA_{492} for normal human serum was in the range −0.05 to 0.05.

The advantage of using the deacylated glycolipid was seen by comparing its performance to that of the native lipid in the ELISA test. At a concentration of 4 μg/ml, the ΔA_{492} observed with lepromatous leprosy serum was 1.2 with the deacylated glycolipid and 0.06 with the native glycolipid. Even at coating concentrations as high as 32 μg/ml, the ΔA_{492} readings did not exceed 0.24 with the native glycolipid. In contrast, the deacylated molecule at a coating concentration of only 1 μg/ml produced a ΔA_{492} of 0.34. The poor performance characteristics of purified phenolic glycolipid in the ELISA may result from antigenic trisaccharide moieties being masked by excess lipid. Even with procedures recommended by other workers [coating in hexane (*15*) or in the presence of sodium deoxycholate (*16*)], we have found that only serums from patients strongly positive for lepromatous leprosy had antibody levels detectable by ELISA when the native glycolipid was used as antigen. This is in marked contrast to the results with the deacylated molecule as antigen.

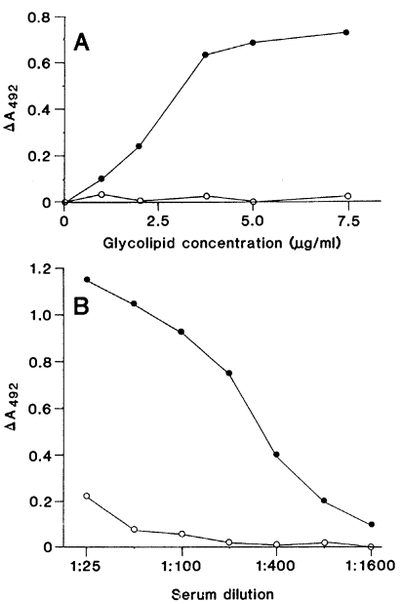

Fig. 1. Effect of variation of (A) coating antigen concentration and (B) serum dilution on the phenolic glycolipid ELISA. Glycolipids from *M. leprae* (closed circles) and *M. kansasii* (open circles) were used. (A) Deacylated glycolipids at different concentrations were used to coat polystyrene microtiter plates as described in the text. Serum from a lepromatous leprosy patient was used in the assay at a dilution of 1:200. (B) Serum from a lepromatous leprosy patient was assayed at different dilutions using plates coated with deacylated glycolipids at 5 μg/ml.

Figure 1A shows the activity observed with serum from a patient with lepromatous leprosy when ELISA plates were coated with the deacylated glycolipids of *M. leprae* and *M. kansasii* at concentrations of 1 to 10 μg/ml. The optimal coating concentration for the *M. leprae* lipid was 5 μg/ml; the *M. kansasii* lipid showed no activity at any of the concentrations used. When high serum concentrations were used, a small amount of activity against the *M. kansasii* lipid was seen with lepromatous leprosy serums (Fig. 1B), while pooled normal human serum had no activity with either lipid at a dilution of 1:20. Incubation of serums with the deacylated glycolipids (1 μg/ml) in aqueous suspension for 1 hour at 37°C before they were added to ELISA plates resulted in inhibition of 70 percent in the phenolic glycolipid titer with the *M. leprae* lipid and an inhibition of only 10 percent with the *M. kansasii* lipid.

The specificity of the ELISA was further tested with serums from groups having different exposure to mycobacteria (Table 1). These were (i) normal individuals from the Seattle area having no known mycobacterial infections; (ii) volunteers who had recently been vaccinated with bacillus Calmette-Guérin (BCG); (iii) patients from Mexico with highly advanced tuberculosis infection, all of whom had high levels of antibodies against *M. tuberculosis* antigens as judged by counterimmunoelectrophoresis; (iv) patients with mycobacterial infections other than leprosy and tuberculosis (including infection with *M. kansasii*, *M. chelonei*, *M. avium-intracellulare*, and *M. fortuitum*), all of whom had elevated titers of antimycobacterial antibodies as judged by an ELISA technique with mycobacterial arabinomannan as antigen (*10*); and (v) leprosy patients, from Seattle and Sri Lanka, who were clinically and histologically characterized according to the Ridley-Jopling criteria (*21*). The results of the phenolic glycolipid ELISA were uniformly negative for the control groups ($\Delta A_{492} < 0.1$), whereas the results in 73 percent of the serums from 60 leprosy patients were positive. Also, the mean antibody levels were directly correlated with the bacterial load in the leprosy patients, with the highest ΔA_{492} observed in lepromatous patients and the lowest levels in patients with tuberculoid disease.

