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A Cardiomyopathy of BALB/c Mice (Superimposed Infection by Coxsackievirus B-3)[1, 2] (40073)

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During studies of murine virus myocardiopathy (1), we inoculated the Nancy strain of coxsackievirus B-3 into BALB/c mice. Unexpectedly, we observed pericarditis in normal controls. On the other hand, we had not noted these changes in virus-free Swiss mice from several sources (Albino ICR, Webster, Hauschka and Marand) or among C3H mice. We could not find an earlier description of pathologic changes in the heart in BALB/c mice (2).[2]

Here, we explore certain pathologic characteristics of BALB/c (C) cardiomyopathy. We also inoculated C mice with coxsackievirus B-3 and contrast the resultant cardiomyopathy with that described earlier in Albino Swiss ICR and C3H mice.

Materials and methods. Mice. BALB/c mice were derived by Bogg from an albino stock in 1913. The strain has been maintained continuously by brother x sister matings. C substrains from laboratories of the Michigan Cancer Foundation (C/MCF); Charles River Laboratory (C/CRL); Jackson Laboratory (C/JL); and Cumberland Laboratory (C/CL) were also used. Additionally, in several experiments noninbred Albino Swiss ICR and inbred C3H mice were employed. Rodents were fed Purina Mouse Chow and given water at will. Each strain or substrain in groups of six to ten animals was kept separately in cages. Infected mice were isolated from uninoculated controls. All mice were examined at least every third day for signs of illness.

Attempts to isolate virus from the hearts of BALB/c mice. An innate vertically transmitted virus of C mice is a possible cause for this inherent pericarditis. Primary mouse embryo fibroblast (MEF) tissue cultures were prepared from decapitated and eviscerated 14- to 18-day old Swiss Albino ICR mouse embryos. Carcasses were minced, trypsinized and grown in 30 ml plastic flasks at a concentration of 25×10^4/ml in Eagle's medium (EM) with 5% fetal calf serum containing 100 μg/ml of penicillin G and 50 μg of streptomycin/ml. Maintenance medium for MEF tissue cultures was EM with 2% fetal calf serum.

At several times from late term to 6 months of age, hearts of C/CRL mice were prepared for inoculation into MEF tissue cultures as 20% cell-free supernates in EM prepared with mortar and pestle; as minced tissue explants; and as single cell trypsinized suspensions (Kasten Method [3]). In each case, 0.1–0.5 ml was inoculated into two MEF 30 ml tissue culture flasks. After washing 3× in calcium and magnesium free Hanks salt solution, minces and whole cells were inoculated as 1:10 suspensions in EM. Experimental cultures and uninoculated controls were incubated (37°), and observed daily for cytopathic effects. Between the 7th and 10th days of incubation both groups of cultures showed rounding, granularity and loss of cells into supernatant fluids. Cells were then scraped from flasks, suspensions centrifuged (300 g, 4°, 5 min) and debris resuspended in EM (1:10). Again, 0.1 ml of these suspensions were inoculated into two new MEF flasks. Two similar blind passages were carried out with each specimen.

Experimental infections. Pregnant mothers were obtained from their breeding laboratories, observed and periodically sacrificed. In experimental infections, 14-day-old mice were inoculated intraperitoneally with 0.05 ml of coxsackievirus B-3 containing $10^{3.5}TCD_{50}$.

Methods for sacrificing; bleeding; autopsy; preparation of specimens for histologic examination and isolation, titration and identification of coxsackievirus B-3 *in vero* or rhe-

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[2] This work was presented in part at the 68th Annual Meeting of The American Society for Microbiology, Detroit, Michigan, May 5–10, 1968.

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sus tissue cultures have been described (1, 4). For virus isolations with infected BALB/c mice, we separated the right and left ventricles by blunt dissection. To remove adhering blood, each ventricle was washed 5x by rinsing in approximately 10 vol of sterile saline and blotted dry. The final wash was titered for residual virus, and the quantity of virus in the myocardium was taken as the difference between that in the specimen and that of the final wash. At midregions of right and left ventricle of each heart three sections were examined for histologic changes.

