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Human muscle-derived, tissue specific, myocytotoxic T cell lines in dermatomyositis

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SUMMARY

Mononuclear cells were isolated from the inflamed muscle tissue of a patient suffering from dermatomyositis (DM). These were expanded in long-term culture and maintained in the presence of IL-2 containing culture medium. Two cell lines were established, one of the helper/inducer ($OKT4^+$) and the other of the suppressor/cytotoxic phenotype ($OKT8^+$). The $OKT4^+$ cell line exhibited a non HLA-restricted, tissue-specific, myocytotoxic effect on rat muscle cell culture. Its lymphoproliferative response to human muscle antigen was HLA-restricted. The $OKT8^+$ cell line exhibited a non HLA-restricted, tissue-specific response to muscle antigens and no myocytotoxic activity in *in vitro* rat muscle cell culture. It is likely that clones of $OKT4^+$ lymphocytes in patients suffering from DM are associated with the pathogenesis of the disease—they probably mediate the diffuse damage to skeletal muscle through their myocytotoxic activity.

Keywords dermatomyositis myocytotoxic T cell line

INTRODUCTION

Polymyositis, or dermatomyositis (DM) if the characteristic rash is present, is an inflammatory myopathy of unknown origin which may be encountered either as a primary disorder or in association with autoimmune diseases or accompanying malignancies (Bohan & Peter, 1975).

Inflammatory infiltrates in the affected skeletal muscle are the diagnostic hallmark of DM (Schwarz *et al.*, 1980). The infiltrates are composed mainly of lymphocytes, the majority of which are T cells with a significant, relative increase in the percentage of cells having the helper/inducer phenotype (Giorno *et al.*, 1984). To date, studies on the functional activity and antigen specificity of muscle lymphocytes in active DM patients have been hampered by the limited number of cells available from the standard muscle biopsy and by the absence of a specific antigen directly involved in the pathogenesis of DM.

These same limitations have also hampered the study of target tissue lymphocytes in patients suffering from other autoimmune diseases. Hence, most of our knowledge of the functions of antigen specific lymphocyte clones in autoimmune diseases is based on studies of animal models (Cohen *et al.*, 1983; Rozenszajn *et al.*, 1986; Caspi *et al.*, 1986).

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However, the development of a technique (Nussenblatt *et al.*, 1984; Ruscetti, Morgan & Gallo, (1977) for growing long-term cultures of T cells in the presence of conditioned medium containing interleukin 2 prepared from phytohemagglutinin-M (PHA)-induced lymphocytes has permitted us to expand and study muscle T cells isolated from the inflamed skeletal muscle of DM patients. In the present study, we established two T cell lines from the inflamed muscle of a DM patient. Phenotypic analyses of these cell lines revealed one to be $OKT8^+$ and the other $OKT4^+$. The muscle derived T cell lines displayed tissue specific myocytotoxic activity and proliferated in the presence of skeletal muscle tissue antigen.

MATERIALS AND METHODS

A 70-year-old woman was admitted to hospital with progressive proximal muscle weakness. On examination, periorbital oedema, an erythematous scaly rash over the forehead, face and upper chest and proximal muscular tenderness were noted. Laboratory findings revealed elevated levels of creatine phosphokinase (CPK) and aldolase in the patient's blood serum.

Muscle biopsy showed multiple focal areas of muscle fibre necrosis, variation in fibre size, large vesicular sarcolemmal nuclei, and focal inflammatory exudates, sometimes perivascular, containing mainly small mononuclear cells.

Using the criteria proposed by Bohan & Peter (1975) the diagnosis of dermatomyositis was definite.

Treatment with a high dose of steroids was initiated but the patient's condition deteriorated rapidly. She died 10 days after admission.

Isolation of lymphocytes from affected muscle tissue

Muscle tissue was obtained from the diagnostic biopsy of the patient's deltoid muscle. The tissue was cut into small pieces under sterile conditions, crushed, passed through a stainless steel mesh screen and suspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY). The mononuclear cells were separated by centrifuging the cell suspension on a Ficoll-Hypaque density gradient (D 1·077) (Böyum, 1968). The cells were then counted and expanded in long-term culture.

