We report here that, in the absence of light, the cells only form irregular aggregates and rarely develop fruiting bodies. However, if aggregates form in the light, fruits develop even if the cultures are placed in the dark.

The myxobacteria were grown vegetatively in shaking liquid cultures containing 1 percent Bactocasitone (Difco) and 8 m*M* $MgSO_4$ at 30°C. Under these conditions the cells grew exponentially until the cell numbers reached 4×10^8 per milliliter. Cultures harvested when the cell concentration exceeded 1×10^8 per milliliter displayed the greatest dependency on light for development. When the cell number reached 1.2×10^8 to 1.4×10^8 per milliliter, the cells were harvested by centrifugation and washed once with Bonner's salts solution (*3*) containing 0.5 g of $CaCl_2 \cdot 2H_2O$ per liter. Washed cells (2×10^8), suspended in 5 μl of Bonner's salts, were placed on Bonner's salts agar plates prepared 2 days before use, and incubated at 30°C at an intensity of 215 lux of incandescent light. The cells aggregated in 10 hours, and formed stalks in 12 to 14 hours and sporangia after 17 hours. Approximately 200 fruiting bodies developed from each 5-μl portion of resuspended cells. During the entire process, there was no detectable growth or increase in the number of cells, respectively (*2*). Consequently, we conclude that morphogenesis is due entirely to the movement of the cells. The morphology of developmental stages of the parent strain CCf has been described (*4*).

A different developmental pattern was observed when the cultures were incubated in the dark. The cells aggregated in 10 hours, forming elongated, interconnected ridges (Fig. 2). The number of sporangia that developed in the dark varied from 0 to 10 percent of control samples incubated in the light. However, the number of sporangia that developed in the dark was often 30 to 40 percent of the number developing in the light when the cells were harvested from cultures whose cell density was between 0.8×10^8 and 1.1×10^8 per milliliter. The few sporangia that did develop in the dark were not borne on stalks but rested directly on the ridges. Figure 3 is a photograph of aggregates that formed in the light and which developed into fruiting bodies. Cells fruit normally if allowed to aggregate in the light and are then transferred to the dark. This suggests that the light may have induced a relatively stable change in the phenotype of the cells.

Photoinducible morphogenesis has been reported in a number of eukaryotic microbial systems, including both acellular and cellular slime molds, and fungi (*5*). Except for a brief statement by Coucke that light stimulated fruiting body formation in the myxobacterium *Sorangium compositum* (*6*), we were not aware of any other publications of photomorphogenetic effects in nonphotosynthetic bacteria. However, it is known that myxobacteria do respond to light in other ways. Aschner and Chorin-Kirsh reported that certain species of myxobacteria are negatively phototactic (*7*), and Burchard and Dworkin reported light-induced lysis and carotenogenesis in the myxobacterium *Myxococcus xanthus* (*8*). The action spectrum for carotenogenesis in *M. xanthus* suggests that a heme compound may be the photoreceptor (*9*). We have found that red light effectively promotes fruiting body formation in *Stigmatella*. We anticipate that the use of light to experimentally alter the course of development will be a valuable aid in analyzing the molecular bases underlying morphogenetic movements and may also lead to the discovery of new pigments involved in morphogenesis in prokaryotes.

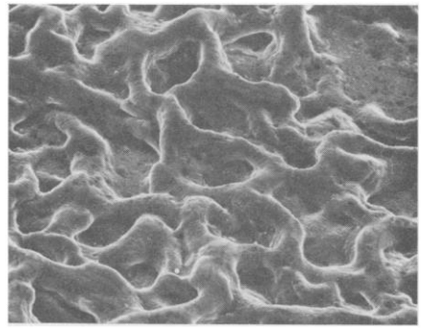

Fig. 2. Development in the dark. Scanning electron micrographs of ridges formed in the dark. Cells were incubated in the dark at 30°C for 24 hours. After 12 hours the ridges had formed. The bar represents 20 μm.

