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Research Article

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Lymphotoxin Formation by Lymphocytes and Muscle in Polymyositis

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ABSTRACT Muscle pieces from 11 patients with dermatomyositis or polymyositis were incubated with autologous peripheral blood lymphocytes and the supernates examined for the production of lymphotoxin, a mediator of delayed hypersensitivity, using human fetal muscle monolayers as the target cell. In the case of all 10 active patients, production of lymphotoxin was demonstrated. This mediator was also demonstrated when muscle alone was incubated from two patients with extensive cellular infiltration. Lymphotoxic activity was not found in supernates obtained by incubation of muscle from nine control subjects with their autologous peripheral blood lymphocytes.

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INTRODUCTION

The pathogenesis of the varied manifestations of muscle injury in polymyositis¹ has not been characterized. A

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Dr. Johnson is the recipient of an Arthritis Foundation Postdoctoral Fellowship and a Merck Faculty Development Award. He was also a Trainee in Arthritis for the period from 1969 to 1971.

Dr. Ziff is the recipient of a U. S. Public Health Service Research Career Award.

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¹"Polymyositis" as used in this paper is intended to include both polymyositis and dermatomyositis.

number of clinical and experimental findings favor an immune mechanism. Positive tests for rheumatoid factor (1-3) and antinuclear antibody (4) have been reported. In some patients, characteristic findings of systemic lupus erythematosus (5-8) or rheumatoid arthritis (2) have accompanied those of polymyositis. Recently focal deposits of C'3, IgG, and IgM have been identified in the vessels of some patients with active polymyositis (9) and in association with degenerating muscle fibers.

Experimental models of myositis induced in rats immunized with allogeneic muscle in Freund's adjuvant (10, 11) and in guinea pigs similarly immunized with rabbit muscle (12) have also suggested an immunologic basis for the disease. Weakness and histologic lesions resembling those of human polymyositis were seen in recipient animals. Kakulas (13, 14) has shown that regional lymph node cells from rats immunized with rabbit muscle homogenate in Freund's adjuvant induce cellular damage when applied to rat fetal muscle explants in vitro. Passive transfer of myositis with sensitized lymphoid cells has been reported in the guinea pig (15) and rat (16). In none of these models were autoantibodies or mediators of delayed hypersensitivity, i.e. lymphokines (17) described.

The muscle infiltrate in typical cases of polymyositis (18) contains largely lymphocytes and monocytes with occasional plasma cells and polymorphonuclear leukocytes, resembling lesions for which a delayed hypersensitivity mechanism has been established. Consistent with this have been the observations of Currie and co-workers (19-21) who showed that lymphoid cells from polymyositis patients, when layered on fetal muscle explants, induce cellular damage. Anti-tissue antibodies directed at either muscle, tumor, or both have been demonstrated in a few cases of polymyositis associated with malignancy (22-24). Antibodies with specificity for muscle have not been demonstrated in cases not

associated with malignancy (25, 26). Thus, in spite of suggestive evidence favoring either a humoral or cellular immune mechanism in polymyositis, neither antibody nor lymphokines have been shown to produce muscle injury in the preponderant patient group.

In the studies described here, evidence has been obtained for the production of a lymphokine, lymphotoxin (27-30), by peripheral blood leukocytes from polymyositis patients when these were incubated with autochthonous muscle in vitro. The activity of the lymphotoxin was measured by its action on muscle cells in a leukocyte-free system in vitro. This mediator was produced by the cells of all of 10 active polymyositis patients examined.

