

A Monoclonal Antibody to *Borrelia burgdorferi* Flagellin Modifies Neuroblastoma Cell Neuritogenesis In Vitro: a Possible Role for Autoimmunity in the Neuropathy of Lyme Disease

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Received 31 October 1996/Returned for modification 6 January 1997/Accepted 27 February 1997

Although *Borrelia burgdorferi* is found at the site of many manifestations of Lyme disease, local infection may not explain all features of the disease. Previous work has demonstrated that the organism's flagellin cross-reacts with a component of human peripheral nerve axon, heat shock protein 60. The cross-reacting epitope is identified by a single anti-*B. burgdorferi* flagellin monoclonal antibody, H9724. We now report that the spontaneous and peptide growth factor-stimulated in vitro neuritogenesis of SK-N-SH neuroblastoma cells and other neural tumor cell lines is suppressed by H9724. In contrast, changes induced by exposure of these cells to optimal and suboptimal concentrations of cyclic AMP, phorbol ester, or retinoic acid are not affected by H9724. H9724 does not decrease cell viability or the ability of the cells to anchor to the culture plate or extracellular matrix and does not block nerve growth factor binding to the cells. These findings are compatible with the premise that anti-axonal antibodies formed during the immune response to *B. burgdorferi* flagellin might modify axonal function in vivo and play a role in the pathogenesis of neurologic features of Lyme disease. A humoral immune response predicated on molecular mimicry could explain persistent or ongoing neurologic dysfunction occurring after elimination of the organism by appropriate antibiotic therapy.

Lyme disease (LD) is a multisystem inflammatory disease, caused by infection with *Borrelia burgdorferi*, transmitted by the bite of an infected tick (39). Clinical features of the infection include the pathognomonic skin rash, erythema migrans, and associated nonspecific systemic symptoms, which comprise early localized disease. Early disseminated LD includes carditis and neurologic disease, e.g., meningoencephalitis and cranial and peripheral neuropathies; these features of LD may occur weeks to months after the onset of infection. Late LD manifestations include arthritis and primarily central nervous system disease, known as tertiary neuroborreliosis (22, 38).

Although *B. burgdorferi* is present in the erythema migrans lesion and has been grown from plasma and cerebrospinal fluid, occasionally from myocardial biopsies, and once from synovial fluid, the organism is seen only rarely in synovial and central nervous system tissue biopsies and has never been found in biopsy material from affected peripheral nerve tissue (10). Electrophysiologic studies of patients with LD peripheral neuropathy usually demonstrate an axonopathy rather than demyelination (22). Nerve biopsies do not reveal demyelination, axon destruction is rare, and there is only a modest cellular infiltration (19). Thus, it is possible that axonal dysfunction might occur in the absence of local infection, inflammation, or frank tissue destruction.

Plausible explanations for later manifestations of LD which proceed despite adequate prior therapy have been proposed previously (35) and include ongoing infection or the persistence of *B. burgdorferi* antigens acting as a focus of ongoing inflammation, similar to the antigen-induced arthritis model of rheumatoid arthritis (40); local cytokine production, due to persistent *B. burgdorferi* antigens, causing tissue modification

(33); and the initiation of autoimmune reactivity. The last might be induced by the liberation of tissue antigens from prior damage or might be due to the mechanism known as molecular mimicry (29, 33).

In support of the possibility that autoimmunity due to molecular mimicry may play a role in LD, we have shown that serum from patients with neurologic LD contains anti-*B. burgdorferi* antibodies which also bind to human peripheral nerve axon (36). A single monoclonal antibody to *B. burgdorferi* flagellin (H9724; developed by Alan G. Barbour, University of California, Irvine [4]) also binds to human nerve (36); H9724 reactivity with human neural tissues has been corroborated by others (1). The patient sera which bound to axon (36) also bind to the H9724-defined flagellar epitope (7). These same sera and H9724 bind to cultured human neuroblastoma cells (lines SK-N-SH, SK-N-MC, and IMR32), with reactivity often predominantly in the neurite or neuritic bud (34). By immunoblot, H9724 binds to a protein with an approximate molecular mass of 64 kDa (p64) in neuroblastoma cells and human nerve and in neural tissues and cell lines of other species; H9724 immunoprecipitates a protein of the same size from both sources (34). The H9724-reactive protein purified from calf adrenal gland has an N-terminal amino acid sequence identical to that of the human 60-kDa heat shock protein, also known as chaperonin 60 (cpn60 [8]) and is bound by the same LD neurologic patient sera which, in earlier studies, bound to axons.

