could germinate as soon as moisture and temperature permit, germination could occur at the end of the dry season (May–June) or later in the wet season. The differences in their patterns of maturation and photoperiod would then control time of flowering.

Because nights at Culiacán (about 9.5 to 12.5 hours) exceed critical night length, flowering may be largely under control of the ripeness-to-flower response. The development of flower buds under experimental night lengths suggests that the plants at Culiacán possibly are inducible on any day of the year. Because the interval when moisture may be available is chiefly between June and September, this factor may have been the most important in timing the flowering requirements of this population.

Matehuala plants are apparently opportunistic, a characteristic of many desert plants. The uncertainty of the time of available moisture and the probable brief nature of its availability may have been an important factor in the selection of the rapid maturity of this population. The critical night length of 10.5 hours probably restricts the time of flowering to the period after 27 August. The chance of induction occurring prior to mid-April due to critical night length seems slight because of the timing of the dry season. Plants flowering in Matehuala in late August and September would have a high probability of producing mature fruit prior to the cool period, from November to January. Because temperatures as low as −4.4°C have been recorded in Matehuala, *Xanthium* development is apt to be confined to the warmer part of the year. Although plants are at various stages of development at Matehuala in November and December, most plants bear mature or nearly mature fruit.

Plants from Ciudad Mante, the ecosystem with the greatest likelihood of moisture from May through October, may have developed their ripeness-to-flower in response to this potentially long growing period. Because of the long dry season and mild winter (temperatures to −1.2°C have been recorded), *Xanthium* is probably induced only after 5 September. The slightly later occurrence, 15 September, of proper night length at Austin, Texas, probably delayed the flowering slightly in the outdoor planting compared to the wild population. Despite this difference, the garden population and the native population near Ciudad Mante were in very similar condition when they were observed in late November.

The ripeness-to-flower responses of the Ciudad Mante plants are similar to, but not as extreme as, those of Hawaiian collections shown by Ray and Alexander (*1*) to perform slowly and erratically under inductive photoperiods. Thus, a combination of differences in critical night length and in ripeness-to-flower response appears to be the basis for reproductive adaptation to different climatic regimes that prevail at the same latitude (and same photoperiodic regime) in *Xanthium strumarium*.

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### References and Notes

1. The terminology follows that of D. Löve and P. Dansereau [*Can. J. Bot.* **37**, 173 (1959)] who regarded all races of *Xanthium* (except *X. spinosum* which was not included in the present study) as being within *X. strumarium* L. As they indicated, morphological intermediates between the named types are common, and the classification is, therefore, somewhat arbitrary. The three Mexican populations are closest to the *italicum* "complex" of Löve and Dansereau. Similarity of different "complex" types in photoperiodism from the same latitude has been shown by P. M. Ray and W. E. Alexander [*Amer. J. Bot.* **53**, 806 (1966)].
2. Calculations of day length (and corresponding night length) for 22.5°N were from Smithsonian Meteorological Tables [*Smithson. Misc. Collect.* **114**, 506 (1951)]. The civil twilight before and after sunset was added to the period between sunrise and sunset for the day-length calculation. The photoperiodic effect of twilight has been reported by V. A. Greulach [*Ohio J. Sci.* **42**, 71 (1942)] and by A. Takimoto and K. Ikeda [*Plant Cell Physiol.* **2**, 213 (1961)].
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4. The chamber was programmed for 10 hours at 30°C during the period of higher light intensity (24,200 to 26,400 lu/m²) and 10 hours at 24°C during the lower light intensity (825 to 1100 lu/m²), allowing 2 hours for transition during morning and 2 hours in the evening. All chambers received 12 hours of higher light intensity from a combination of fluorescent and incandescent lamps. Light period extensions were with incandescent lamps only.
5. Supported by NSF grant GB-6097. I thank T. K. Moore, who participated as a summer student, and P. N. Dixon for assistance. P. M. Ray offered helpful comments on the manuscript.

