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Impaired autologous mixed lymphocyte reaction with normal concanavalin A-induced suppression in adult polymyositis/dermatomyositis

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SUMMARY

Polymyositis/dermatomyositis (PM/DM) is an autoimmune disorder of unknown aetiology. In order to study whether immunoregulatory abnormalities might be involved in this autoimmune state, we investigated the autologous mixed lymphocyte reaction (AMLR) and concanavalin A-induced suppressor cell function (Con A-induced suppression) in adult patients with primary PM/DM. We found the AMLR to be significantly depressed in patients; responsiveness could not be enhanced by increasing the numbers of non-T stimulator cells in culture, nor by varying the day on which cultures were harvested. Con A-induced suppression of T cell proliferative responses to mitogenic stimuli was normal. These findings implicate abnormal immunoregulation in the pathophysiology of PM/DM. Further, the dissociation of AMLR reactivity from Con A-inducible suppression suggests that events important for immunoregulatory competence may occur in the AMLR culture, despite the absence of an observed proliferative response.

Keywords polymyositis/dermatomyositis Con A-induced suppression autoimmunity immunoregulation

INTRODUCTION

The inflammatory myopathies comprise a variety of disorders whose common hallmark is weakness, accompanied by degeneration and mononuclear infiltration of muscle (Pearson & Currie, 1974). In adults, polymyositis/dermatomyositis (PM/DM) may be encountered as a primary disorder, in association with defined autoimmune syndromes, or accompanying malignancies.

It is thought likely that cell-mediated autoimmunity to muscle is important in the pathophysiology of PM/DM (Ziff & Johnson, 1970). Previous work by a variety of investigators has established that lymphocytes sensitized to skeletal muscle are present in patients (Partridge & Smith, 1976; Esiri, MacLennan & Hazleman, 1973). Johnson, Fink & Ziff (1972) have shown that these cells elaborate a myotoxic lymphotoxin like lymphokine on incubation with autologous muscle. Several techniques have been used to demonstrate that patients' lymphocytes can mediate injury to cultures of muscle cells (Haas & Arnason, 1974; Dawkins & Mastaglia, 1973; Currie, 1970; Kakulas, 1966). The role of cell-mediated immunity in PM/DM is supported by animal studies. Through regimens of immunization with homogenized muscle in adjuvant, investigators have induced weakness and cellular infiltrates in animals. The induced myositis is transferrable with lymphoid cells, but not with

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serum (Dawkins, 1975). Experimental myositis differs significantly from its human counterpart in being self-limited (Pearson & Currie, 1974).

Analysis of an autoimmune state such as that seen in PM/DM requires consideration of a diversity of aetiological possibilities, including genetic predisposition, viral induction and immunoregulatory abnormalities (Allison, 1977; Chou, 1972). To investigate various immunoregulatory functions of lymphocytes in this autoimmune state, we have studied the autologous mixed lymphocyte reaction (AMLR) and suppression of lymphocyte proliferation by concanavalin A treated lymphocytes (Con A-induced suppression) in adult patients with primary PM/DM. We have studied only those patients in whom the effect of medications, including corticosteroids, on these *in vitro* assays would be nil (MacDermott & Stacey, 1981).

The AMLR denotes proliferation of T lymphocytes co-cultured with autologous non-T lymphocytes (Kuntz, Innes & Weksler, 1976; Weksler & Kozak, 1977), and is considered to reflect a variety of recognition events important for immunoregulation, including the maintenance of self tolerance (Sakane & Green, 1979). It is impaired in several autoimmune states (Sakane, Steinberg & Green, 1978a; Miyasaka *et al.*, 1980), and in some conditions affecting lymphoid tissues (Moody *et al.*, 1979; Quesada, Reuben & Murphy, 1981).

The presence of autoreactive cells in normal individuals has recently been questioned following experiments which demonstrated reactivity to xenogeneic antigens absorbed to autologous stimulator cells (Huber *et al.*, 1982). However, human autoreactive T cell clones have been established (Hellman & Stobo, 1982), and these investigators have confirmed specific self-reactivity of individual clones, some to B cells and others to macrophages (Richardson & Stobo, 1982). It has also been demonstrated that autoreactive murine T cell hybridomas can be activated by syngeneic Ia positive cells in serum free medium, thus excluding the possibility that adsorbed foreign serum components were responsible for the observed reactivity in this system (Glimcher & Shevach, 1982).