METHODS

Patients. Patients with polymyositis or dermatomyositis were admitted to Parkland Memorial Hospital, Children's Medical Center and the Dallas Veterans Administration Hospital. Their salient clinical features are listed in Table I. Three patients had uncomplicated polymyositis (type I of Walton and Adams [18]); six had dermatomyositis (type II); one, D. M., had type III polymyositis with polyarthritis and an SSCA² titer of 1:112; and one, A. P., had polymyositis associated with a ductal carcinoma of the breast (type IV). All had abnormal findings on electromyography which were compatible with the diagnosis of polymyositis. Muscle biopsy carried out in 11 patients showed a wide range of histologic findings, characteristic of polymyositis in 9. The biopsy was read as normal in one case (S. L.). In A. P., the patient with carcinoma of the breast, the major alteration noted was marked swelling of the muscle spindles. With the exception of K. B., who did not appear clinically active at the time of assay, all cases showed elevations of muscle enzymes. In 8 of the 10 active patients at least two enzyme levels were elevated.

Three patients had been under treatment with Prednisone (McKesson Laboratories, Bridgeport, Conn.). G. R. had received varying dosage levels for 2 yr, but was in relapse at the time of study on 15 mg daily; B. G. had received 60 mg daily for 3 wk before study, but was responding poorly; and P. R. had received 60 mg daily for 2 days. ESR was elevated in 5 of 10 patients; the latex fixation test was positive in 3 of 11; antinuclear fluorescence tests were negative in all.

The control group consisted of seven women undergoing elective abdominal surgery: tubal ligation in the case of four; and cholecystectomy, common bile duct exploration, and hysterectomy, one each. The lymphocytes of all responded to phytohemagglutinin (31) and to one-way mixed lymphocyte culture (32). Supernates from these stimulated peripheral blood leukocytes showed lymphotoxin activity in all cases.

Two other control patients were studied. One, G. B., was an 8 yr old girl with generalized weakness and edema of

the face, hands, and legs; SGOT 25 U, CPK 4 U (normal < 30), and aldolase 10 U (normal < 8). Biopsy of muscle showed typical vascular lesions of polyarteritis nodosa. The second, B. R., a 64 yr old man with adult onset muscular dystrophy, gave a 13 yr history of progressive weakness. He had a stork-limb type of atrophy peripherally in all extremities, centrifugal weakness, and selective hypertrophy of the deltoid muscles; SGOT, 50 U, CPK 56 U (normal < 71), and aldolase 1.9 U (normal < 2.5).

Incubation of leukocytes and muscle. 35-60 ml of heparinized blood and 2-5 g, wet weight, of skeletal muscle were obtained from each patient and control subject and incubated as outlined in Fig. 1. Leukocyte suspensions in the case of the patients 1-4 and four of the control patients were prepared by sedimentation in 10% Dextran (Cutter Laboratories, Inc., Berkeley, Calif.). All other patient and control leukocyte suspensions were separated by the Hypaque-Ficoll technique (33) which produced populations of lymphocytes and monocytes containing 1% or less of polymorphonuclear leukocytes. Muscle was obtained from the rectus femoris of seven polymyositis patients, the deltoid of three, and the gastrocnemius of one. It was obtained from the rectus abdominus in the case of all of the control patients. Fat was resected from the muscle specimen and it was then cut into small pieces (1 × 1 mm) immediately after excision and teased apart in culture medium to exposure a maximum surface area. For each measurement two to four replicate samples of 100-150 mg of autochthonous muscle and 5 × 10⁶ leukocytes were incubated both alone and together in 1 ml of medium as shown in Fig. 1. The culture medium, either minimal essential medium (MEM) or RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), was enriched with vitamins and glutamine and contained either 10% inactivated human agammaglobulinemic serum (Hyland Laboratories, Los Angeles, Calif.) in the case of leukocytes prepared by the Dextran sedimentation technique or 10% inactivated fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) in the case of leukocytes purified by the Hypaque-Ficoll technique (33). In all instances, penicillin, 100 U/ml, and streptomycin, 90 µg/ml, were added to the culture media. The various lots of agammaglobulinemic serum and fetal calf serum used were screened before use and shown to be free of cytotoxicity in the assay system described below. Test supernates were dialyzed against culture medium and centrifuged before