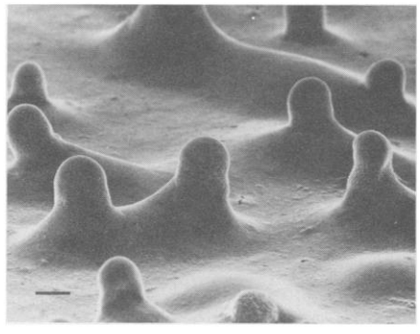

Fig. 3. Development in the light. Scanning electron micrographs of fruiting swarms that formed in the presence of light. Plates were incubated in 215 lux of incandescent light. Temperature was held constant at 30°C. The photograph represents samples 12 hours after the initiation of development. The bar represents 20 μm.

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Suppression of Chronic Allergic Encephalomyelitis: Relevance to Multiple Sclerosis

Abstract. *The expression of chronic relapsing experimental allergic encephalomyelitis in strain 13 guinea pigs was suppressed with a single series of injections of myelin basic protein in incomplete Freund's adjuvant. The suppression appeared permanent, and subsequent rechallenge with central nervous sytem antigen failed to elicit exacerbations.*

Chronic relapsing experimental allergic encephalomyelitis (EAE) is a model for demyelinating disease (*1*) that simulates in many ways the clinical and pathologic courses of the human demyelinating disease, multiple sclerosis (MS) (*2*). The application of acute EAE as a direct model of MS is limited due to its short-term monophasic pattern, fatal outcome, and microscopic lesions. These are in contrast with the chronic relapsing course and grossly visible central nervous system (CNS) lesions of MS (*2*). Injections of myelin basic protein (MBP),

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the encephalitogenic component of CNS myelin (*3*), suppress the acute manifestations of EAE (*4, 5*). A similar approach was attempted in MS, but the results were inconclusive (*6*). The similarities between chronic relapsing EAE and MS led us to examine the effectiveness of MBP suppression in this model system, the permanence of such suppression, and whether suppressed animals were resistant to rechallenge with CNS tissue by clinical, morphologic, and immunologic evaluations (*7*) of chronic EAE animals up to 27 months after inoculation with CNS tissue. Our findings suggest that in this model, MBP suppression is highly effective and permanent and, in the majority of cases, rechallenge of long-term suppressed animals with the original antigen failed to elicit subsequent changes.

Four groups of animals (Table 1) consisting of 57 juvenile (weighing less than 250 g) strain 13 guinea pigs were sensitized in the nuchal area with isologous spinal cord in complete Freund's adjuvant (CFA) (*1*). Light and electron microscopy were performed on brain and spinal cord tissue embedded in epoxy resin. We also studied lymphocytes for estimations of circulating B cells, early (high-affinity-rosetting) T cells, and late (total, 24-hour-rosetting) T cells (*8*).

Unsuppressed chronic EAE was studied in 27 animals for up to 27 months after injection without any further treatment after their sensitization as juveniles (Table 1). From this group, morphologic studies were performed on 12 animals, of which four were used for morphologic and lymphocyte evaluation. The remaining 15 animals were studied for clinical evaluation and lymphocyte fluctuations only. In a second group (unsuppressed, chronic EAE, rechallenged), four guinea pigs were sensitized in the same manner as the first group, maintained for up to 12 months, and then rechallenged in the nuchal area with 0.5 ml of an emulsion of CNS tissue in CFA. All four were examined for clinical and morphologic changes and lymphocyte fluctuations. The suppressed, chronic EAE group was composed of 20 strain 13 guinea pigs injected as juveniles for chronic EAE. From 1 to 5 weeks after inoculation, these animals were given a series of ten intramuscular injections of bovine MBP in incomplete Freund's adjuvant (IFA) (*4*) at 3-day intervals. Each animal received a total of 1.4 mg of MBP. The group was also followed for up to 27 months after the initial inoculation. Morphologic study was performed on 15, morphology and lymphocyte values were examined in seven, and clinical and lymphocyte evaluation only were studied in the remaining five. Suppressed, chronic, rechallenged EAE was studied in six guinea pigs, sensitized and suppressed as in the latter group. At 12 months after the primary injection, two were rechallenged with CNS tissue in CFA. The remaining four were similarly rechallenged 26 months after injection. Morphologic and lymphocyte estimations were carried out on all six animals.