Preparation of conditioned medium (CM) containing interleukin 2

Venous blood mononuclear cells (MNC) from two healthy donors were separated on a Ficoll-Hypaque density gradient (D 1·077), mixed together and incubated ($1{\cdot}5 \times 10^6$ cells/ml) with 0·0125 ml/ml PHA (Difco, Detroit, MI) and 5 ng/ml phorbol 12-myristate 13-acetate (Sigma, St Louis, MO) in complete RPM1-1640 (C-RPMI), which contains 100 U/ml penicillin, 10 μg/ml streptomycin, 1% 200 mM glutamine, 1% 100 mM sodium pyruvate, 1% non-essential aminoacids (Biological Industries, Bet Haemek, Israel), and 5×10^{-2} mM 2-mercaptoethanol (ME) (Merck), and to which 10% inactivated (56°C, 30 min), pooled human serum was added.

After 72 h incubation at 37°C in a fully humidified atmosphere containing 7·5% CO_2 in air, the cells were centrifuged and the supernatant, the CM, stored at −20°C (Rozenszajn *et al.*, 1984).

Long-term culture of lymphocytes—expansion and maintenance

Mononuclear cells isolated from muscle tissue were seeded in round-bottomed microtitre plates (Nunc, Denmark), 1×10^3 cells per well, in 0·2 ml C-RPMI-1640 culture medium containing 10% inactivated pooled human serum. Cells were cultured with or without the addition of 20% CM and/or 0·0125 ml/ml PHA. The cells were incubated at 37°C in a fully-humidified atmosphere containing 7·5% CO_2 in air. Each week, one-half of the culture medium was replaced by fresh medium, with or without CM and/or PHA. Two weeks after seeding, irradiated (4000 rad) peripheral blood mononuclear cells from healthy donors were added as feeder cells, 1×10^3/well. For further expansion the cultures were transferred to 24-well multidishes (Nunc, Denmark) and maintained in C-RPMI containing 10% pooled human serum and 20% CM. Cell lines were cultured and grown for months, proliferating *in vitro* in culture medium one half of which was replaced every 4 days. In

addition, irradiated (4000 rad) human mononuclear cells, at a ratio of 1 irradiated cell/6 cultured cells, and 0·0125 ml PHA/ml culture medium, were supplemented once a month.

Determination of the surface markers and phenotypes of the cell lines

E rosette formation according to the method of Wybran was used as a marker for T cells (Wybran, Carr & Fudenberg, 1972).

T cell subsets were classified according to surface antigen specificity using monoclonal antibodies, OKT3 for mature peripheral T cell populations, OKT4 for inducer/helper T lymphocytes and OKT8 for the suppressor/cytotoxic T cell subset (Rosenszajn *et al.*, 1984; Janossy *et al.*, 1981). B cells were identified by the presence of surface membrane immunoglobulins (SIg) employing a direct detection method in which the cells were incubated with the $F(ab)_2$ fraction of FITC-conjugated polyspecific antiserum (Kallestad, Austin, TX) (Whitemeyer, Bankhurst & Williams, 1977).

Control T cell lines

Venous blood mononuclear cells from healthy donors were separated on Ficoll-Hypaque gradient (D 1·077). The cells were seeded for T lymphocyte colony formation under culture conditions described elsewhere (Rozenszajn *et al.*, 1981). Lymphocyte colonies were picked from the agar and cultured in flat-bottomed microplates, one colony/well, in C-RPMI containing 10% pooled, inactivated human serum and 20% CM. After extensive proliferation the cells were transferred to 24-well multidishes for further expansion and maintenance under the same conditions as described above. The T cell lines, each developed from one individual T cell colony, were characterized using monoclonal antibodies. Two cell lines served as controls: one had the OKT4 phenotype and the other the OKT8 phenotype.

Culture of skeletal muscle cells of rat fetus

Cultures of skeletal muscle cells were prepared from the thigh muscles of embryonic (18 to 21 days old) or neonatal (1 to 2 days old) rats (Shainberg, Yagil & Yaffe, 1971; Shainberg *et al.*, 1984). The limbs were severed and washed in phosphate-buffered saline (PBS) to remove excess blood cells. The muscle tissue was mechanically dispersed and transferred to a Ca-free 0·25% solution containing EDTA (1 mM) and incubated with continuous stirring at 37°C. The cells were collected after successive 10 min periods of incubation until all the tissue was dispersed.