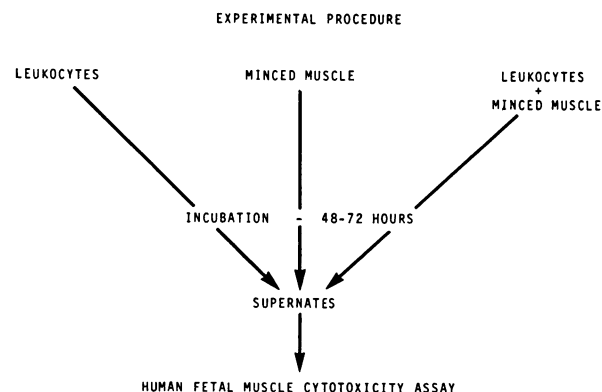


FIGURE 1 Experimental procedure for incubation of minced muscle and peripheral blood leukocytes in production of lymphotoxic supernates.

² Abbreviations used in this paper: CPK, creatine phosphokinase; ESR, erythrocyte sedimentation rate; MEM, minimal essential medium; PHA, phytohemagglutinin; RPMI, Roswell Park Memorial Institute; SGOT, serum glutamic oxaloacetic acid transaminase; SSCA, sensitized sheep cell agglutination.

TABLE I
Clinical Findings in Polymyositis Patients

Subject	Age	Sex	Duration of symptoms	Type of polymyosities	Duration prednisone therapy	Enzyme levels*	
						Enzyme	U
	yr		months				
B. C.	43	F	12	I	—	SGOT	257
						CPK	586
						Aldolase	16
S. L.	13	F	14	II	—	SGOT	110
						CPK	7*
						Aldolase	2
K. L.	13	F	12	II	—	SGOT	250
						CPK	4*
						Aldolase	4
W. A.	50	M	4	I	—	SGOT	221
						CPK	390
						SGOT	60
C. F.	9	F	12	II	—	CPK	55
						Aldolase	8
						SGOT	70
G. R.	8	M	21	II	12 months	CPK	36*
						Aldolase	5
						SGOT	45
K. B.	10	F	67	II	—	SGOT	61
						CPK	59
						SGOT	370
A. P.	60	F	6	IV	—	CPK	2300
						SGOT	250
						CPK	626
B. G.	57	M	24	I	3 wk	Aldolase	4
						SGOT	404
						CPK	692
P. R.	60	F	0.8	II	2 days	Aldolase	25
						SGOT	404
						CPK	692
D. M.	39	F	18	III	—	Aldolase	25
						SGOT	404
						CPK	692

* Normal values: SGOT <50 U, CPK <50 U, Aldolase <8 U. In the case of K. L., the normal CPK value was <25 U and in the case of S. L. and G. R. it was <30 U.

cytotoxicity assay. After dialysis, specimens were made up to 10% serum concentration, using either inactivated human agammaglobulinemic serum or fetal calf serum depending on the original culture conditions.

Cytotoxicity assay. The assay for cytotoxic activity was similar to that described by Kolb and Granger (27) and Kolb, Williams, and Granger (31) for lymphotoxin with the important exception that human fetal muscle (Grand Island Biological Co., Grand Island, N. Y.), which was maintained as a stock culture in this laboratory, was used instead of mouse L-929 fibroblasts. It was determined by utilizing dilutions of lymphotoxin containing supernates that this cell line had greater sensitivity than several commercially available L-929 cell lines. Before the utilization of this target tissue, a lymphotoxin-containing supernate was prepared by PHA stimulation of normal lymphocytes and purified by the methods of Granger et al. (27-29, 31) as described in the next section. This preparation produced cytotoxicity as demonstrated both histologically and by inhibition of incorporation of ¹⁴C-labeled amino acids into cellular protein in both L-929 fibroblast and human fetal muscle monolayers.