Con A-induced suppression is thought to parallel *in vivo* activity of a variety of suppressor cells (Sakane & Green, 1977; Shou, Schwartz & Good, 1976), and deficiencies of their performance have been shown in several autoimmune diseases (Sakane, Steinberg & Green, 1978b). There is evidence derived from normal healthy volunteers linking Con A-induced suppression to that subset of T lymphocytes which proliferates in the AMLR (Sakane & Green, 1979; Innes *et al.*, 1979).

In this study, we demonstrate significant impairment of the AMLR in our patients, while Con A-induced suppression in this group appears normal.

MATERIALS AND METHODS

Patients with PM/DM. Diagnosis was based on the criteria of Bohan and Peter (Bohan *et al.*, 1977). All patients included in the study had 'probable' or 'definite' disease by these criteria. Patients with malignancy, overlap syndromes (as defined by the presence of a second established rheumatologic diagnosis), and with childhood DM were excluded. All patients described as 'active' were studied after diagnosis was established, and before treatment was begun. These patients had elevated muscle enzymes and weakness at the time of study. All, however, were ambulatory and without other intercurrent illness. Patients described as 'inactive' were studied upon return to their physicians for routine check-up. These latter patients were in clinical and biochemical remission at the time of study. Inactive patients on pharmacological doses of corticosteroid (greater than 7·5 mg/day prednisone equivalent) were excluded; patients on smaller doses of corticosteroid (i.e. 7·5 mg/day or less) were studied at least 24 h after a dose of medication since it has been demonstrated that the AMLR returns to normal levels within 24 h of administration of an oral dose of prednisone (Hahn *et al.*, 1980). Patients on anti-malarial or anti-inflammatory medication were studied at least 24 h after a dose of medication. Patients on cytotoxic immunosuppressive medications were excluded. No patients studied had light or electron microscopic criteria for the diagnosis of inclusion body myositis (Carpenter *et al.*, 1978). All patients recently diagnosed had electron microscopic study of their biopsy specimens. Peripheral venous blood was obtained from all patients and normal healthy controls between 8 and 10 a.m. Patients were age, sex and race matched with normal healthy controls receiving no medications. For technical reasons, not all assays were

performed on each patient. Each patient is assigned a patient number, which is used consistently in all tables. Paired normal subjects are similarly identified. Except where noted, assays on patients and their paired normal controls were performed concurrently.

Preparation of lymphocytes and purification of T cells and non-T cells. Peripheral blood mononuclear cells (PBM) were isolated by centrifugation on a Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA) gradient. T and non-T cells were separated by rosetting twice with sheep erythrocytes (SRBC). Briefly, 100×10^6 cells/ml were incubated with 5 ml SRBC absorbed heat-inactivated fetal calf serum and 5 ml of 10% SRBC in RPMI 1640. The mixture was incubated 1 h at 4°C and then gently resuspended and sedimented over Ficoll-Hypaque. Rosetted and non-rosetted fractions were subjected to repeated rosette formation overnight and subsequent density gradient centrifugation. Cells recovered from the rosetted pellet by lysis with 0·14 M NH_4Cl were used as responder cells and were greater than 98% E rosette positive. Non-T fractions contained 55–65% sIg positive cells, 25–35% esterase positive cells and less than 5% E rosette positive cells.

Autologus MLR. One hundred thousand T cells were cultured with 1×10^5 mitomycin C treated (50 μg mitomycin C/2×10^6 cells/ml) non-T cells in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human AB serum, 1 mM L-glutamine, 25 mM HEPES buffer, 100 u/ml penicillin and 100 μg/ml streptomycin. Triplicate cultures were carried out in round bottom microtitre plates (Linbro Chemical Co., Hamden, Connecticut, USA) in 5% CO_2 and 95% air at 37°C. On day 5 of the culture, 0·5 μCi ^{3}H-thymidine (3 Ci/mMol, New England Nuclear, Boston, Massachusetts, USA) was added per well and the cultures were harvested 16 h later using a MASH harvester. Uptake of ^{3}H-thymidine was measured by liquid scintillation counter. The results are expressed as counts per minute (ct/min) and as delta (Δ ct/min) where:

$$\Delta \text{ ct/min} = (\text{ct/min of AMLR cultures}) - (\text{ct/min of responders cultured alone}).$$

Generation of suppressor cells. Suppressor T cells were generated by incubating 3×10^6 unseparated PBM/ml of complete culture medium with 40 μg/ml of Con A (Miles-Yeda, Rehovot, Israel) in 16 mm flat bottomed plates (Costar, Cambridge, Massachusetts) in 5% CO_2 and 95% air at 37°C for 48 h. Control cultures were incubated under the same conditions without Con A. After 48 h the cells were washed thoroughly, irradiated with 3,000 rad utilizing an X-ray source and assayed for suppressor cell function. To control for the effect of residual Con A, 40 μg/ml Con A was added to some control cultures immediately before washing.