They were then centrifuged for 5 min at 500 ***g***. The pellets were resuspended in growth medium (DMEM 83%, horse serum 15%, chick embryo extract 2%) and preplated for 20–30 min in 10 ml Petri dishes to remove fibroblasts. The supernatant containing mainly myoblasts was collected and diluted with growth medium to a concentration of $0{\cdot}8 \times 10^6$ cells/ml for plating in 35 mm collagen-coated, plastic tissue dishes (1·5 ml/dish). The cultures were grown at 37°C in a water-saturated atmosphere of 90% air and 10% CO_2.

Culture of heart muscle cells of rat fetus

Whole hearts were removed from rat fetuses 18 to 21 days old and washed in PBS to remove excess blood. The hearts were dissected mechanically and disaggregated with a Ca-free 0·25% trypsin solution containing EDTA (1 mM). The cells were collected following a 10–15 min period of incubation at 37°C with continuous stirring and then centrifuged for 5 min at 500 ***g***. The cells in the pellet were resuspended in growth medium and the percentage of myoblasts in the suspension increased by preplating for 30 min. The heart muscle cells were then plated in collagen-coated multi-well dishes, 1×10^6 cell/ml (Harary & Farley, 1963).

Preparation of antigen

Human skeletal muscle, obtained during a surgical procedure, was homogenized in a glass homogenizer and suspended in C-RPMI-1640. Rat skeletal muscle cells which had differentiated *in vitro* were obtained from a 10-day-old muscle culture. The supernatant was discarded, the monolayer of muscle cells scraped from the Petri dish with a rubber policeman and homogenized with 1 ml PBS in a glass-tephlon homogenizer.

The protein content of the homogenate was determined using the method of Meulemans (Meulemans, 1960).

Proliferation test

Cells from the long-term cultures were washed three times and incubated in 0·2 ml of C-RPMI-1640 in 96-well, round-bottomed microtitre plates, 5×10^4 to 2×10^5 cells/well, with the various concentrations of human and rat skeletal muscle homogenate. Homogenates of protein concentrations ranging from 1 mg to 7 mg/well were used for lymphocyte activation. After 48 h, the cells were pulsed for 18 h with 1 μCi/well of ^{3}H-thymidine (Nuclear Research Center, Hanegev, Israel) and harvested.

In some experiments, irradiated (4000 rad) mononuclear cells from the patient's offspring, 5×10^4 cells/well, were added as antigen-presenting cells to the test culture.

Effect of cell lines on cultures of skeletal and heart muscle cells

Morphological study. Cells from T cell lines grown for 1 and 3 months were added, 7×10^4 cells/plates, to cultures of rat skeletal or heart muscle cells 5 and 10 days old. The cytotoxic effect of the cell lines on the muscle cultures was monitored under an inverted microscope ($\times$ 100).

Enzyme activity studies. A 10-day-old culture of skeletal muscle cells was co-cultured for 8 days with T cell lines. The supernatant was removed and the monolayer of muscle cells washed three times with 2 ml PBS. The monolayer was then separated from the Petri dish with a rubber policeman and homogenized with 1 ml PBS in a glass-Tephlon homogenizer. The homogenate was centrifuged for 10 min at 1800 rev/min and the activities of CPK, lactic dehydrogenase (LDH) and aldolase in the supernatant were determined (Rosalki, 1967; Wacker, Ulmer & Vallee, 1956; Bergmeyer, Ganehn & Grassel, 1974). These served as indicators of the mass of intact residual muscle cells in the cultures (Cohen, Buckingham & Gros, 1978; Shainberg *et al.*, 1971).

HLA typing. HLA-A, -B, -C, -DR typing was done using the standard complement-dependent microcytotoxicity technique.

Statistical analysis. Student's *t*-test was used to determine the statistical significance of our results.

RESULTS

Lymphocytes which had infiltrated the inflamed muscle of the DM patient were expanded in long term cultures. Two cell lines were established, E/7 and F/7.

The phenotype of cell line E/7 was predominantly OKT4$^+$ and that of F/7 mainly OKT8$^+$, as determined by monoclonal antibodies using an indirect immunofluorescence technique.