Results. Pericarditis. Uninoculated mice remained well. However, during routine histologic studies of coxsackievirus B-3 myocarditis, several uninoculated control C/MCF mice had mononuclear infiltrates in the right ventricular pericardium along with minimal subepicardial myocardial necrosis in subjacent areas. These changes were exclusively limited to the right heart. In some of the mice there was only pericarditis, while in others, there was also spotty myocardial necrosis immediately beneath the pericardial exudate.

In order to determine if these changes were limited to C mice bred at the Michigan Cancer Foundation, we obtained BALB/c mice from three other colonies, namely, substrains C/CRL; C/JL/ and C/CL. Subsequently, we observed and periodically sacrificed 55 C/MCF mice (from birth through 1-year of age); 16 C/CRL and 14 C/JL mice (up to 90 days old); 26 C/CL (just before delivery); and 107 C/CL mice (birth to 107 days old). Mice from three of the sources showed an identical acute right ventricular pericarditis. Similar histologic changes occurred in 42% (C/MCF), 50% (C/CRL and 43% (C/JL) mice. On the other hand, we found that hearts of BALB/c mice up to 45 days old which were bred at the Cumberland Laboratory were normal (Table I, A).

Thereafter, we followed C/MCF mice consecutively over two years, an interval approaching their normal life expectancy. When these rodents were 17, 28, 56, 110 and 180 days old 1 of 4; 0 of 5; 5 of 5; 3 of 5; and 11 of 26 mice had characteristic right ventricular pericarditis (Table II, A). At 10–12 months of age, 3 of 10 of these mice had pericarditis. No mouse older than a year showed these changes. Thus, in the C/MCF substrain, inherent pericarditis usually appeared from the second through the sixth month of life, its incidence declining thereafter, and disappeared by the time these mice were a year old. We did not find acute mononuclear pericarditis in the C/CL substrain, but it is possible that pericardial changes may have been seen if we had extended our observations beyond 1½ months.

Chronic calcific fibrotic pericardial changes.

TABLE I. PERICARDITIS AMONG SUBSTRAINS OF BALB/c MICE.

BALB/c substrain	Age of mice[a] (Mos.)	No. of mice with pericarditis ÷ No. of mice examined
A. Mononuclear pericarditis		
C/MCF	0–12	23/55 (42%)
C/CRL	1½	8/16 (50%)
C/JL	1½	6/14 (43%)
C/CL	fetal	0/26 (0%)
	0–1½	0/107 (0%)
B. Chronic fibrotic calcific pericardial changes		
C/MCF	6–24	8/42 (19%)
C/CRL	1½	0/16 (0%)
C/JL	1½	0/14 (0%)
C/CL[b]	8	6/29 (21%)

[a] C/CRL and C/JL mice were studied at 6 weeks of age; C/MCF mice were examined at several times through 24 months (see Table III). C/CL, fetuses were examined just before delivery (26 mice), at 1 week (22 mice), 2 weeks (37 mice), 4 weeks (15 mice), at 6 weeks (7 mice), and at 8 months (29 mice).

[b] Sex was noted only in the C/CL substrain at 8 months. There were 15 males. Three of each sex had right ventricular fibrotic pericardial scars.

TABLE II. AGE AT APPEARANCE OF PERICARDIAL CHANGES IN THE C/MCF SUBSTRAIN OF BALB/c MICE.

	Age of mice (Mos.)	No. of mice with pericarditis ÷ No. of mice examined
A. Mononuclear Pericarditis	(17/30) 0.57	1/4
	(28/30) 0.93	0/5
	(56/30) 1.87	5/5
	(110/30) 3.67	3/5
	6	11/26
	10	2/5
	12	1/5
B. Chronic fibrotic calcific pericardial changes	6	2/21
	12	2/5
	13	4/10
	24	0/6

In continuing observations, we noted at autopsy gross whitish stippling over the right ventricle in a number of the older C/MCF mice. On microscopic review this older lesion was in the exact location as the acute mononuclear pericarditis, and was fibrosis with spotty calcification (von Kossa's stain). This calcifying pericardial scar was not seen in younger mice which were less than 6 months old; occurred less frequently than the acute pericardial lesions (Table II, B); and never accompanied the earlier mononuclear pericarditis.

Similar right ventricular calcific fibrotic pericardial changes were also seen in older animals from the C/CL substrain, and occurred equally in both sexes. We did not see these fibrotic scars in either the C/CRL or C/JL substrains, but our studies with these animals did not extend beyond 45 days (Table I, B).