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## Myotonic Muscular Dystrophy: Abnormalities in Fibroblast Culture

Abstract. *Skin fibroblasts in culture, derived from four unrelated patients with myotonic muscular dystrophy, contain abnormally large amounts of material with the staining characteristics of acid mucopolysaccharide. These cells also differ from normal cells in their pattern of growth at a high density in culture.*

Myotonic muscular dystrophy, which is inherited in an autosomal dominant pattern, is a disorder that affects skeletal, cardiac, and smooth muscle, the central nervous system, the reproductive system, the lens of the eye, the skull, and scalp hair (*1*). No single metabolic aberration has been adduced to explain the many different physiological and anatomical abnormalities (*2–4*) observed in this disorder. These widespread manifestations made it appear likely that fibroblast cultures derived from patients with myotonic muscular dystrophy (MMD) would express the inherited disorder in some way. The first MMD cell strain that we studied in culture grew in a sheet that was unusually dense and cohesive for a human diploid fibroblast strain. When microelectrode punctures were made for transmembrane measurements of membrane potential (*5*), the MMD cells often adhered tenaciously to the electrode as it was withdrawn. Because these observations suggested that some product of the cells might be abnormal or excessive, four MMD cell strains were grown in culture and stained for acid mucopolysaccharide.

Table 1. Acid mucopolysaccharide staining in fibroblast cultures. A total of 200 cells per cover slip were stained and scored according to the method described in the text.

| Patient | Percentage of cells with alcian blue–positive granules covering more than 20 percent of cytoplasmic area |
|---|---|
| *Patients with myotonic muscular dystrophy* | |
| A1 | 36 |
| B6 | 55 |
| B7 | 59 |
| B17 | 60 |
| *Normal individuals and patients with other neurological or muscle diseases* | |
| Mean | 11 ± 7 (8 cases) |
| Range | 3 to 26 |

Cultures were initiated from skin obtained by punch biopsy or in the course of muscle biopsy; control cultures were derived from normal individuals or patients with other neurological or muscle diseases. The explants and subsequent subcultures were grown in the Dulbecco-Vogt modification of Eagle's medium, supplemented with 10 percent calf serum. Between the fourth and tenth passages in cultures, cells were grown in

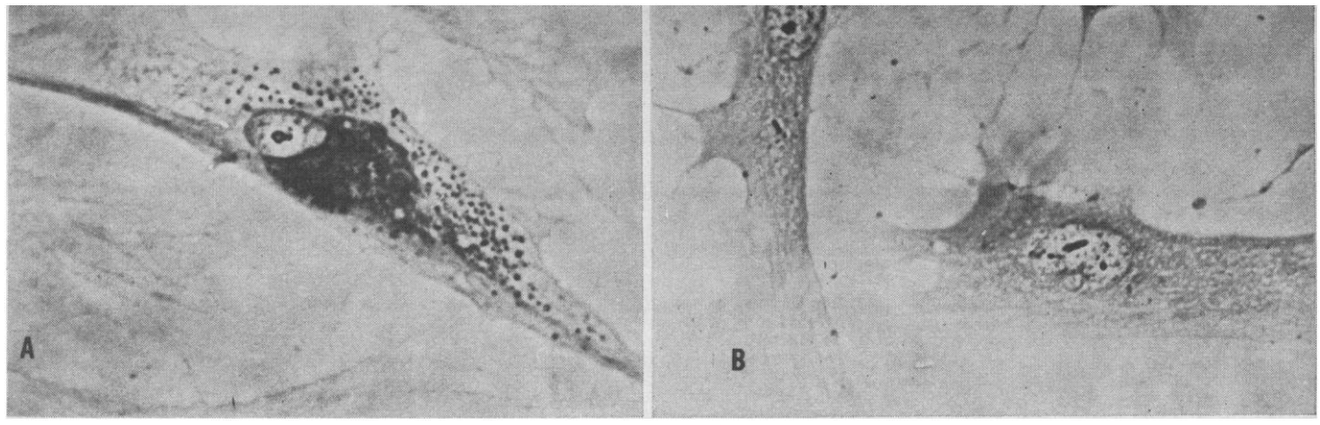


Fig. 1. Skin fibroblasts in cell culture. Cells fixed in lead nitrate-formalin and stained with alcian blue–chromotrope R. Photographed with × 40 phase objective. (A) Patient with myotonic muscular dystrophy; (B) normal person (× 1360).

60-mm petri dishes for 28 days with changes of medium made twice weekly. The cell sheets were then trypsinized onto cover slips; they were fixed and stained 24 hours later. The fixative was a mixture of lead nitrate, ethanol, and formalin for 30 minutes, and the stain was alcian blue (*p*H 2.5, in 3 percent acetic acid) with chromotrope R counterstain. In some experiments we compared the results of the method of Danes and Bearn (*6*) (methanol fixation and toluidine blue staining) with the results of fixation with lead nitrate and staining with alcian blue.