The clinical manifestations of all four groups were compared (Table 1). In the first group, all animals demonstrated late-onset EAE (at 8 to 12 weeks after injection) as evidenced by mouth-wetting, weight loss, incontinence, and paraparesis. The appearance of these signs was followed by some recovery and one or more relapses, characterized by worsening of the neurological signs. Morphologically, the CNS of these animals revealed evidence of recent (acute), ongoing, and chronic disease activity. Acute disease was evidenced by large infiltrates of inflammatory cells around blood vessels (perivascular cuffing) associated

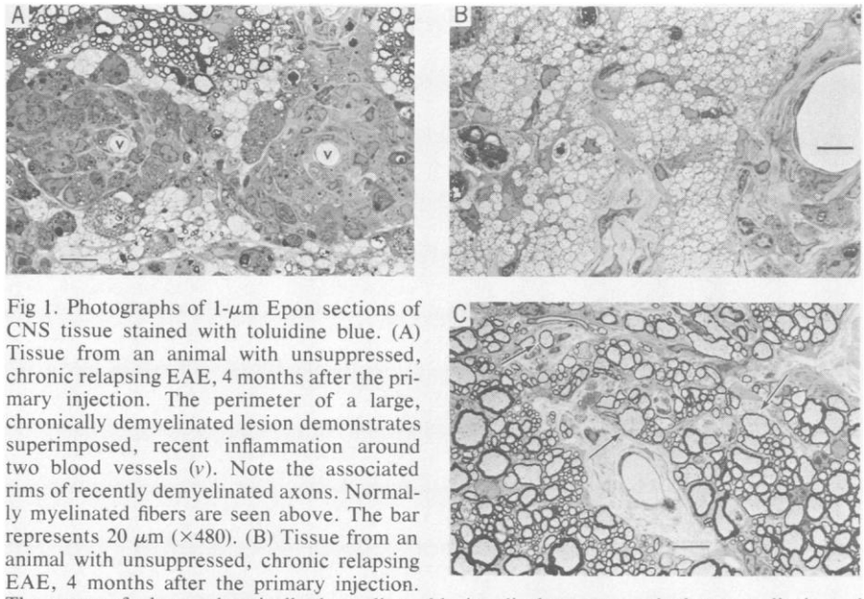

Fig 1. Photographs of 1-μm Epon sections of CNS tissue stained with toluidine blue. (A) Tissue from an animal with unsuppressed, chronic relapsing EAE, 4 months after the primary injection. The perimeter of a large, chronically demyelinated lesion demonstrates superimposed, recent inflammation around two blood vessels (*v*). Note the associated rims of recently demyelinated axons. Normally myelinated fibers are seen above. The bar represents 20 μm (×480). (B) Tissue from an animal with unsuppressed, chronic relapsing EAE, 4 months after the primary injection. The center of a large, chronically demyelinated lesion displays many naked axons, gliosis, and fibrosis of a blood vessel (right). The bar represents 15 μm (×760). (C) Tissue from an animal with suppressed, chronic EAE, 25 months after the primary injection. A small area from an old lesion located in the subpial region of a dorsal column. Note the uniform remyelination of all previously affected fibers (arrows) and the residual fibrosis around a blood vessel (center). The bar represents 15 μm (×760).