Assay of suppressor cell function. Suppression of phytohaemagglutinin (PHA)-induced lymphocyte proliferation was measured by culturing 1×10^5 X-irradiated Con A activated or control cells and 1×10^5 fresh allogeneic responder cells with a 1:100 final dilution of a stock solution of PHA (GIBCO Laboratories, Grand Island, New York, USA). Cultures were performed in triplicate in round bottom microtitre plates in a final volume of 0·2 ml. Proliferation of the responder cells was measured by the incorporation of ^{3}H-thymidine (3 Ci/mMol; 0·5 μCi/well; New England Nuclear) for the final 16 h of a 3 day culture. The cells were processed on a MASH unit and ^{3}H-thymidine uptake was measured on a liquid scintillation counter (Beckman, Inc., Irvine, California, USA). All results are expressed as mean counts per minute (ct/min) per culture ± standard deviation and as % suppression calculated as follows:

$$\% \text{ suppression} = 1 - \frac{\begin{array}{c}\text{Mean ct/min of PHA stimulated} \\ \text{cultures containing Con A} \\ \text{activated cells}\end{array} - \begin{array}{c}\text{Mean ct/min of unstimulated} \\ \text{cultures containing Con A} \\ \text{activated cells}\end{array}}{\begin{array}{c}\text{Mean ct/min of PHA stimulated} \\ \text{cultures containing} \\ \text{control cells}\end{array} - \begin{array}{c}\text{Mean ct/min of unstimulated} \\ \text{cultures containing} \\ \text{control cells}\end{array}} \times 100.$$

Statistical analysis. In our study, patients were paired with age and sex matched normal subjects. Because our study population was not a random sample from the population at large, there was no presumption that our results would follow a normal distribution. All data were therefore analysed without assumptions about the distribution of data; the Wilcoxon signed rank test for paired data

(Hollander & Wolfe, 1973) was utilized to test for significant differences between patients and controls, and between active and inactive patients. Results were analyzed for normality by means of the Wilk–Shapiro test; where results were found to follow a normal distribution, the *t*-test for paired data was also performed; there were no differences between results of the Wilcoxon signed rank test and the *t*-test for paired data. All data are presented as analysed with the Wilcoxon signed rank test.

RESULTS

AMLR reactivity of normal subjects and patients with PM/DM

The AMLR proliferative response was studied in nine PM/DM patients and their age, race, and sex matched normal controls. As illustrated in Table 1, this AMLR proliferative response was significantly depressed in patients, with a P value of less than 0·01 using the Wilcoxon signed rank test for paired data. This non-parametric test was used because the population of delta cpm was shown not to follow a normal distribution by the Wilk–Shapiro test. The variation in Δ ct/min of patients and normals is accounted for partially by the wide variation in their ages.

A time course study of the AMLR in normal individuals and in PM/DM patients revealed a continuous low reactivity by PM/DM lymphocytes throughout the culture period (Fig. 1). Thus, a

Table 1. Autologous MLR in normal subjects and patients with PM/DM

Subject*	Age	Activity of disease†	^{3}H-thymidine incorporation ct/min in cultures containing: Responders alone‡	Responders + stimulators§	Δ ct/min¶
PM patient 1	21	I	7,965±3,823	17,098±2,827	9,133
Normal 1	26		750± 568	10,012±6,738	9,262
PM patient 2	23	A	3,060± 591	4,028±2,865	968
Normal 2	23		4,528± 888	20,202±3,115	15,674
PM patient 3	45	I	363± 228	1,145± 261	782
Normal 3	45		62± 74	1,072± 596	1,010
PM patient 4	47	A	3,185± 233	11,646±1,135	8,461
Normal 4	48		3,924± 314	14,546±1,668	10,722
PM patient 5	50	A	800± 80	1,038± 345	238
PM patient 6	55	I	698± 260	329± 193	—
Normal 5	51		1,148± 299	3,893± 36	2,745
PM patient 7	57	A	1,669± 265	425± 263	—
Normal 6	60		2,954± 188	6,934± 226	3,980
PM patient 8	62	I	406± 468	211± 13	—
Normal 6	60		2,954± 188	6,934± 226	3,980
PM patient 9	67	I	730± 670	1,088± 722	358
Normal 7	62		594± 389	2,253± 658	1,659

* Normal controls were age, sex and race matched with each patient and were tested concurrently.