Therefore, we found that BALB/c mice of each of four substrains showed parts of what appears to be an identical pericardial and subepicardial cardiomyopathy specifically localizing to the right heart. It begins as a fine mononuclear pericarditis, progresses to a denser infiltrate, and completes its evolution with mixed calcification and fibrosis.

Attempts to isolate indigenous viruses from the hearts of BALB/c mice. Hearts from the four brood mothers several days before term and from five C/CRL mice at each of several ages from 2–24 weeks were prepared as cell free supernates, tissue minces, or washed-trypsinized cells. In this experiment 39 mice were sacrificed. Each preparation was inoculated or cocultivated onto two MEF tissue culture flasks and cultures were observed daily for cytopathic changes. Degenerating cultures and uninoculated controls were blindly-passaged twice. No viruses were recovered.

Coxsackievirus B-3 infection in BALB/c mice. When Nancy strain of coxsackievirus B-3 was inoculated intraperitoneally into each of four substrains of C mice available to us, subclinical infection resulted. The kinetics of coxsackievirus in blood and heart was studied in C/MCF mice. There was viremia on day 1, but not on day 3. Virus was present in the heart on days 1 and 4, but was absent in most hearts by the 6th day (Fig. 1). In order to compare the capacity of the right and left ventricles to support coxsackievirus B-3 replication, 9 additional C/CRL mice were inoculated and 4 days later were sacrificed. Mean virus titers in the right and left ventricles are similar (Fig. 2).

On histologic section of hearts from BALB/c weanling mice 14 days after infection with coxsackievirus B-3, there was a focal calcific fibrotic pericardial scar which again was limited to the right ventricle (Fig. 3, A). This pericardial scar in young mice recovering from coxsackievirus B-3 myocarditis could not be distinguished from the inherent chronic lesions of uninoculated older C mice.

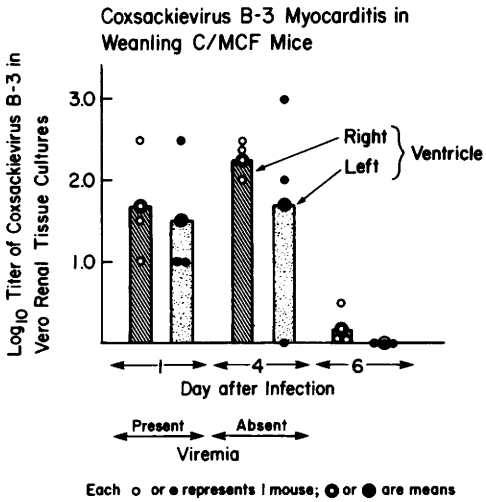

FIGURE 1

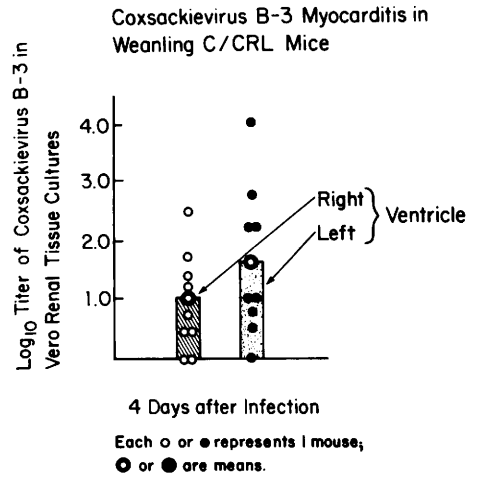

FIGURE 2

Likewise, some infected weanlings had isolated foci, or a more diffuse calcification in both left and right ventricular myocardium (Fig. 4, B).

In order to find the relative frequency of pericardial and myocardial lesions in infected young C mice, we inoculated 43 C/MCF, 19 C/CRL, 5 C/JL and 13 C/CL 14-day-old sucklings with Nancy virus. All mice were sacrificed 2 weeks later. We found right ventricular pericardial scars in each substrain, but it was predominant among C/MCF and C/CRL mice. Myocardial calcification was focal in C/MCF, diffuse in C/CL, and mixed in the others (Table III).