In culture, neuroblastoma cells elongate and send out cell processes known as neurites, representing the cells' attempt at differentiating and producing an axon. This occurs in serum-free medium cultures with or without exogenous stimulants, e.g., nerve growth factor (NGF) or retinoic acid (RA). PC12 pheochromocytoma cells do not spontaneously differentiate or undergo neuritogenesis, requiring the presence of NGF or other stimulants.

We performed studies with cultured neural cell lines grown

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with or without H9724; the cells were either untreated or stimulated with NGF, basic fibroblast growth factor (bFGF), dibutyryl cyclic AMP (db-cAMP), phorbol 12-myristate 13-acetate (PMA), RA, the calcium ionophore A23187, and potassium chloride. We found that H9724, the anti-*B. burgdorferi* flagellin monoclonal antibody which binds to cpn60, blocked changes in cell morphology and neurite formation occurring in unstimulated cells and in cells treated with certain of the stimulants noted. Antibodies to the H9724-defined epitope of flagellin produced during the human immune response to *B. burgdorferi* might bind to this human axonal protein in vivo and modify axonal function. Thus, molecular mimicry might provide an explanation for some cases of the neuropathy seen in LD.

(Parts of this work were previously presented at the American College of Rheumatology national meetings in Boston, Mass. [October 1991], and Atlanta, Ga. [October 1992], and at the Vth International Symposium on Lyme Borreliosis, Crystal City, Va. [June 1992].)

MATERIALS AND METHODS

Cell culture. SK-N-SH human neuroblastoma cells (American Type Culture Collection, Rockville, Md.) were maintained in culture in supplemented medium (SM): RPMI 1640 without L-glutamine supplemented with 10% fetal calf serum and 200 mM L-glutamine (GIBCO, Grand Island, N.Y.) at 37°C in 5% CO₂, as previously described (34). Rat neuroblastoma cell line B103 (courtesy of David Schubert, Salk Institute, La Jolla, Calif.), murine neuroblastoma cell line Neuro2a, and rat pheochromocytoma cell line PC12 (American Type Culture Collection) were maintained in culture with supplemented media as follows: B103 in Dulbecco modified Eagle medium (DMEM) (GIBCO)-5% heat-inactivated horse serum (HyClone)-2.5% fetal calf serum-ampicillin (8 mg/liter) (GIBCO), Neuro2a in minimal essential medium (GIBCO)-10% fetal calf serum (GIBCO), and PC12 in RPMI-10% heat-inactivated horse serum (HyClone)-5% fetal calf serum (GIBCO).

Cell lines used in these experiments were grown in 250-ml tissue culture flasks (Falcon, Lincoln Park, N.J.) and then replated for morphology studies in 35-mm (Corning, Corning, N.Y.) or 100-mm (Falcon) culture dishes; for growth of PC12 cells, the plates were precoated with calf skin collagen (Sigma, St. Louis, Mo.). Cells were harvested by replacing the SM with phosphate-buffered saline (PBS) plus 0.25% trypsin plus 1 mM EDTA (GIBCO) and incubating for 20 min at 37°C. The cells were removed by gentle pipetting, washed three times in 50 ml of PBS, and then replated for passage or studies.

SK-N-SH cells were also grown in serum-free medium. Cells were maintained in a 1:1 mixture of Ham's F-12 medium and Dulbecco-Vogt's modification of Eagle's medium (DMEM/F-12) containing 1.2 g of NaHCO₃ per liter and 15 mM HEPES buffer (GIBCO). This basal medium was supplemented with 5 µg of bovine insulin per ml, 100 µg of human transferrin per ml, 20 nM progesterone, 100 µM putrescine dihydrochloride, and 30 nM sodium selenite (Sigma). The substratum was modified for serum-free medium experiments: 35-mm Falcon plastic tissue culture dishes were precoated with poly-D-lysine (Sigma) as previously described (6). Fibronectin was added separately to dishes containing medium at a concentration of 5 µg/ml. Cells were harvested as described above, with the following change: after a 1-min incubation with trypsin, the trypsin solution was aspirated and replaced with 1 ml of a 1% soybean trypsin inhibitor (Sigma) solution in DMEM. The detached cells were then collected, centrifuged, and resuspended in serum-free medium.

Reagents. H9724 and 6TS, immunoglobulin G2a (IgG2a) murine monoclonal antibodies to *B. burgdorferi* flagellin, were purchased from Alan Barbour, then at the University of Texas at San Antonio (4), as was H5332, an IgG2a monoclonal antibody to the organism's outer surface protein A (OspA [5]); both H9724 and H5332 stock solutions contained approximately 20 µg of antibody/ml.

NGF, bFGF, db-cAMP, butyric acid, PMA, calcium ionophore A23187, RA, and potassium chloride (KCl) were purchased from Sigma.