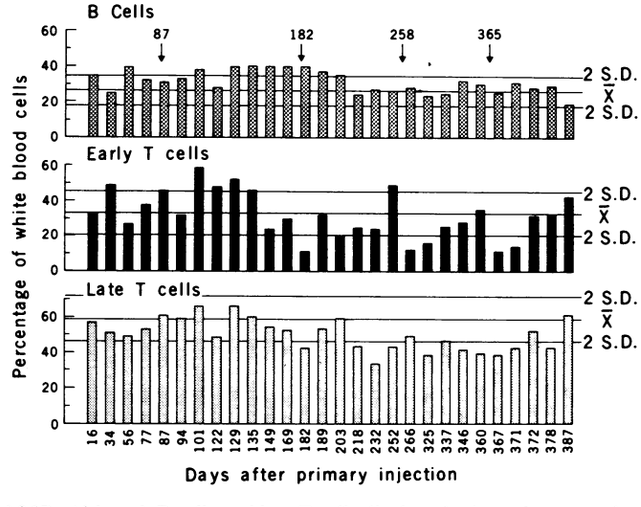

Fig 2. Histograms of circulating lymphocyte levels from one representative unsuppressed, chronic EAE strain 13 guinea pig, based upon 28 separate bleedings. The actual values are compared with the mean ($\bar{X}$) ± two standard deviations (*S.D.*) obtained from 11 uninoculated, normal strain 13 guinea pigs (*8*). This animal had four clinical exacerbations, noted by the arrows. A delayed onset of disease occurred on day 87, followed by three relapses (days 182, 258, and 365). Although B cells and late T cells displayed minor changes only, early T cells demonstrated significant decreases ($P < .01$) coinciding with relapses. During remissions, early T cells return to normal levels.

with rims of tightly packed, naked axons (Fig. 1A). Ongoing disease was represented by an abundance of myelin-laden macrophages and other cells that were engaged in the removal of myelin from axons. Long-term changes consisted of fibrosis of the leptomeninges and the areas around parenchymal blood vessels, in addition to intense gliosis and masses of naked axons (Fig. 1B). At the periphery of plaques, some of which were grossly visible, scattered axons with thinner than normal myelin sheaths were seen, appearances characteristic of remyelination (*4*). The second group, unsuppressed, chronic rechallenged EAE animals, demonstrated a clinical course similar to the first group (Table 1). However, 2 to 4 weeks after the second sensitization, they developed signs of acute disease. Upon morphologic examination, areas of acute disease of the CNS could be seen, superimposed on chronically demyelinated lesions.

For up to 27 months after injection, suppressed, chronic EAE animals (third group) failed to develop clinical signs. Large CNS lesions were not seen. Short-term suppressed animals showed some chronic meningeal inflammation and CNS remyelination, features indicative of previous subclinical disease. These changes probably corresponded to those known to occur during the latent period of chronic EAE (*1*). Animals examined more than 2 years after their primary injection still displayed evidence of a single episode of previous disease, manifested by uniformly remyelinated axons (Fig. 1C). Fibrotic and gliotic changes were present but not prominent. Suppressed, chronic, rechallenged EAE animals (fourth group) displayed lesions identical to the third group with no evidence of recent changes, but with some residual evidence of disease in the form of narrow zones of subpial remyelination.

Lymphocyte population study of 14 unsuppressed, chronic EAE animals demonstrated significant decreases in early T cell values ($P < .01$) that coincided with clinical relapses (Fig. 2). Unsuppressed, rechallenged EAE animals demonstrated similar patterns of early T cell populations up to the second inoculation with CNS in CFA, 2 weeks after which they displayed an acute episode of disease that was accompanied by a marked decrease in the level of early T cells. In the third group (suppressed, chronic EAE animals), the number of early T cells underwent a transient increase between 4 and 6 weeks after injection. Later, the number of early T cells remained within the normal range or was slightly elevated. In the suppressed, rechallenged group, the amount of early T cells was similar to the latter group, but showed a mild decrease after rechallenge. The B cells and late T cells in all four groups showed minor fluctuations only.

Table 1. Clinical course and outcome of chronic EAE. All times given are from the date of the primary injection with spinal cord, except as noted. *N* is the number of animals.

Group	*N*	Time of suppressive injection (weeks)	Onset of signs (weeks)	Time rechallenged (months)	Time killed (months)
Unsuppressed	27		8 to 12*		≤ 27
Unsuppressed, rechallenged	4		8 to 12*	12	1 after rechallenge
Suppressed	20	1 to 5	†		≤ 27
Suppresed, rechallenged	6	1 to 5	†	12 or 26	1 after rechallenge

*Animals in these groups continued to show signs throughout the duration of the experiment. †Animals in these groups showed no symptoms throughout the duration of the experiments.