In the assay adopted for the study of supernates of muscle-leukocyte incubation mixtures, 4- to 5-day old monolayers were selected for uniformity of growth. Supernates were applied to the monolayers in Leighton tubes for 48 hr at 37°C in an atmosphere of 95% oxygen and 5% CO₂. Supernates were then removed and 0.5 μCi of ¹⁴C-labeled amino acid hydrolysate (Schwarz Bio-Research, Inc., Orangeburg, N. Y.) in an amino acid-free medium (27) was added for a 50 min labeling period. Incorporation of ¹⁴C-labeled amino acids was then halted by immersing the Leighton tubes containing the monolayers in ice water, and the medium was removed by aspiration. Cellular protein was extracted from the monolayers with three hot trichloroacetic acid extractions using 1 drop of fetal calf serum per tube as a carrier. The precipitated protein was then dissolved in 0.5 M potassium hydroxide and counted by standard liquid scintillation techniques. In each test series a group of 6-10 nontoxic supernates were tested as controls to determine the reproducibility of the method. These consisted of samples of culture medium from the same batch used in the test supernates, incubated without cells or muscle for 3-4 days. The serum contained in the con-

trol medium was identical to that present in the supernates in each test series.

In a few cases in which supernates tested before and after dialysis were found to show significant cytotoxicity, this activity was not eliminated by dialysis.

Preparation and purification of lymphotoxin. Mononuclear cell populations were obtained by the Hypaque-Ficoll technique (33) using normal human peripheral blood or human spleen cell suspensions obtained from trauma patients subjected to emergency splenectomy. These were stimulated with PHA as described by Kolb and Granger (28). Supernates were harvested on the 4th or 5th day of incubation, immediately precipitated with 60% NH_4SO_4 , and the precipitates so obtained reconstituted in $\frac{1}{4}$ the original volume in sterile water prepared for injection. After dialysis overnight in the cold against 1000 vol of Hartmann's solution for intravenous use (Cutter Laboratories, Inc., Berkeley, Calif.), samples of 4 ml were applied to a sterilized Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column (1.5×90 cm) poured under aseptic conditions and equilibrated with the same buffer. Eluted fractions of the lymphotoxin preparation were dialyzed against sterile water and lyophilized. Portions of the lyophilized column fractions were reconstituted with RPMI-1640 (Grand Island Biological Co.) to which 10% inactivated fetal calf serum and antibiotics were added and assayed for cytotoxicity. Fractions showing major cytotoxicity were pooled and stored at -70°C for use in lymphotoxin assays as positive controls. To render dialysis tubing (Union Carbide Corp., New York) nontoxic it was sequentially extracted with 5% acetic acid and boiling 1% sodium bicarbonate, then thoroughly rinsed in sterile water and autoclaved for 10 min at 15 lb. per in² at 120°C . Dialysis of cytotoxic supernates before testing was performed in dialysis membranes which had been similarly treated. Between fractionations the column was equilibrated with normal saline containing sodium azide and disodium EDTA in a concentration of 1×10^{-3} M to prevent bacterial or fungal contamination. Immediately before fractionation of toxic supernates, it was washed with 2 column volumes of Hartmann's solution without sodium azide or disodium EDTA.

Characterization of cytotoxic supernates. Chromatography of the purified preparation of lymphotoxin described above and several cytotoxic supernates obtained from muscle-lymphocyte incubation mixtures, was performed in a sterilized glass column containing autoclaved Sephadex G-200 which was poured and equilibrated as described above. Hartmann's solution for intravenous use (Cutter Laboratories, Inc., Berkeley, Calif.) was employed as the equilibrating buffer in the manner described for purification of lymphotoxin. Column fractions were dialyzed against sterile water (Cutter Laboratories, Inc.) using dialysis tubing (Union Carbide Corp.) which had been pretreated to remove cytotoxic materials as described above.

RESULTS

Histologic changes produced by cytotoxic supernates. When supernates obtained from incubation of autochthonous muscle with lymphocytes of patients with polymyositis were added to muscle cell monolayers, evidence of muscle cell injury was observed histologically. Fig. 2A demonstrates the appearance of a human fetal muscle monolayer after 7 days of culture. A relatively

uniform growth of spindle-shaped fibers with elongated processes is seen. Fig. 2B is a high power view of this culture line showing myofibrils within each muscle cell. Fig. 2C shows the appearance of a monolayer which had been exposed for 48 hr to a toxic supernate containing a purified preparation of lymphotoxin. Numerous cells have disappeared or have rounded up and their nuclei take a dark stain. These changes are similar to those described by Granger and Kolb (27) in the L-929 mouse fibroblast strain. Fig. 2D shows similar changes occurring after 48 hr exposure of the muscle monolayer to a culture medium containing dialyzed toxic supernate from leukocytes incubated with autochthonous muscle of a polymyositis patient (G. R.).