Modification of cell neuritogenesis by addition of reagents. In initial experiments, 2 × 10⁶ cells were plated in 35-mm tissue culture dishes and allowed to attach for 1 day. In some experiments, 5 × 10⁴ cells were plated in order to have a less crowded cell population on the final days of observation.

Cells either were treated with H9724, 6TS, or H5332 (1:100 dilution of culture supernatant) or were cultured in unmodified SM or serum-free medium. In initial experiments, H9724 was added only to the initial culture medium, but later experiments included the addition of monoclonal antibodies daily throughout the experiment. In some experiments, cells were treated with stimulants at concentrations determined by others to be optimal: 1 mM (20), db-cAMP, 1 mM butyric acid, 50 ng of NGF per ml (17), 10⁻⁸ M PMA (30), 10⁻⁷ M RA (30), 400 nM (24), A23187 (26), or 30 mM potassium chloride (31) with or without monoclonal antibody H9724 or H5332, or the monoclonal antibodies alone. In preliminary studies, 1 ng of bFGF per ml was found to be optimal in stimulating neuritogenesis (data not shown); 0.3 and 1 ng/ml were used in experiments. Serial

TABLE 1. Neuroblastoma cell growth in presence of reagent^a

Reagent added	% SK-N-SH cells bearing long neurites after days (mean of triplicate):			
	1	3	5	7 ^b
Control	23.7 (4.4)	24.0 (3.8)	55.3 (5.7)	61.3 (8.2)
H9724	21.0 (5.3)	18.3 (3.1)	11.3 (1.9)	14.0 (4.0)
H5332	13.7 (4.4)	21.3 (5.7)	49.7 (8.3)	57.7 (7.9)

^a Results of a typical experiment in which SK-N-SH neuroblastoma cells were grown in the presence of H9724 or H5332 or with no addition to the SM. Results are shown as the percentages of cells with long neurites after the noted number of days in culture; the numbers in parentheses represent the standard deviations for each data set.

^b On day 7, the difference between H9724-treated and control cultures was significant at $P < 0.001$ and the difference between H9724-treated and H5332-treated cultures was significant at $P < 0.001$.

10-fold dilutions from the optimal concentrations of db-cAMP, RA, PMA, and A23187 were used in other experiments. Following a 24-h period to allow cell attachment to the plates, medium was replaced every 2 to 3 days over the 5- to 8-day duration of each experiment; the fresh medium contained the same reagent(s) (stimulants and/or monoclonal antibodies) originally present in the medium.

The morphology of the cells were observed by phase-contrast microscopy (Olympus Model IMT-2; Lake Success, N.Y.). The percentage of cells observed to be round, irregular, or elongated and bearing no neurites, short neurites, or long neurites (more than twice the soma length) was determined by counting 50 cells per culture dish (20). All experiments were done six times or more, except as noted; the results appearing in tables describing results of a typical experiment are the means of such experiments done in triplicate, unless otherwise noted.

In order to determine possible interference with anchoring of cells to the plate, cells were incubated with monoclonal antibodies prior to plating.

In certain experiments, cells were grown with H9724 for 1 or 2 days and then cultured in SM alone. These cells were then passaged or tested for viability, measured by their ability to exclude trypan blue dye. SM was discarded, the cells were washed with PBS (pH 7.2), and trypan blue solution (0.4%) (Sigma) was applied. One hundred cells were evaluated per culture.

In certain experiments, the cells were subjected to heat shock (from 0 to 30 min per day at 41°C) and treated either with H9724 or no antibody to determine if such stress might cause changes which interfere with the effects of H9724. Cells were examined daily; data reported are for day 6 after initiation of the culture. One hundred cells were evaluated per culture plate, and each experiment was run in triplicate.

Statistical analysis. Comparison of neuritogenesis induced by different stimuli was by one-way analysis of variance with Scheffé's multipoint post hoc test (3, 41).

RESULTS

H9724 suppresses spontaneous, NGF- and bFGF-stimulated morphologic changes and neurite formation. Neuroblastoma cells spontaneously elongated and produced the cellular process called a neurite. In initial experiments, we added H9724 only at the time of initiation of neuroblastoma cell cultures. This resulted in a transient decrease in spontaneous cell elongation and neurite production. By the fourth to fifth day of culture, there was no residual effect of the H9724 treatment on the first day; the subsequent growth of and neurite production by the cells proceeded as if they had not been exposed to H9724 previously. Furthermore, H9724-treated cells were no less viable, as measured by trypan blue exclusion (data not shown). Treatment with H5332 or 6TS had no effect on SK-N-SH cells' neurite outgrowth or viability (data not shown).