Two normal cell lines were selected as controls, one, N/7/2, with OKT4$^+$ phenotype and the other, N/9/1, with OKT8$^+$ phenotype (Table 1).

When 6×10^4 cells from the patient's E/7 or F/7 cell lines were added to 5-day-old skeletal muscle cultures which had just undergone myoblast fusion, the morphology of the myotubes was unaffected, remaining similar in appearance to that of the control N/7/2 and N/9/1 cell lines (Fig. 1b). However, when the patient's OKT4$^+$ cell line was added to 10-day-old muscle cultures, obvious morphological alterations were soon seen—the myotubes, originally thick, cylindrical and contracting spontaneously became, within 4–5 days, flat, narrow, granulated in the cytoplasm, and almost completely devoid of spontaneous contractions (Fig. 1a). Increasing the number of the OKT4$^+$ cell line lymphocytes in the co-culture caused the changes in the morphology to become more rapid.

The patient's OKT8$^+$ line and the control cell line, N/7/2 and N/9/1, exerted no significant effect on the structure of the myotubes in the culture. Also, the addition of supernatant from the patient's E/7 and F/7 line culture to the muscle cultures caused no evident morphological changes in the myotubes and no alterations in the spontaneous contraction activity.

In 10-day-old skeletal muscle cultures to which the patient's OKT4$^+$ line cells had been added, the activities of CPK, LDH and aldolase (monitors of the residual muscle cell mass in the culture)

Table 1. T cell lines derived from inflamed muscle tissue—phenotype and proliferative response to homogenate of rat muscle cell cultures

	% of positive cells		^{3}H thymidine uptake	
Cell line	OKT3	OKT4	OKT8	ct/min s.d.
E/7†	80	84	0	681±152
F/7†	96	0	100	1593±96*
N/7/2‡	100	100	0	ND
N/9/1‡	100	5	96	526±133
Muscle cells				315±85

* $P<0{\cdot}001$ relative to proliferation of the normal cell line N/9/1.

† T cell line expanded from mononuclear cells derived from the DM patient's inflamed muscle tissue.

‡ T cell line, expanded from T lymphocyte colonies derived from normal mononuclear blood cells served as control cell line.

Homogenate from 10-day-old culture of rat skeletal muscle cells was added, 1 mg/well, to the T cell lines (1×10^5 cells/well). Muscle antigen-induced proliferation was determined by ^{3}H-thymidine (1 μCi/well) uptake after 66 h of incubation.

were 40% to 60% of those of the control cultures. This is indicative of muscle damage and of a decrease in the mass of intact muscle cells remaining in the culture dish (Table 2).

Neither of the patient's cell lines had a significant effect on the morphology and/or spontaneous contractions of dissociated heart muscle cells grown in cell culture.

When studying the proliferative response of the cell lines to human muscle homogenate or rat muscle culture homogenate, it was found that the patient's OKT4$^+$ cell line, which had a cytotoxic effect on rat skeletal muscle cell culture (Table 2, Fig. 1a) was able to proliferate only when antigen-presenting cells from the patient's offspring were supplemented to the culture together with the human muscle homogenate (Fig. 2). The patient's OKT8$^+$ cell line responded to both antigens (Tables 1, 3). The proliferative response of this cell line increased 2-fold when the system was supplemented with irradiated MNC from all the patient's offspring.

Mononuclear cells from the patient's offspring were added in some experiments in order to supply the system with antigen-presenting cells displaying the deceased patient's major histocompatibility complex (MHC) gene products. When the antigen-presenting cells from each offspring were tested separately, it was found that only those from M.I. were capable of triggering a statistically significant proliferative response from the patient's OKT4$^+$ positive cell line in the presence of human muscle homogenate (Fig. 2).

Analyses of the MHC gene products of the patient's family and reconstruction of the deceased patient's MHC genes showed that M.I. was the only offspring who had inherited the patient's HLA-2-40-4 haplotype (Fig. 3).

DISCUSSION

Considerable evidence has accumulated to date which suggests that the immune system plays a major role in the pathogenesis of DM mainly through cell mediated mechanisms.

Peripheral blood lymphocytes from patients with DM have been shown to proliferate in culture in response to a stimulus from normal muscle (Garlepp & Dawkins, 1984) while their cytotoxicity to muscle culture is controversial (Dawkins & Mastaglia, 1973; Haas, 1980).