Establishment of variability of amino acid- ^{14}C incorporation into monolayers. Amino acid- ^{14}C incorporation into protein, a sensitive and objective measure of viability, gave variable results even though monolayers were selected for histologic uniformity. To take account of this variability, 6–10 control incubations of human fetal muscle monolayers were carried out simultaneously with each set of cytotoxicity measurements under identical conditions. The results of a representative set of control measurements are given in Table II. As shown, with the monolayer employed, a decrease of 888 cpm or 28% from the mean value of 3136 cpm, representing a fall of more than two standard deviations from the mean of the control values, would be significant at the 95% confidence level. In the experiments described, decreases in amino acid- ^{14}C incorporation significant at this level ranged between 24 and 30% in various series of control measurements.

Assay of supernates produced by incubation of muscle and leukocytes of control subjects. When monolayers were exposed to supernates obtained from the various test incubations, two to four replicate determinations

TABLE II
Standardization of Control Human Fetal Muscle Monolayers
Incubated with Amino Acid- ^{14}C Mixture

Tube No.	cpm
1	3861
2	3626
3	3042
4	3029
5	2608
6	2912
7	2876
Average	3136

Counts per minute of monolayers ± 2 SD = 3136 ± 888 . A decrease of $\geq 28\%$ represents the 95% confidence level of reproducibility of the method.



FIGURE 2A Human fetal muscle monolayer after 7 days of culture. Masson trichrome stain. $\times 400$.

were made and the values given represent averages of these determinations. Table III summarizes the results of incubation of leukocytes alone and of muscle alone from normal subjects and the combination of leukocytes and autochthonous muscle of these subjects. Incubation periods varied between 48 and 72 hr. Culture in excess of 72 hr resulted in increasing scatter of replicate values, presumably due to release of non-

specific toxic agents. In no case was significant cytotoxicity observed in the dialyzed supernates from the seven control subjects undergoing elective surgery in the case of supernate from cells alone, muscle alone, or of cells and autochthonous muscle incubated together. It should be noted that the patients with polyarteritis nodosa and muscular dystrophy likewise did not produce significant cytotoxicity. Histologic preparations

of incubated muscle pieces from the control and patient groups alike showed deterioration after the 1st day in tissue culture which was striking after 72 hr.

Assay of supernate produced by incubation of muscle and leukocytes of patients with polymyositis. In contrast to the control subjects, 10 out of 11 patients with polymyositis showed significant cytotoxic activity in the supernates obtained when their leukocytes were in-

cubated with autochthonous muscle (Table IV). The disease of the single patient without cytotoxicity, K. B., had been quiescent for some months, and no longer required corticosteroid therapy. Her muscle biopsy showed mainly atrophy and fibrosis (Table IV). All the other patients (Table I) had active disease. Corticosteroid therapy had been initiated previously in three patients of this group; of these, one