Our results suggest that the ELISA using deacylated phenolic glycolipid is

potentially a highly specific assay for detection of a humoral response to leprosy infection and, for the first time, represents a direct method for studying such a response without complications due to previous exposure to environmental mycobacteria or BCG vaccination. It will be of particular interest to apply such a test to detection of the very early stages of leprosy infection. Early detection and treatment of leprosy has the potential benefits of reducing the incidence of deformities due to the disease and also of reducing the transmission of leprosy in the community.

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Substance P and Somatostatin Regulate Sympathetic Noradrenergic Function

Abstract. *Peptidergic-noradrenergic interactions were examined in explants of rat sympathetic superior cervical ganglia and in cultures of dissociated cells. The putative peptide transmitters substance P and somatostatin each increased the activity of the catecholamine-synthesizing enzyme tyrosine hydroxylase after 1 week of exposure in culture. Maximal increases occurred at 10^{-7} molar for each peptide, and either increasing or decreasing the concentration reduced the effects. Similar increases in tyrosine hydroxylase were produced by a metabolically stable agonist of substance P, while a substance P antagonist prevented the effects of the agonist. The data suggest that the increased tyrosine hydroxylase activity was mediated by peptide interaction with specific substance P receptors and that peptides may modulate sympathetic catecholaminergic function.*

The demonstration of putative peptide neurotransmitters in sympathetic ganglia (*1*, *2*) suggests that the biochemical organization of the autonomic nervous system is considerably more complicated than previously thought. For example, the peptide neurotransmitters substance P (SP) and somatostatin (SS) are present in mammalian autonomic ganglia (*1*, *2*), and electrophysiologic evidence suggests that they subserve a physiologic role in the sympathetic system (*3*, *4*). Repetitive stimulation of presynaptic nerves to sympathetic ganglia elicits not only a burst of action potentials, but also slow depolarization which is insensitive to cholinergic antagonists (*5*). Iontophoretic application of SP to sympathetic neurons mimics this noncholinergic, slow excitatory postsynaptic potential (EPSP) (*3*, *4*). Moreover, the slow EPSP elicited by repetitive stimulation is blocked by antagonists of SP (*4*). Finally, SP is released from sympathetic ganglia by a high potassium stimulus in a calcium-dependent manner (*3*). These observations suggest that SP release elicits the slow EPSP in sympathetic neurons. Although the function of peptide transmitters in autonomic ganglia is unclear, the prolonged nature of this electrophysiologic response suggests that they may subserve modulatory functions.

We studied the influence of SP and SS on catecholaminergic function in autonomic ganglia and found that both peptides regulate noradrenergic phenotypic characters in sympathetic neurons in culture.

To examine peptidergic influences on sympathetic, noradrenergic function, we studied the effects of SP and SS on the catecholamine-synthesizing enzyme tyrosine hydroxylase (TOH) in the rat sympathetic superior cervical ganglion (SCG) in vitro. TOH, the rate-limiting enzyme in catecholamine synthesis (*6*), is localized in the principal neurons of sympathetic ganglia, and is a convenient index of noradrenergic function. Two separate tissue culture techniques were used, SCG organ transplants and dissociated cell cultures of the SCG.

First we found that cultures rapidly metabolized exogenous peptide introduced into the medium. However, addition of a mixture of bacitracin and captopril, potent peptidase inhibitors, reduced this loss to less than 10 percent of added SP and 35 percent of added SS after 24 hours. Consequently, peptidase inhibitors were routinely added to the culture medium. The medium was also changed every other day to maintain the original peptide concentrations.

Addition of bacitracin and captopril to the medium significantly increased TOH activity both in ganglion explants (33 percent increase) and in dissociated cell cultures after 1 week (29 percent increase) (Fig. 1A). Moreover, addition of SP led to further significant 40 percent and 34 percent increases in explant and cell cultures, respectively; addition of SS led to 41 and 30 percent increases, respectively (Fig. 1). By contrast, the peptides had no effect on TOH activity in ganglion homogenates, excluding a direct effect on the TOH enzyme or radiochemical catalytic assay (data not shown). Consequently, SP and SS apparently increased TOH activity through processes that transpired in culture and not through direct interaction with the apo- or holoenzyme.

Dose-response relationships were examined by culturing explants in the presence of different peptide concentrations (Fig. 1B). Both SP and SS exerted maximal effects at $10^{-7}M$. Diminishing concentrations below $10^{-7}M$ reduced the stimulatory action on TOH activity, with no effect at $10^{-9}M$. Similarly, increasing concentrations above $10^{-7}M$ reduced the effect on TOH, with loss of action at $10^{-4}M$. Submaximal responses at concentrations above $10^{-7}M$ may reflect a variety of effects, including receptor desensitization or down regulation, as defined for other transmitters (*7*). Although