When H9724 was added daily, progressive suppression in neurite development was noted (Table 1) without the escape phenomenon seen in the experiments using only a single treatment with H9724. Daily addition of H5332 and 6TS had no effect on cell growth or neurite outgrowth. 6TS was not used in later experiments; H5332 was used as the isotype and subclass control in all further experiments. The suppressive effect of H9724 on spontaneous neuritogenesis (Table 2) was highly

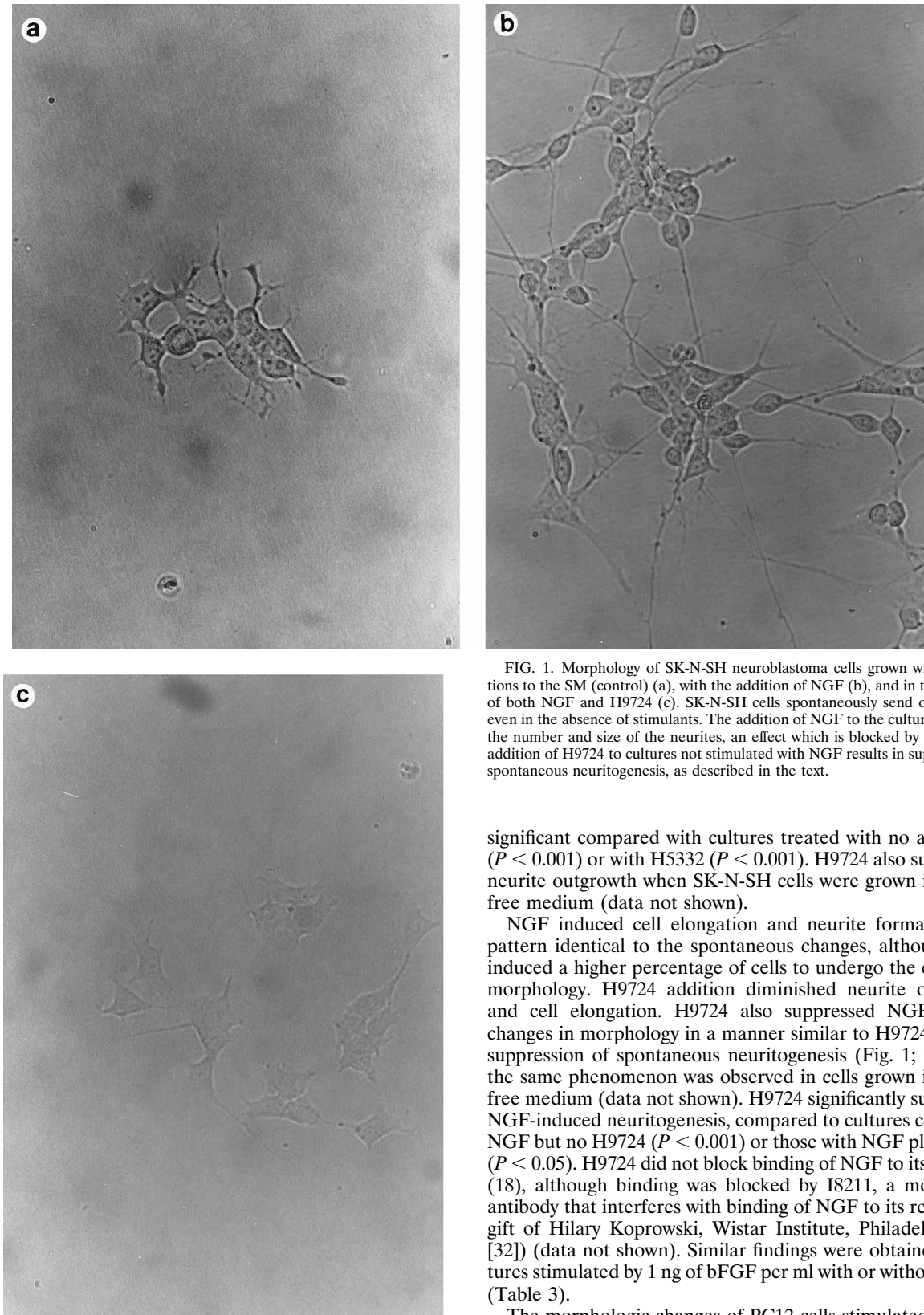


FIG. 1. Morphology of SK-N-SH neuroblastoma cells grown with no additions to the SM (control) (a), with the addition of NGF (b), and in the presence of both NGF and H9724 (c). SK-N-SH cells spontaneously send out neurites, even in the absence of stimulants. The addition of NGF to the cultures increases the number and size of the neurites, an effect which is blocked by H9724. The addition of H9724 to cultures not stimulated with NGF results in suppression of spontaneous neuritogenesis, as described in the text.

significant compared with cultures treated with no antibodies ($P < 0.001$) or with H5332 ($P < 0.001$). H9724 also suppressed neurite outgrowth when SK-N-SH cells were grown in serum-free medium (data not shown).