Immunizing various animal species with skeletal muscle results in a diffuse inflammatory

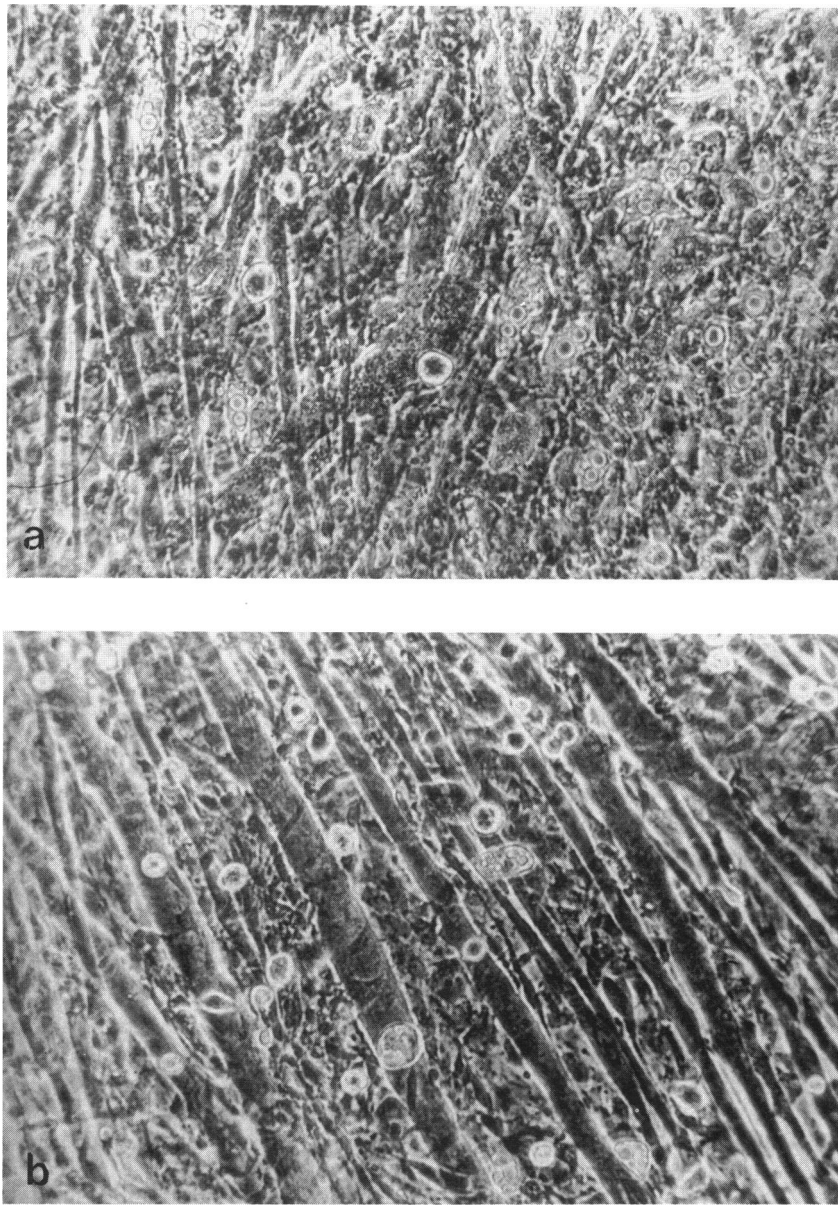

Fig. 1. (a) A tissue-specific myocytotoxic effect was observed when the OKT4$^+$ cell line derived from the DM patient's inflamed muscle was co-cultured for 8 days with rat muscle cell culture. The myotubes became flat, narrow and devoid of spontaneous contractions. (b) When the muscle cell culture was incubated with a normal OKT4$^+$ T cell line, no significant effects on its structure were evident.

myopathy which can serve as animal models of DM. Suspensions of sensitized cells from these animal models were found to be cytotoxic to normal muscle cultures (Kakulas, 1968); the disease could be passively transferred by lymphocytes but not serum (Morgan, Peter & Newbould, 1971).