Prevalence of Right Ventricular Pericardial Lesions in Coxsackievirus B-3 Infected C/MCF Mice and in Their Controls. To determine if the right ventricular calcific pericardial scars in coxsackievirus B-3 infected BALB/c mice occur more frequently in infected C mice than do similar calcified or noncalcified lesions in uninoculated controls, 29 C/MCF infected mice and the same number of age-matched uninoculated controls were sacrificed at intervals after birth from 3 to 296 days. (Calcification, of course, does not occur in the uninoculated C mice until they are at least 6 months old.) After sectioning and staining with hematoxylin and eosin, hearts were examined histologically. From 14 days onward each right ventricular pericardial lesion in infected mice contained deposits of calcium, but uninfected controls showed inflammatory exudates without mineralization through the 179th day of this experiment

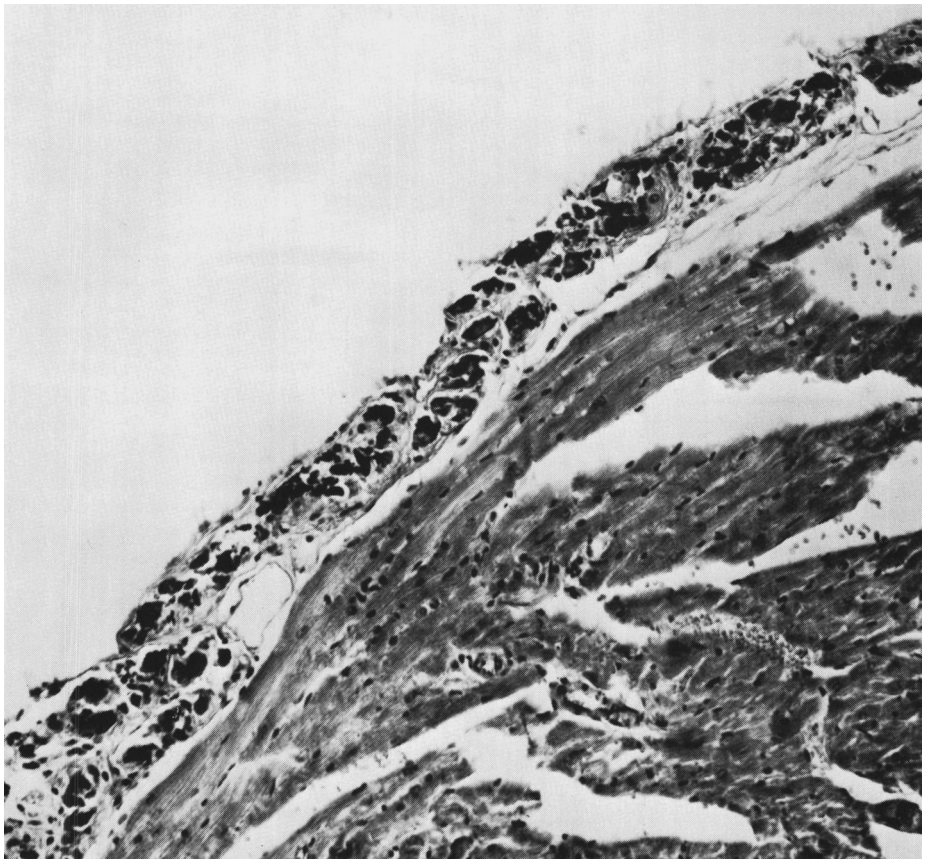

FIGURE 3

FIGS. 3 and 4. Pathologic findings in weanling 4 week-old C/MCF mice 14 days after infection with the Nancy strain of coxsackievirus B-3 are shown. Sections are stained with hematoxylin and eosin (× 415). Fig. 3. A right ventricular calcific fibrosing pericardial scar is seen. Fig. 4. Focal myocardial calcification is shown. The arrow indicates a calcium deposit.

(Table IV). On the 296th day, two of five noninoculated mice also contained inflammatory noncalcified pericardial lesions. In both groups, pericarditis affected only the right heart.