NGF induced cell elongation and neurite formation in a pattern identical to the spontaneous changes, although NGF induced a higher percentage of cells to undergo the change in morphology. H9724 addition diminished neurite outgrowth and cell elongation. H9724 also suppressed NGF-induced changes in morphology in a manner similar to H9724-induced suppression of spontaneous neuritogenesis (Fig. 1; Table 2); the same phenomenon was observed in cells grown in serum-free medium (data not shown). H9724 significantly suppressed NGF-induced neuritogenesis, compared to cultures containing NGF but no H9724 ($P < 0.001$) or those with NGF plus H5332 ($P < 0.05$). H9724 did not block binding of NGF to its receptor (18), although binding was blocked by I8211, a monoclonal antibody that interferes with binding of NGF to its receptor (a gift of Hilary Koprowski, Wistar Institute, Philadelphia, Pa. [32]) (data not shown). Similar findings were obtained in cultures stimulated by 1 ng of bFGF per ml with or without H9724 (Table 3).

The morphologic changes of PC12 cells stimulated by NGF were similar to those of SK-N-SH and were also suppressed by the addition of H9724 (Table 4). PC12 cells do not elongate or produce neurites in the absence of NGF, so no studies of spontaneous changes can be performed. B103 and Neuro2a

TABLE 2. Neuroblastoma cell growth in presence of reagent after stimulation^a

Antibody added:		% of SK-N-SH cells bearing long neurites after 8 days (mean of sextuplicate) with differentiation stimulus added to cultures:				
H9724	H5332	None	NGF	db-cAMP	PMA	RA
–	–	55.2* (9.4)	85.2* (4.2)	51.5 (12.2)	57.7 (15.6)	98.0 (4.4)
+	–	4.7 (2.8)	59.7 (4.2)	47.3 (11.0)	51.8 (7.1)	94.5 (6.7)
–	+	43.3* (9.4)	86.5** (10.9)	66.2 (9.7)	54.5 (12.9)	98.0 (8.3)

^a Results of a typical experiment in which SK-N-SH neuroblastoma cells, stimulated by NGF, db-cAMP, PMA, or RA, were grown in the presence of H9724 or H5332 or with no monoclonal antibody added to the SM. Results are shown as the percentages of cells with long neurites after 8 days in culture. Numbers in parentheses indicate standard deviations for each mean; no standard deviation was calculated for RA studies, each done only twice, rather than six times, as per the rest of the studies reported. *, difference from H9724-treated cultures was significant at $P < 0.001$; **, difference from H9724-treated cultures was significant at $P < 0.05$.

cells also elongated and produced neurites in culture spontaneously and in response to NGF. Both spontaneous and NGF-induced changes in both cell lines in culture were diminished by H9724 (data not shown).

H9724 does not suppress db-cAMP-, PMA-, or RA-induced changes in SK-N-SH cell morphology. H9724 did not suppress the growth of neurites from SK-N-SH cells stimulated with db-cAMP at the optimal (1 mM) concentration (Table 2). Addition of 0.1 mM db-cAMP (confirmed to be a suboptimal concentration) caused less neuritogenesis, but this too was not blocked by H9724; no changes were seen in cultures supplemented with 1 mM sodium butyrate, the morphology being identical to culture in SM with no additions (Table 5).

Stimulation of cells by RA or PMA at optimal concentrations (10^{-7} and 10^{-8} M, respectively) was not blocked by H9724 (Table 2). The morphology of cells treated with RA and PMA was somewhat different from that of unstimulated cells or cells stimulated with NGF, bFGF, or db-cAMP in that they had a smaller cell body, longer neurites, and multiple neurites often forming net-like complexes (data not shown). Suboptimal concentrations of RA and PMA (Table 6) caused fewer cells to produce neurites (although the morphology of these cells was typically that seen in the cultures stimulated with optimal concentrations of these additives), but this stimulation was also not suppressed by H9724 beyond the nonspecific suppression seen in H5332-treated cultures.

Use of A23187 (26) at concentrations noted in the literature for other cell types (400 nM) led to death of SK-N-SH cells.

TABLE 3. Neuroblastoma cell growth after stimulation with growth factor and with or without H9724^a

H9724 added		% of SK-N-SH cells bearing long neurites after 6 days (mean of sextuplicate) with differentiation stimulus added to cultures:		
		None	NGF	bFGF
–		21.3 (7.6)	40.6 (2.8)	39.0 (7.0)
+		6.0 (6.0)	7.0 (1.4)	5.3 (4.2)

^a Results of a typical experiment in which SK-N-SH neuroblastoma cells, stimulated by bFGF or NGF or not stimulated, were grown in serum-free medium in the presence or absence of H9724. Results are shown as the percentages of cells with long neurites after 6 days in culture. Numbers in parentheses indicate standard deviations for each mean. Differences between H9724-treated and control cultures were significant at $P < 0.001$ for cultures stimulated with either NGF or bFGF. Differences between H9724-treated and control cultures were significant at $P < 0.05$ for cultures not stimulated.