These results indicate that chronic relapsing EAE can be successfully suppressed with injections of MBP in IFA. Furthermore, this suppression is of long duration; it appears to be permanent since rechallenge with antigen 1 to 2 years later failed to cause an exacerbation. Not only was the disease suppressed at the clinical and morphological level, but the observed lymphocyte fluctuations complemented the clinical and morphological data and also supported the notion of suppression. Similar lymphocyte fluctuations have been reported during unsuppressed and suppressed acute EAE in adult strain 13 guinea pigs (*8*). It has been suggested that the elevation of early T cells during long-term suppressed acute EAE is indicative of a population of circulating, MBP-generated suppressor cells (*8, 9*). The role of memory cells in the different responses between unsuppressed and suppressed animals remains unknown.

The observed suppression of chronic EAE might be a stress-related phenomenon caused nonspecifically by multiple injections. However, in our experiments, all groups were repeatedly bled by cardiac puncture under anesthesia to obtain lymphocytes and this procedure did not affect the disease course. Also, one animal sensitized for chronic EAE received sham injections of saline, and this did not suppress the disease.

Treatment of MS subjects with MBP has been considered for many years but, to date, such an approach has been inconclusive (*6*). This concept in MS stemmed largely from the successful suppression of acute EAE (*3*). We have shown that chronic relapsing EAE can also be suppressed with MBP. Furthermore, it was found that although some subclinical lesions did develop in suppressed animals, they became completely remyelinated. Chronic EAE is a disease vastly different from acute EAE (*1*). Like MS, chronic EAE has a protracted course with exacerbations, is age-dependent, and shows genetic predisposition.

Although MBP trials on humans may not utilize IFA in favor of other depot-forming reagents such as liposomes, the successful suppression of this relapsing animal disease model and the apparent permanence of this suppression are encouraging for future MBP trials on MS subjects.

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Mutant Strains of *Rhizobium japonicum* with Increased Ability to Fix Nitrogen for Soybean

Abstract. *A strain of* Rhizobium japonicum *used in commercial inoculants was mutagenized and screened by a rapid effectiveness assay with soybean plants. Two mutant strains nodulated the roots earlier than the wild type and also expressed greater symbiotic nitrogen-fixing activity than the wild type in the presence and absence of fixed nitrogen. In addition, one of the mutants formed more root nodules than the wild type. Plants inoculated with these strains had increased dry weights (~60 percent) and nitrogen content (~100 percent) when grown in growth chambers.*

Mutagenesis is used as a method for obtaining strains of bacteria that synthesize increased levels of microbially produced metabolites. We wished to determine whether mutagenesis of the bacterium *Rhizobium japonicum* would produce strains capable of improving the N_2-fixing root nodule symbiosis between this organism and soybean [*Glycine max* (L.) Merr.]. Current efforts for obtaining more effective *Rhizobium* strains rely on isolating strains from the soil or from nodules of vigorous plants (*1*).

A culture of *R. japonicum* 61A76 (obtained from J. Burton, Nitragin Co., Milwaukee) was selected for this work because it has been used in a commercial inoculum for over a decade. The bacteria were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and subcultured for many generations in a minimal medium (*2*). The resulting culture was diluted and plated on agar medium. Each colony was tested for symbiotic acetylene reduction by a rapid screening effectiveness assay with 14-day-old vial-grown soybean plants (*3*). Acetylene reduction is an index of N_2 fixation (*4*).

Twenty-five hundred colonies were tested, and two isolates (SM31 and SM35) had significantly greater symbiotic acetylene-reducing activities on 14-day-old plants (cultivar Hodgson) as measured by the effectiveness assay (Table 1). In addition, SM35 formed more nodules than the wild type on soybean roots. The higher activities of SM31 and SM35 (and the greater number of nodules by SM35) also were observed on three other soybean cultivars tested (Corsoy, Chippewa, and Dunn).