† Assessed as described under Materials and Methods.

‡ Two hundred thousand T cells cultured alone.

§ One hundred thousand T cells were cultured with 1×10^5 mitomycin C-inactivated autologous non-T cells.

¶ Significantly depressed in patients, $P < 0{\cdot}01$, Wilcoxon signed rank test for paired data.

A = active disease; I = inactive disease.

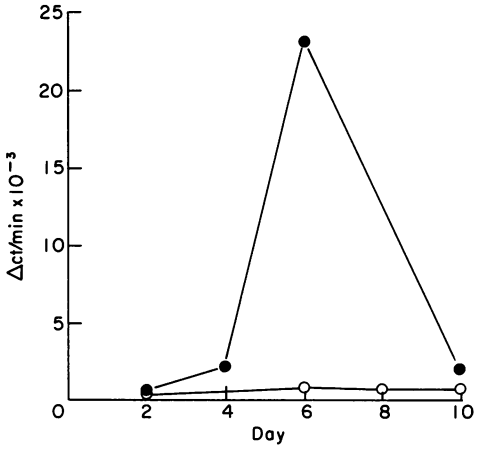

Fig. 1. Kinetic study of the AMLR in two age matched normal controls (●) and three patients with PM/DM (○). One hundred thousand responder T cells were cultured with 1×10^5 stimulating non-T cells over varying periods of time. Mean values for normal controls and PM/DM patients are plotted. The PM/DM patients showed low reactivity throughout the culture period. These assays, for technical reasons, were not performed concurrently.

difference in the time course of the proliferative response cannot account for the reduced AMLR in patients.

A dose–response study performed by culturing 1×10^5 PM/DM T cells with an increasing number of PM/DM non-T stimulator cells showed that increased stimulator cell numbers were unable to provoke an AMLR response (Table 2). Thus, the decreased AMLR in PM/DM patients cannot be ascribed to subthreshold numbers of stimulator cells. Nor was the AMLR defect in these patients due to an abnormal proportion of monocytes in the stimulator population, as the percentage of peripheral blood monocytes in our patients ranged from 4–9% (normal 2–10%).

Finally, patients were demonstrated to possess reactivity in allogeneic MLR which was similar to that of their controls (Table 3); this finding argues against a non-specific depression of all T lymphocyte functions in these patients. Comparison of active with inactive patients showed similar impairment of AMLR proliferative response in both groups.

Con A-induced suppression by lymphocytes from normal subjects and patients with PM/DM

Con A-induced suppression of proliferative responses of lymphocytes was studied in four patients

Table 2. Dose–response study of the AMLR in PM/DM patients

Subject	Number of non-T stimulating cells*	ct/min	Δ ct/min
PM patient 7	None	1,669±265	—
	1×10^5	425±263	—
	2×10^5	322±100	—
	3×10^5	151±100	—
PM patient 8	None	406±468	—
	1×10^5	211± 13	—
	2×10^5	189± 90	—

* One hundred thousand responding T cells were cultured with varying numbers of mitomycin C treated autologous non-T cells for 6 days.

Table 3. Allogeneic MLR in PM/DM patients

Subject	Responders alone	Responders + autologous stimulators*	Responders + allogeneic stimulators†
PM patient 3	363 ± 228	1,145 ± 261	19,473 ± 618
Normal 3	62 ± 74	1,072 ± 596	9,032 ± 1,370
PM patient 9	730 ± 670	1,088 ± 722	26,883 ± 2,266
Normal 8	594 ± 389	2,253 ± 658	41,082 ± 5,654

* One hundred thousand responding T cells were cultured with 1×10^5 mitomycin C treated autologous non-T cells for 6 days.

† One hundred thousand responding T cells were cultured with 1×10^5 mitomycin C treated allogeneic non-T cells for 6 days.