TABLE 4. Pheochromocytoma cell growth^a

NGF	Reagent added:		% of cells bearing long neurites after days (mean of quadruplicate):				
	H9724	H5332	1	3	5	6	8
–	–	–	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
+	–	–	3.3 (1.1)	28.5 (6.7)	44.5 (4.6)	51.3 (3.1)	59.8 (2.7)
–	+	–	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
+	+	–	3.8 (1.1)	34.3 (4.2)	38.5 (8.3)	28.5 (11.7)	41.3 (6.9)
+	–	+	9.3 (2.5)	34.3 (6.1)	52.5 (8.1)	64.3 (9.0)	63.8 (3.7)

^a Results of a typical experiment in which PC12 pheochromocytoma cells were grown in the presence or absence of NGF plus either H9724 or H5332 or no stimulant. Results are shown as the percentages of cells with long neurites after the noted number of days in culture.

Even when used at lower concentrations (40 and 4 nM), A23187 induced sufficient cell death that determination of the H9724 effect was not possible (data not shown). The addition of 30 mM KCl to cultures, another method of increasing intracellular calcium in order to stimulate neural cells (31), did not induce morphologic changes in SK-N-SH cells (in a typical experiment, 54% of untreated cells bore long neurites after 6 days of culture, compared with 57% of cells stimulated with 30 mM KCl).

H9724 does not alter anchoring of SK-N-SH neuroblastoma or PC12 pheochromocytoma cells to plate or matrix, respectively. Pretreatment of cells with H9724 prior to plating did not prevent SK-N-SH cells from anchoring to the plate or PC12 cells from anchoring to the extracellular matrix provided. The cultures started from these pretreated cells elongated and developed neurites in a manner indistinguishable from that of cultures started with untreated cells (data not shown).

Heat stress of SK-N-SH cells abrogates H9724 suppressive effects on neuritogenesis. In a typical experiment (Table 7), cells not subjected to heat shock had spontaneous neuritogenesis blocked by H9724 but not by H5332. Daily heat shock caused a slight increase in the proportion of cells undergoing spontaneous neuritogenesis and a decrease in the H9724 effect.

DISCUSSION

Electrophysiologic studies have shown that the peripheral neuropathy of LD is usually an axonopathy; demyelination is

TABLE 5. Neuroblastoma cell growth in presence of db-cAMP^a

Reagent	% of cells bearing long neurites after days (mean of quadruplicate):				
	1	3	5	6	8
1 mM db-cAMP	20.3 (1.1)	36.3 (2.4)	44.0 (5.1)	52.3 (8.0)	64.5 (9.1)
1 mM db-cAMP plus H9724	48.5 (3.3)	36.3 (4.9)	38.8 (6.1)	50.3 (5.4)	58.5 (6.2)
1 mM db-cAMP plus H5332	42.3 (5.1)	28.5 (8.3)	50.0 (9.9)	74.0 (11.2)	66.0 (4.3)
0.1 mM db-cAMP	16.0 (1.7)	44.8 (4.1)	68.5 (1.9)	44.8 (3.1)	60.0 (5.6)
0.1 mM db-cAMP plus H9724	50.3 (5.1)	40.5 (4.8)	56.5 (1.4)	58.8 (3.1)	52.8 (9.2)
0.1 mM db-cAMP plus H5332	24.0 (4.4)	46.3 (5.2)	64.5 (2.3)	70.3 (6.9)	66.8 (6.2)
No addition	5.3 (3.7)	18.0 (5.1)	22.5 (3.3)	24.5 (4.7)	35.3 (4.9)
1 mM butyric acid	4.0 (2.3)	16.5 (2.5)	24.8 (5.3)	22.3 (3.9)	32.5 (5.5)

^a Results of a typical experiment in which SK-N-SH neuroblastoma cells were grown in the presence of 1 mM or 0.1 mM db-cAMP plus either H9724 or H5332 or with no added monoclonal antibody. Control cultures contained either no addition or the addition of 1 mM sodium butyrate. Results are shown as the percentages of cells with long neurites after the noted number of days in culture.