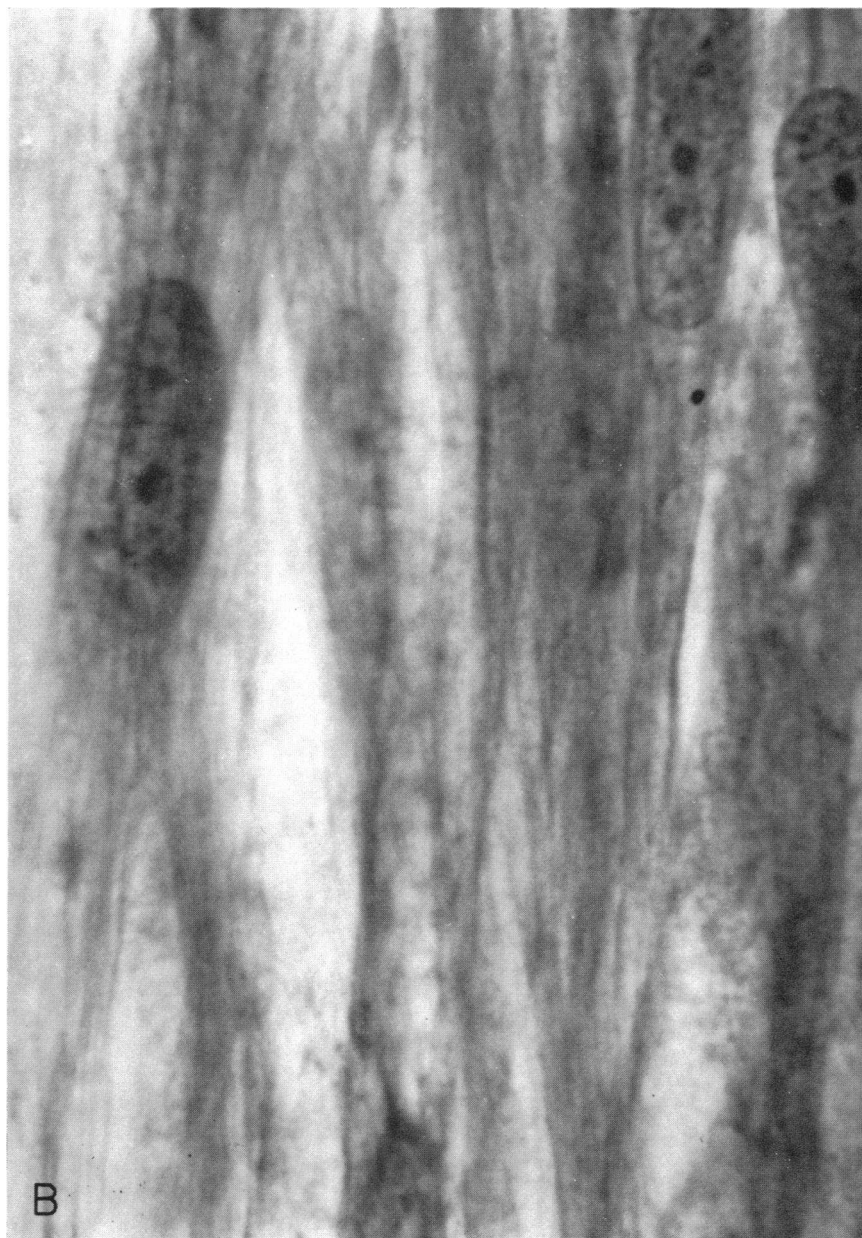


FIGURE 2B Magnification of human fetal muscle monolayer after 7 days of culture. Myofibrils are seen within muscle cells. Masson trichrome stain. $\times 1500$.