Cover slips from MMD cell strains were readily distinguished from normals, without any overlap between the two groups. The alcian blue method was so sensitive that a few blue granules could be seen in up to 40 percent of normal cells. The difference between the MMD and normal cells was most evident in the proportion of cells that had a large quantity (covering more than 20 percent of their cytoplasmic area) of alcian blue–positive material (Fig. 1). Four MMD cell strains, derived from unrelated patients, contained from 36 to 60 percent of such strongly positive cells. Seven control strains contained from 3 to 15 percent of cells in this category. One control strain, derived from a patient with an undiagnosed myopathy, contained 26 percent of these strongly positive cells in one experiment (Table 1). If a culture grew poorly, as happens from time to time for diverse reasons, an MMD strain, otherwise strongly positive, would contain less alcian blue–positive material and fall into the normal range. When duplicate cover slips were stained with the methanol–toluidine blue method of Danes and Bearn (*6*), the percentage of cells with metachromatic material was approximately that of the cells, fixed with the mixture of lead nitrate, ethanol and formalin, that were strongly positive for alcian blue. Thus our control cells were similar to those in the much larger series of Danes and Bearn (*6*), and the MMD cell strains are clearly outside the normal range.

In two experiments, the alcian blue–positive material in the MMD cells was digested by a prior incubation of 1 hour in testicular hyaluronidase, which further suggests that the material is acid mucopolysaccharide. In several preliminary experiments, MMD cells were not clearly distinguished from controls by staining for fat with oil red O or by measurement of glycogen content (alcohol precipitation–anthrone reaction).

The MMD cell cultures often show a distinctive pattern of growth when examined under low-power magnification in the inverted microscope. Normal human diploid cells, after they reach confluence in the petri dish, heap up in a regular way (*7*). Successive layers grow so that the long axes of cells above and below are oriented in different directions, and each layer preserves the swirling pattern characteristic of the first monolayer. When MMD cells have become several layers thick there is a disorganized pattern to the upper layers. In addition, on several occasions MMD cell strains have grown to a cell density, per plate, higher than any normal human adult diploid strain grown in this laboratory under the same conditions.

Abnormally large amounts of metachromatic or alcian blue–staining material have been found in cultured fibroblasts derived from patients with several different genetic disorders (*6*), and the excess material has been chemically characterized for some of these diseases (*8*). However, for none of these is the basic enzyme defect established, nor is it known whether the excess acid mucopolysaccharide, so prominent in the cultured cells, is important in the actual pathogenesis of these diseases.

An abnormal amount or distribution of acid mucopolysaccharide has not yet been sought in tissues of MMD patients. It is interesting to speculate, however, how excess acid mucopolysaccharides in vivo could cause some of the unusual features of this disorder. Because acid mucopolysaccharides are polyelectrolytes with many anionic sites on each molecule, an excess near the MMD muscle membrane could lower the calcium ion concentration at the membrane and produce the partial depolarization and spontaneous oscillations of membrane potential that have been recorded from MMD muscle (*3*). Measurements of ion content and $^{22}Na$ efflux have shown a large excess of sodium (and of total monovalent cation) in MMD muscle biopsies, without any detectable abnormality in the sodium pump (*4*). These excess cations are bound, and could be bound to acid mucopolysaccharide if it were present in unusual amounts in MMD muscle. Deposition of acid mucopolysaccharide in the lens could lead to the MMD cataract and in the skull to the hyperostosis frontalis interna commonly seen in MMD patients.

Whether or not excess acid mucopolysaccharide is implicated in the pathogenesis of MMD, the histochemical abnormality that we are reporting is evidence that the cultured dermal fibroblasts express the disorder and provides a marker to guide further analysis in the cell culture system.

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## Tumor-Specific Antigens Detected by Inhibition of Macrophage Migration


Abstract. *Tumor-specific antigens of a guinea pig hepatoma induced by diethylnitrosamine were detected by the inhibition of migration of specifically sensitized macrophages from capillary tubes, and by the local passive transfer of delayed skin hypersensitivity and the suppression of growth of intradermally injected tumor.*


Tumor-specific transplantation antigens of a hepatoma induced in guinea pigs (strain 2) by diethylnitrosamine were detected by methods for measuring cellular immunity to tumors. These methods were: (i) inhibition of migration of macrophages from capillary tubes and (ii) local passive transfer of both delayed skin reactivity and the ability to suppress the growth of intradermally injected tumor cells. All three methods were studied simultaneously with peritoneal exudate cells from the same animals.