TABLE 6. Neuroblastoma cell growth with RA and PMA^a

Antibody	RA				PMA			
	Control	100 nM	10 nM	1 nM	Control	10 nM	1 nM	0.1 nM
No antibody added	44.0 (5.7)	76.8 (4.2)	68.0 (2.0)	39.3 (5.0)	42.7 (6.1)	59.3 (6.1)	59.3 (6.1)	41.3 (11.0)
H9724	22.0 (8.7)	55.0 (0.0)	56.0 (5.7)	38.5 (5.0)	22.7 (10.0)	52.7 (5.0)	55.3 (9.2)	43.3 (1.2)
H5332	48.8 (8.1)	58.0 (5.5)	59.3 (4.2)	49.0 (1.0)	42.7 (6.1)	48.7 (4.2)	55.3 (2.0)	42.0 (0.0)

^a Results of typical experiments in which SK-N-SH neuroblastoma cells were grown in the presence of 100 nM (optimal concentration) or 10 or 1 nM (suboptimal concentration) of RA plus either H9724 or H5332 or no added monoclonal antibody or in the presence of 10 nM (optimal concentration), 1 nM, or 0.1 nM PMA plus either H9724 or H5332 or no added monoclonal antibody. Control cultures contained no addition. Results are expressed as the percentages of cells with long neurites after the noted number of days in culture; the standard deviations are noted in parentheses.

very uncommon (19, 22). The inability to find the organism in biopsies of affected nerve tissue may indicate that very few organisms are present but that they are nonetheless capable of producing significant functional disruption; paucity of organisms has been the case at other sites of LD damage, as well (10, 23). Vasculitis in affected nerves has been reported elsewhere and may be part of the neuropathologic process (25). Alternatively, or as a contributing factor, it may be that the organism is no longer, or was never, present at the site of the neuropathy and that immune mechanisms may be the basis for the axonopathy; we have previously suggested that autoimmunity might be an active principle in the pathogenesis of LD (34, 36). Previous studies have demonstrated that patients with LD-associated neuropathy have serum and cerebrospinal fluid antibodies to *B. burgdorferi* flagellin, often binding to the H9724-defined epitope (7); this epitope cross-reacts with human peripheral nerve axon (36). These antibodies bind to a specific axonal target, a protein with an approximate molecular mass of 64 kDa (34), now known to be cpn60, or a very closely related protein (8).

We demonstrated that H9724, a monoclonal antibody to the shared flagellin-cpn60 epitope, modifies in vitro neurite outgrowth in neuroblastoma cells with suppression of spontaneous and NGF- or bFGF-induced cellular changes. In a system not containing serum or active complement components, the H9724 effect was not associated with evidence of cellular damage or death, in that treated cells were capable of excluding trypan blue and of normal growth and neuritogenesis if H9724 was removed after the initial 24 h of culture.

Gajdusek suggested previously that antibodies to axon might induce axonal dysfunction without causing histologically apparent damage (15). Thus the immune response to *B. burgdorferi* flagellin, which induces antibodies capable of binding to a human axonal protein, might cause axonal dysfunction, perhaps without obvious histologic damage, based on the molecular mimicry between the H9724-defined flagellar epitope and the axonal protein.

It is known that the function of other heat shock-stress proteins is to choreograph protein folding and aid in oligomerization of protein subunits (11). The fact that the effect of H9724 is overcome by applying heat stress to the cells suggests that, in fact, H9724 is binding to and blocking the function of cpn60 or a closely related heat shock protein. H9724 blocks spontaneous, NGF-, and bFGF-stimulated morphologic changes but has no effect on the actions of the other stimulants studied, even when they are used at suboptimal concentrations. This suggests that the H9724 blocking effect is not localized to the emerging neurite but must be proximal to the construction of that structure during in vitro neurite outgrowth. Spontaneous and peptide growth factor-mediated neuritogenesis is diminished by H9724, but the other stimulants were not blocked by H9724. We assume that the emergence of the neurite ultimately represents a final pathway common to all stimulants. Our data are

compatible with the premise that H9724 has its effect at a site proximal to effects mediated by cAMP and protein kinase (activated directly by phorbol esters) or that the effect of H9724 is on a parallel, more physiological pathway. Heat shock also appears to increase spontaneous neuritogenesis, an observation suggesting that cpn60, or a related protein, may play a role in neuritogenesis.

cpn60 is known to be an intracellular protein, although in some circumstances it, or a homolog, can be expressed on the surface of cells (14). Based on other studies, including surface radioiodination-autoradiography, we have concluded that the target of H9724 in unstimulated SK-N-SH cells is intracellular (data not shown). Certainly, it would be difficult to explain interference with neurite formation on the basis of surface binding of H9724, but that remains a possibility. Our results are compatible with the premise that H9724 is capable of entering the live cultured cells being studied without permanently damaging the cells. Passage of antibody into living neuronal cells has been documented previously (12, 13, 21) and in the case of SK-N-SH cells might be mediated by surface Fcγ receptors (16). Since H5332 had no effect on neuritogenesis, the effects of H9724 are antigen specific and do not represent nonspecific effects of murine IgG on SK-N-SH cells.