In infected mice the cumulative prevalence of right ventricular calcific pericarditis was 35% (10 of 29 mice). Inflammatory noncalcific pericarditis at the same sites were seen in 48% (14 of 29 mice) in the age-matched controls (Table IV). Since the incidence of pericarditis is not increased in infected C mice, it is possible but not certain that pericarditis in the infected mice occurred only in those animals with a predilection for the later development of the inherent right ventricular cardiomyopathy. When coxsackievirus B-3 infection supervenes, however, it is apparent that the appearance of calcific pericarditis is markedly hastened.

Discussion. BALB/c mice have been used extensively in biological research for over 60 years, but we could find no previous report of the spontaneous continuing right heart cardiomyopathy described here in C mice obtained from four separate breeding colonies (2). The etiology of the BALB/c lesions, which we have not seen in Swiss mice from several sources, or in inbred C3H mice remains unknown. We have been unable to recover a vertically transmitted virus (5).

In about a third of several hundred BALB/c mice which we have examined and some time after the second month of life a mononuclear infiltrate appears in the pericardium of the right ventricle. This may be associated with minimal subjacent myocardial necrosis without leukocytic infiltration. Beginning after 6 months of age, these C mice have only a partially calcified pericardial scar remaining. Early and late lesions are strikingly limited to the pericardium of the right

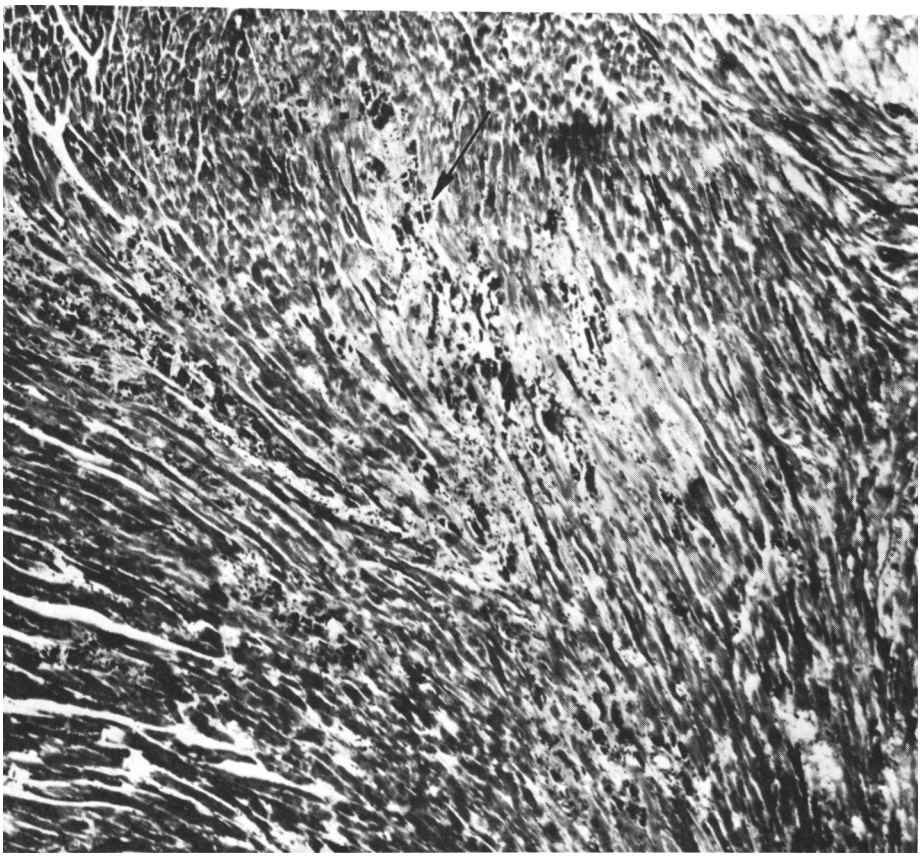

FIGURE 4

ventricle and no calcification is seen before the 180th day of life.

Spontaneous pericarditis and myocarditis with calcification has been reported in DBA inbred (6, 7) and, occasionally, in Swiss mice (8). Myocardial calcification (without pericardial involvement) has been induced in several species of rodents including BALB/c mice by stress, hydrocortisone or low protein diets (9–11). None of these changes resemble those described here.