Different colony types obtained from a single strain of *Rhizobium* may have different symbiotic properties (*5*). However, SM31 and SM35 had the same colony appearance as the wild type even when dilutions were made in Tween 40 (*5*). Strains SM31 and SM35 had the same growth rate as the wild type in both rich and minimal media and both were lysed by phage specific for the wild type (*2*). The higher activities caused by these strains were not due to resistance to the commercial bleach used for seed sterilization. The plants continued to show higher activities than the wild type when the seeds were sterilized with mercuric chloride (*6*). Three colonies from a diluted culture of the wild type and three colonies from the mutagenized culture were less active than SM 31 and SM35 when tested by the effectiveness assay.

Bacteria were isolated from nodules taken from plants inoculated with either the wild type or one of the two mutant strains. In all cases, the bacteria from the nodules had the same symbiotic properties (tested by the effectiveness assay) as the original strains inoculated on the seeds.

In order to learn more about the nature of this increased acetylene-reducing activity, nodules produced by SM31 and SM35 were picked from the roots and weighed. Total nodule weight per plant was greater with these strains than with the wild type. This wild-type strain of *R. japonicum* is capable of reducing acetylene asymbiotically (*7*) so that it was possible to test for acetylene reduction without the host plant. Using conditions previously described (*2*), we were unable to demonstrate that the two mutants had more asymbiotic activity than the wild type.

Table 1. Comparisons between Hodgson soybeans inoculated with the wild-type and mutant strains. Plants (7, 8, and 14 days old) were grown in plastic bag–covered vials used in the effectiveness assay (*3*). Values are based on a total of 20 plants. The 21-day-old plants had root systems in cellophane pouches (Seed-Pack Growth Pouch; Scientific Products). The remainder of the plant was open to air. Values are the means of four pouches, each containing three plants. The 7-, 8-, 14-, and 21-day-old plants were grown in a plant-growth chamber with an 18-hour photoperiod. Warm and cool fluorescent bulbs and 100-W incandescent bulbs supplied 300 μE m^{-2} sec^{-1}. The plants grown for 38 days were in 7-inch clay pots containing a sterile mixture (equal parts) of vermiculite and perlite with two plants to a pot. These plants were grown in a Biotron growth chamber with a relative humidity of 70 percent, average light intensity of 600 μE m^{-2} sec^{-1} at the top of the pot, and a photoperiod of 16 hours. The day and night temperatures were 28° and 24°C, respectively. The position of the pots in the chamber was changed daily. Each seed was inoculated with approximately 1×10^9 cells at the time of planting. The medium for growth of the plants in the vials was RBN, brought to *p*H 7.0 with NaOH (*3*). This medium, without sucrose, was used in the pouches. The pots were watered with distilled water and an N-free solution described by Evans *et al.* (*14*). The values are means of at least 15 plants.

Measurement	Age of plants (days)	Strain		
		Wild type	SM31	SM35
Active/nodulated plants (No.)	7	0/0	0/9	0/5
	8	0/13	6/18	4/20
C_2H_2 reduced per plant (nmole/hour)	14			
No N		309	530*	623*
10 m*M* KNO_3		44	96*	90*
1m*M* NH_4^+ acetate		66	125*	123*
Mean number of nodules per plant	14			
No N		9.2	12.0	15.6*
1 m*M* NH_4^+ acetate		7.2	9.0	11.7*
Nitrogen increase	21			
Per plant (milligrams)		4.0	4.8	6.3*
Per pouch (micrograms)†		46	59	189‡
Dry weight of plants (grams)	38	1.88	3.26*	2.69‡
Nitrogen per milligram, dry weight (micrograms)	38	22.3	27.5*	28.6*

*With *t*-test analysis values exceed wild type value at 99 percent level of confidence. †Liquid remaining in pouch after the plants were removed. ‡With *t*-test analysis values exceed wild type values at 95 percent level of confidence.

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