The mechanism of spontaneous neuritogenesis of neural cells in vitro is unknown. It is possible that neural cells produced autocrine or paracrine stimulants. It is known that bFGF is produced by certain neural cells (28) and neural organs (2) and that bFGF can induce PC12 pheochromocytoma cells to differentiate in vitro (27). Nakafuku et al. (27) suggest "...the presence of signaling pathways which are unique to NGF and FGF and are not shared with (other) growth factors." Our findings are compatible with the premise that H9724 interferes with in vitro neuritogenesis at the level of transduction of information from peptide hormone receptors on the cell membrane to the nucleus, with cpn60 a part of a common pathway for the effects of both NGF and bFGF.

One potential means by which H9724 might interfere with

TABLE 7. Neuroblastoma cell growth after heat shock^a

Duration of daily heat shock (min)	% of cells bearing long neurites after 6 days (mean of triplicate) with monoclonal antibody:		
	None	H9724	H5332
None	47.3 (5.5)	18.0 (3.7)	36.7 (6.4)
10	57.0 (4.7)	47.3 (3.2)	51.3 (2.9)
20	59.7 (5.8)	46.3 (5.3)	45.3 (3.9)
30	62.3 (4.8)	58.7 (6.0)	54.3 (3.1)

^a Results of a typical experiment in which SK-N-SH neuroblastoma cells were cultured with daily heat shock at 41°C for 0, 10, 20, or 30 min plus either H9724 or H5332 or no antibody. Results are shown as the percentages of cells with long neurites after 6 days in culture. Similar results were noted after 4 days in culture. The standard deviations are noted in parentheses.

neuritogenesis was by blocking the anchoring or binding of the extending neurite to the plate or extracellular matrix provided. Such a mechanism would not explain how neuritogenesis stimulated by the nonpeptide growth factors was resistant to H9724, however. The possibility that H9724 might act by such was initially raised by the fact that the laminin receptor is of the same approximate molecular mass as the axonal protein bound by H9724 (9, 34). Subsequent work established that the cross-reacting protein was cpn60, an intracellular protein unrelated to the laminin receptor (8).

One possible interpretation of our findings is that H9724 might bind to the NGF receptor in a manner which does not block the binding of NGF but does block transmission of the resultant signal to its intracellular target. However, H9724 has only a single identified target in these cells: cpn60. Its N-terminal sequence is not related to the NGF receptor or to other members of the NGF receptor family (37). Direct interference with the NGF receptor would not explain H9724's ability to block spontaneous neuritogenesis (unless NGF is an autocrine stimulant). Furthermore, there is no evidence that bFGF binds to the NGF receptor. More likely explanations would be that cpn60 may be involved in signal transduction and that H9724 binding interferes with the signal initiated by NGF- and bFGF-receptor interactions or that H9724 interferes with a process related, but immediately distal, to the receptor mechanism. Attempts at determining if calcium influx-induced changes (a step distal to NGF binding to its receptor) could be blocked by H9724 were unsuccessful; we would predict that calcium influx-induced changes should be relatively resistant to H9724 blockade, just as cAMP-induced effects were not hindered by H9724.

We have previously suggested that antibodies reactive with the shared flagellin-cpn60 epitope may play a role in the immunopathogenesis of LD-associated neuropathy (33, 34, 36). The studies reported here provide a possible clue to the mechanism underlying such a phenomenon. Our data are compatible with the premise that H9724 binds to, and may enter, living cells in vitro and binds to a protein, cpn60, which is involved in *in vitro* neuritogenesis, in a manner not elucidated by our studies. The axonopathy of LD could be the result of antibodies made by the host to flagellin which then bind to cpn60 in the axon *in vivo*. We speculate that this immune reactivity might be involved in modification of axonal maintenance or function *in vivo*, as hypothesized by Gajdusek (15). The function and location of cpn60 and how it is related to the transduction of peptide hormone signal and involved in neuritogenesis and perhaps other changes of these cells are topics currently under investigation.

ACKNOWLEDGMENTS

Thanks to Ira Black for reviewing the manuscript and providing us with many helpful comments, to Emanuel DiCicco-Bloom for helpful discussions, to Robin Radziewicz for her assistance in the laboratory, and to John M. Dwyer for originally showing one of us (L.H.S.) the excitement of studying potential immunologic mechanisms of inflammatory disease.

This work was supported by grants from the Foundation of UMDNJ, the New Jersey Chapter of the Arthritis Foundation, and the Morris L. Sigal Memorial Medical Research Foundation.

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Editor: R. E. McCallum