Age-matched pairs of male guinea pigs (Sewell-Wright NIH strain 2) were immunized to line 1 tumor by three courses of injections. Each course consisted of intradermal injections of $3.0 \times 10^6$ live tumor cells once a week for 3 weeks. There was a 2- or 3-week pause between courses. Peritoneal exudate cells were obtained from these animals in the following manner. Thirty milliliters of a light mineral oil (Drakeol 6 VR, Butler Refining Co., Gettysburg, Pa.) was sterilized and injected into the peritoneal cavity. The animal was killed 4 days after injection of Drakeol, and the peritoneal cavity was washed once with 80-ml, and three times with 40-ml portions of medium 199. The exudate and washings were placed in a separatory funnel at 4°C for 45 minutes, and the cell-rich fraction was then withdrawn from the bottom. Cells were washed three times with medium 199, and a sample was tested for viability with trypan blue (GIBCO, 0.4 percent). Differential cell counts of the peritoneal exudate (PE) usually showed about 50 percent macrophages, 40 percent lymphocytes, and 10 percent granulocytes.

Ascites-variant forms of two separate transplantable hepatomas, lines 1 and 7, were harvested (*1*). These lines have different tumor-specific transplantation antigens as shown by cross-protection studies (*2*). Cells were washed three times with medium 199 and then tested for viability with trypan blue. The tumor cells in these suspensions consisted mostly of single cells; only a few small clumps were present.

Tumor-cell suspensions (line 1 or line 7) were mixed with equal volumes of peritoneal exudate cells from animals immunized to line 1. For macrophage-migration tests, the following mixtures were made—$60 \times 10^6$ PE cells per milliliter with $60 \times 10^6$ tumor cells per milliliter, and $60 \times 10^6$ PE cells per milliliter with $6 \times 10^6$ tumor cells per milliliter. These mixtures were incubated for 10 minutes, with shaking, at 37°C.

Cell mixtures were placed in capillary tubes and sealed in pairs into tissue chambers, after the method of David (*3*). The chambers were filled with McCoy's 5a medium (modified) with 30 percent fetal bovine serum (GIBCO) and incubated for 5 days at 37°C. The areas covered by the migrating cells in 24 hours were measured with a planimeter (Table 1).

Migration of PE cells from animals immunized to line 1 tumor is inhibited by an equal number of line 1 tumor cells, but not by an equal number of line 7 tumor cells. Mixtures of PE and tumor cells (10 : 1) showed no inhibition of migration when tumor cells of either line 1 or line 7 were used. There was no inhibition of migration of PE cells from nonimmunized animals when the cells were mixed with line 1 or line 7 tumor cells (Table 2).

Cell mixtures were also prepared for use in local passive transfer of delayed skin sensitivity. One-tenth of a milliliter of these mixtures contained $3 \times 10^6$ PE cells and $1.5 \times 10^6$ tumor cells of either line 1 or line 7. Age-matched pairs of male guinea pigs (strain 2) were injected intradermally with 0.1 ml of each of these mixtures and at a separate site with $1.5 \times 10^6$ cells from each tumor line. Delayed skin reactions were measured with a caliper at 24 hours (Table 3). Because each intradermal injection contained live tumor cells, it was possible to mea-

Table 1. Areas of migration (measured by planimetry from a projected image and expressed in square millimeters) of peritoneal exudate (PE) cells from animals immunized with line 1 tumor. Line 7 shares no tumor-specific transplantation antigen with line 1.

| Cells (No./$10^6$) per mixture | | | Migration area* ($mm^2$) |
|---|---|---|---|
| PE | Line 7 | Line 1 | |
| 60 | 60 | | 21.51 ± 0.42 |
| 60 | | 60 | 10.15 ± 0.68 |
| 60 | 6 | | 19.92 ± 0.55 |
| 60 | | 6 | 20.25 ± 0.67 |
| 60 | | | 18.47 ± 0.51 |

* Mean area ± standard error of the mean for eight capillary tubes.

Table 2. Areas of migration (measured by planimetry from a projected image and expressed in square millimeters) of peritoneal exudate (PE) cells from nonimmunized animals.

| Cells (No./$10^6$) per mixture | | | Migration area* ($mm^2$) |
|---|---|---|---|
| PE | Line 7 | Line 1 | |
| 60 | 60 | | 15.47 ± 0.27 |
| 60 | | 60 | 15.68 ± 0.34 |
| 60 | | | 14.40 ± 0.17 |
| | 60 | | 0.99 ± 0.05 |
| | | 60 | 0.91 ± 0.08 |

* Mean area ± standard error of the mean for six capillary tubes.