Table 4. Con A-induced suppression mediated by lymphocytes from normal subjects and patients with PM/DM

Subject	Con A activation of cells in first culture	PHA stimulated proliferation (^{3}H-Tdr Uptake) Unstimulated ct/min	PHA stimulated ct/min	% Suppression*
Patient 6	−	9,573 ± 1,107	139,061 ± 18,587	32
	+	2,032 ± 812	90,357 ± 10,406	
Normal 9	−	4,487 ± 1,056	133,894 ± 1,870	10
	+	1,215 ± 287	118,090 ± 10,337	
Normal 6	−	11,609 ± 2,268	134,504 ± 2,126	37
	+	3,851 ± 531	80,723 ± 9,962	
Patient 4	−	19,296 ± 1,825	112,910 ± 2,019	47
	+	2,936 ± 275	52,382 ± 3,337	
Normal 4	−	26,138 ± 511	100,289 ± 2,298	20
	+	2,663 ± 474	62,144 ± 5,188	
Patient 10	−	1,148 ± 172	21,936 ± 6,364	14
	+	486 ± 87	18,266 ± 1,052	
Normal 10	−	2,010 ± 185	19,839 ± 2,023	20
	+	507 ± 105	14,684 ± 2,568	
Normal 11	−	2,228 ± 255	20,814 ± 1,923	25
	+	1,045 ± 277	15,029 ± 2,012	
Patient 11	−	7,291 ± 953	53,452 ± 4,911	46
	+	1,968 ± 366	26,930 ± 2,270	
Normal 12	−	5,486 ± 515	75,212 ± 3,362	43
	+	1,890 ± 377	41,429 ± 3,312	
Normal 15	−	4,900 ± 667	68,931 ± 6,766	86
	+	400 ± 78	9,458 ± 3,329	

* Calculated as described in Materials and Methods.

with PM/DM (Table 4). All subjects studied (patients and normals) demonstrated levels of suppression comparable to those found in normal subjects in the literature (Fineman, Mudawwar & Geha, 1979; Smith & Svejgaard, 1981). Con A-induced suppression of PHA driven proliferation by lymphocytes from the two groups of subjects differed very little, with a mean suppression of 35% for four patients and 34% for seven controls ($p > 0{\cdot}05$). These populations were shown by the

Wilk–Shapiro test to follow a normal distribution, and were analysed by the *t*-test for paired data as well as the Wilcoxon signed rank test for paired data. Results of the two methods of statistical analysis were not different. Comparison of active with inactive patients showed no differences between the two groups for these functional assays.

Dissociation of AMLR reactivity and Con A-induced suppressor cell function

Con A suppressor cell function was normal in Patient 6, in whom the AMLR proliferative response was clearly deficient. Furthermore in all the patients we have studied, Con A-induced suppressor function has been normal whereas in all patients except one, the AMLR was significantly reduced. There thus appears to be a dissociation between AMLR proliferative response and Con A-induced suppression in our group of PM/DM patients.

DISCUSSION

The AMLR, which denotes proliferation of T cells in response to autologous non-T cells, appears to be a complex and important example of autoreactivity or autorecognition (Weksler, Moody & Kozak, 1981; Battisto & Ponzio, 1981). T cell recognition of cell surface structures encoded by Ia/DR genes of the major histocompatibility complex is the basis of the AMLR (Weksler *et al.*, 1981), and such recognition has been shown essential for the afferent limb of normal immune responses to foreign antigens (Shevach, 1976). It has further been shown that immunoregulatory activities of T cells are activated in AMLR cultures. It has therefore been hypothesized that T cell recognition of self, as reflected in appropriate AMLR reactivity, is essential for the maintenance of normal immune homeostasis (Smith & Talal, 1982).

The finding of an impaired AMLR response in our group of patients with PM/DM thus suggests the presence of abnormal immunoregulation in this expression of autoimmunity. It is currently unclear whether this defect precedes disease expression or results from it, through the depletion of AMLR reactive T cell subsets by their commitment as part of the disease process. We do not feel that the decrease in AMLR reactivity could be due to nonspecific illness as those patients with inactive disease who were clinically well also showed the AMLR defect. Further, in another study, the AMLR has been shown to be unhindered in at least one group of normal individuals who were non-specifically ill (Moody *et al.*, 1979).

Sakane & Green (1979) have shown that the Con A-inducible subset of T suppressor cells resides within the AMLR reactive subset of T cells. In normal individuals, Innes *et al.* (1979) have demonstrated an inability to generate Con A-induced suppressors from cultures containing only T cells and postulate that events in the AMLR are necessary for this induction to occur. Taken together with our results, these findings suggest strongly that functions dependent on recognition events occurring in AMLR cultures (e.g., the activation of precursors of the Con A-induced suppressor cell) may proceed unhindered despite the absence of observed T cell proliferation in the AMLR.

Smolen *et al.* (1982) have recently clarified interactions between T cell subsets and specific non-T mononuclear cell types in AMLR cultures. Although T cell subset numbers in our patients with PM/DM are normal (data not shown), the interaction between specific cell types may be abnormal, thus producing the observed impairment in AMLR proliferative response. Clarification of these issues is in progress.

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