Experimental animal models of different autoimmune diseases have yielded copious and relevant information concerning the role of the immune cellular mechanisms in these diseases. Antigen-specific T cell lines established from animal models of autoimmune diseases are able to

Table 2. Changes in enzyme levels of rat muscle cell cultures due to cytotoxic effect of T cell lines

Cell line	Enzyme activity (IU/mg protein)		
	CPK	LDH	Aldolase
E/7 (OKT4$^+$)	3·89	10·96	1·79
N/7/2 (OKT4$^+$) (control)	9·61	18·25	2·81

T cell lines (7×10^4/well) were added to 10-day-old rat muscle cell cultures. The activities of the enzymes of the muscle cell homogenate, which are gauges of the mass of residual muscle cells in the culture, were determined after 8 days of co-culture.

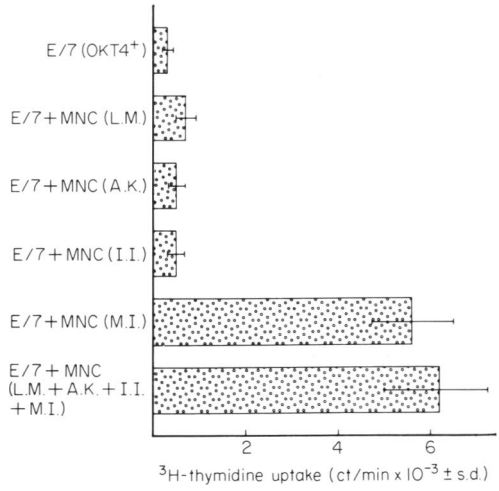

Fig. 2. Proliferative response of DM patient's OKT4$^+$ T cell line to human muscle homogenate in the presence of offspring's mononuclear cells (MNC). Irradiated (4000 rad) blood MNC from her offspring (L.M., A.K., I.L., M.I.) were added (10^4 cells/well) to the patient's cell line (5×10^4 cell/well). The cells were incubated in microtitre plates for 66 h, being pulsed with ^{3}H-TdR 18 h before harvesting. $P < 0{\cdot}01$.

mediate similar autoimmune lesions when inoculated into naive rats. These autoimmune lesions are accompanied by the accumulation of immunologically specific cell lines in the target organ. The stable lines which are able to mediate autoimmune diseases all bear the PAN T cell marker and those of helper cells (Cohen *et al.*, 1983; Rozenszajn *et al.*, 1986).

A diagnostic characteristic of DM is the marked infiltration of the inflamed skeletal muscle by lymphocytes (Schwarz *et al.*, 1980). The majority of the infiltrating cells are T cells, with a significant relative increase in the percentage of the helper/inducer phenotype lymphocyte (Giorno *et al.*, 1984). OKT4$^+$ cells were also observed to be prevalent in the affected tissue in other autoimmune diseases; in the synovial membrane in rheumatoid arthritis (Duke *et al.*, 1982) and in the active lesion within the human central nervous system in multiple sclerosis (Traugott, Reinherz & Rains, 1983).

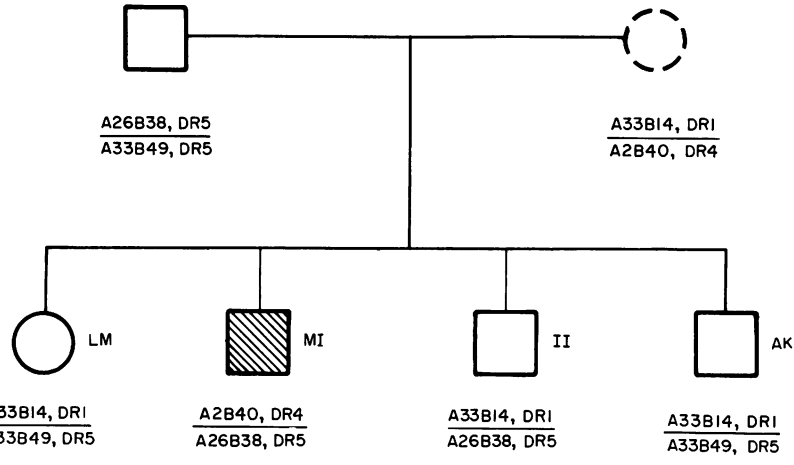

Fig. 3. Pedigree of MHC genes of DM patient's family. The MHC genes of the deceased patient (broken circle) were reconstructed from analyses of the MHC genes of her offspring. The patient's haplotype HLA 2-40-4, was inherited only by M.I. (shaded square) whose mononuclear cells were essential for evoking a proliferative response from the OKT4$^+$ cell line due to the presence of human muscle antigen.