Coxsackievirus B-3 myocarditis in Swiss or C3H mice conspicuously spares the pericardium (12, 13). Nevertheless, when BALB/c weanlings from four distinct breeding colonies are inoculated with the Nancy strain of coxsackievirus B-3, pericardial changes localized to the right ventricle, as well as myocarditis results. Coxsackievirus B-3 (Nancy) multiplies to equally high titers in both the right and left ventricles, but pericarditis occurs only in the right heart. After one month coxsackievirus B-3 recovered C mice have calcific fibrotic pericardial scars which are identical to those which can only be seen in much older (6 months or more) uninfected siblings. The prevalence of pericarditis is not increased in infected BALB/c mice, and it may be that pericarditis develops only in BALB/c mice with a predilection for the cardiomyopathy. Coxsackievirus B-3 infection, however, remarkably accelerates the "natural" cardiomyopathy. The right heart, thus, is already "injured" when coxsackievirus B-3 infection ensues. That injured tissues harboring inflammatory exudates are more susceptible to virus infection has been noted before, but the mechanism remains unexplained (14). One may speculate that leukotactic components of complement such as C5 are present at areas of genetically determined injury in the right ventricular pericardium. Coxsackievirus B-3, complexed with antibody might be attracted to complement in the right heart, augmenting and accelerating inflammation and, ultimately, calcification (15).*

TABLE III. PATHOLOGIC CHANGES OF COXSACKIEVIRUS B-3 MYOCARDITIS IN SEVERAL SUBSTRAINS OF BALB/c MICE.

	Histologic findings	
Substrain	Pericardium	Myocardium
C/MCF	29/43[a] (67%)	15/43 (35%) focal
C/CRL	9/19 (47%)	5/19 (26%) focal and diffuse
C/JL	2/5 (40%)	5/5 (100%) focal and diffuse
C/CL	3/13 (23%)	11/13 (85%) diffuse

[a] Number of mice with designated finding ÷ number of mice examined.

Summary. We describe progressive cardiomyopathy affecting three, and probably all four substrains of BALB/c (C) mice examined. Cardiac changes are limited to the pericardium and immediately subjacent epicardium of the right ventricle. By 2 months of age, mice have mononuclear infiltrates which progress to a dense exudative pericarditis. Whitish flecks on the pericardial surface can be seen at 6 months, and a calcifying scar persists.

When $10^{3.5}TCD_{50}$ of coxsackievirus B-3 (Nancy) is inoculated intraperitoneally into weanling Swiss or C3H mice, myocarditis without pericarditis results. If the infected mice are BALB/c, myocarditis and right ven-

* While this manuscript was being reviewed for publication, some of the original observations reported in abstract in 1968 were reported (16).

TABLE IV. PREVALENCE OF PERICARDIAL LESIONS IN COXSACKIEVIRUS B-3 INFECTED C/MCF MICE AND THEIR AGE-MATCHED CONTROLS.

	Coxsackievirus B-3 calcific pericarditis		Inherent noncalcified inflammatory pericarditis	
Age of mice (Days)	No. with disease ÷ No. examined	Cumulative prevalence (%)	No. with disease ÷ No. examined	Cumulative prevalence (%)
3	0 of 4 mice	0/4 (0%)	1 of 4 mice	1/4 (25%)
14	1 of 5 mice	1/9 (11%)	0 of 5 mice	1/9 (11%)
42	3 of 5 mice	4/14 (29%)	5 of 5 mice	6/14 (43%)
95	0 of 5 mice	4/19 (21%)	3 of 5 mice	9/19 (47%)
179	1 of 5 mice	5/24 (21%)	3 of 5 mice	12/24 (50%)
296	5 of 5 mice	10/29 (35%)	2 of 5 mice	14/29 (48%)

tricular pericarditis ensues. A calcified fibrotic scar over the right ventricle identical to the end-stage of the inherent lesion appears in coxsackievirus B3 infected mice at about one month of age.

Coxsackievirus B-3 multiplies equally well in the right and left ventricles of C mice, but pericarditis is limited to the right heart. In age-matched controls, the prevalence of calcific right ventricular pericarditis in BALB/c mice inoculated with coxsackievirus B-3 is the same as that of the inherent cardiomyopathy, and it is possible that the early calcific lesion in infected mice occurs in animals who later would have developed a right ventricular pericardial cardiomyopathy.

We thank Mrs. Mary Jane Shippey and Mr. Fred Smith for their help in these experiments.

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