Table 3. Proliferative response of cell lines to human muscle homogenate

	^{3}H-thymidine uptake (ct/min ± s.d.)			
Cell line (2×10^5/well)	Medium	(1 mg muscle protein/well)	(4 mg muscle protein/well)	(7 mg muscle protein/well)
E/7 (OKT4$^+$)§	319 ± 127	243 ± 216	463 ± 105	350 ± 163
F/7 (OKT8$^+$)§	760 ± 86	2650 ± 166‡	1213 ± 96†	1140 ± 133*
N/9/1 (OKT8$^+$)¶	567 ± 52	607 ± 151	759 ± 394	ND

* $P < 0{\cdot}05$.
† $P < 0{\cdot}01$.
‡ $P < 0{\cdot}001$.
§ T cell line derived from the DM patient's inflamed muscle tissue.
¶ T cell lines expanded from T lymphocyte colonies derived from normal mononuclear blood cells (control).

We have succeeded in isolating the lymphocytes infiltrating the inflamed skeletal muscle in DM, expanding and growing them by using polyclonal stimulation with PHA and an IL-2-containing culture medium. Two long-term cell lines derived from muscle lymphocytes were established. The two cell lines were either OKT4$^+$ or OKT8$^+$. No changes were noted in the phenotypic characteristics of the cell lines during the 10 months in which they were maintained (Table 1).

The patient's OKT4$^+$ cell line was found to be tissue specific and HLA-restricted in its proliferative response, the cell line responded only when the muscle homogenate, exposed to antigen, contained a specific single parental DR antigen—DR 4. This antigen was shared only by the deceased patient and her offspring, M.I., and only the MNC from this offspring were able to trigger the proliferative response in the OKT4$^+$ cell line (Fauci, Lane & Volkman, 1983) (Tables 1, 3; Figs 2, 3). Also, the OKT4$^+$ cell line exhibited a non-HLA restricted tissue-specific myocytotoxic activity against rat skeletal muscle cultures and an insignificant effect on heart muscle cell cultures,

despite the histological proximity between these tissues. This was shown by the morphological alterations (Fig. 1a), and the obviously greater damage to the cell line-treated skeletal muscle culture (Table 2). The fact that the cell line supernatant does not affect muscle cultures and that line cells were observed to adhere to muscle cells may indicate that a cell-to-cell interaction may be operative in the mechanism of muscle damage (Mastaglia, Dawkins & Papadimitriou, 1974). The differences in response of the 5-day and the 10-day-old muscle cultures may be due to the fact that the 10-day-old cultures contain myotubes which are more differentiated and thus more susceptible to the action of the sensitized lymphocytes (Martonosi *et al.*, 1977).

The patient's $OKT8^+$ cell line exhibited a non-HLA restricted tissue specific proliferative response to muscle homogenate (Tables 1, 3) but no myocytotoxic activity in a muscle culture *in vitro*. Our results lead us to conclude that $OKT4^+$ lymphocyte clones are associated with the pathogenesis of DM, probably causing the diffuse damage to skeletal muscle through their tissue-specific myocytotoxic activity. Our findings concur with accumulated data which relate cytotoxic T lymphocytes of the helper phenotype with autoimmune disorders (Cohen *et al.*, 1983; Rozenszajn *et al.*, 1986; Fauci *et al.*, 1983; Meuer, Schlossman & Reinhertz, 1982; Moretta *et al.*, 1984).

We have shown that in autoimmune disease such as DM, the search for and the isolation of the pathogenic lymphocytes from the target tissue where they have homed is of central value. A study of the growth of these cells, their expansion and characteristics will no doubt shed light on the mechanisms of the activation and behaviour of human T cells in autoimmune diseases. The striking similarity between our DM patient, 'a human model', and animal models used till now to study autoimmune diseases mediated by T lymphocytes seems to validate the relevance of the animal models and to emphasize the strong correlation between such models and autoimmune disease in man.

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