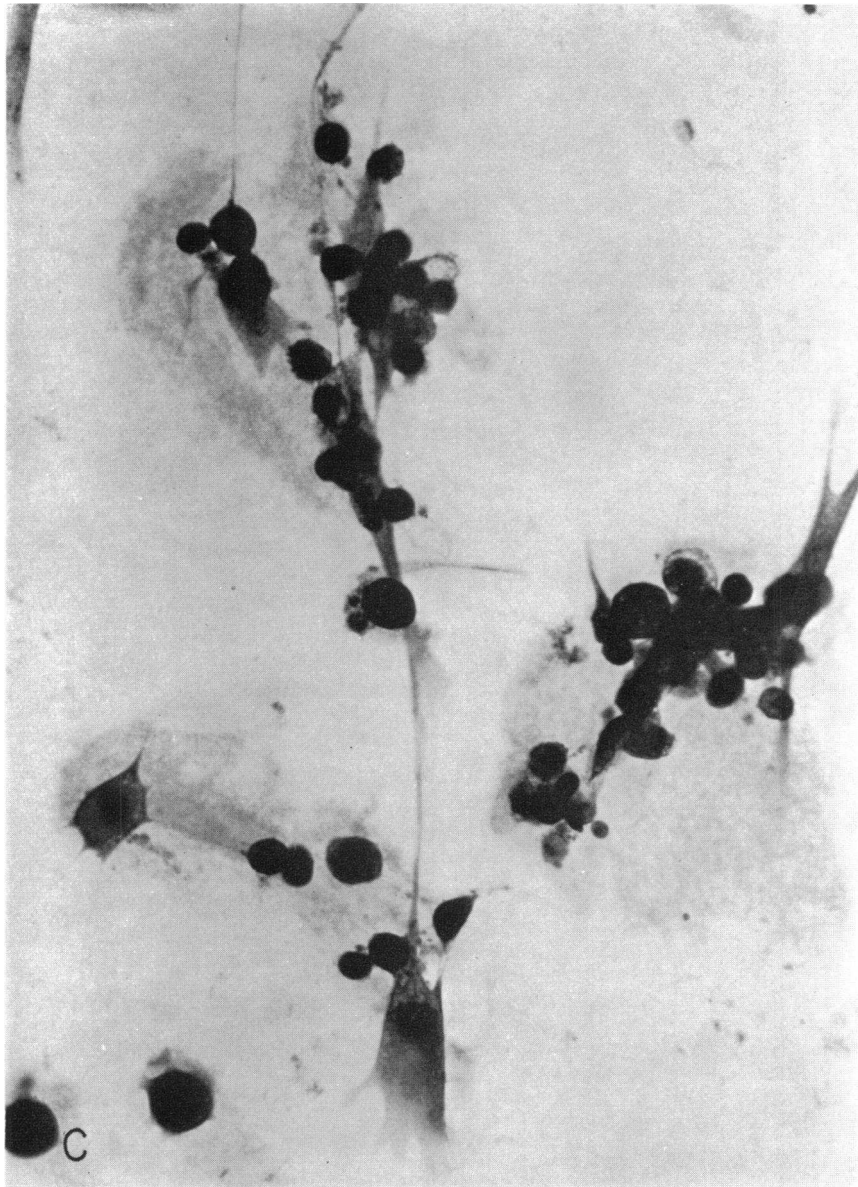


FIGURE 2C Human fetal muscle monolayer after 48 hr exposure to culture medium containing purified lymphotoxin prepared by treatment of human lymphocytes with PHA and chromatography of the supernate on Sephadex G-200. Many cells have either disappeared or rounded up. The nuclei take a dark stain. Masson trichrome stain. $\times 400$.

(P. R.) had received prednisone for only 2 days before biopsy. The other seven active patients had not received steroids.

The supernates of the leukocytes alone of C. F. and G. R., both of whom had severe polymyositis, as manifested by fever, severe weakness, and extensive muscle infiltration (Table IV) produced significant cytotox-

icity. In the case of these two patients incubation of muscle alone also produced cytotoxicity. It is pertinent to note (Table V) that the muscle of these patients contained prominent infiltrates. Impressive, however, was the finding that in the other seven active cases where comparisons of supernates from leukocytes alone, muscle alone, and leukocytes plus muscle were all

performed, only the latter combination produced significant cytotoxicity. Furthermore, the cytotoxicity did not appear, as seen in Table IV, to be merely an additive effect of the individual cytotoxicities of the leukocyte and muscle supernates. Table V shows the correlation between histologic changes in the muscle specimens and cytotoxicity results. It is of interest that

necrosis was not a necessary characteristic of the muscle utilized to stimulate the production of lymphotoxin by autologous lymphocytes as observed in the case of patients S. L. and K. L. In fact, the muscle of S. L. was free of all histologic abnormalities. Only in four patients, B. C., W. A., C. F., and G. R. was necrosis a significant aspect of the histologic changes.

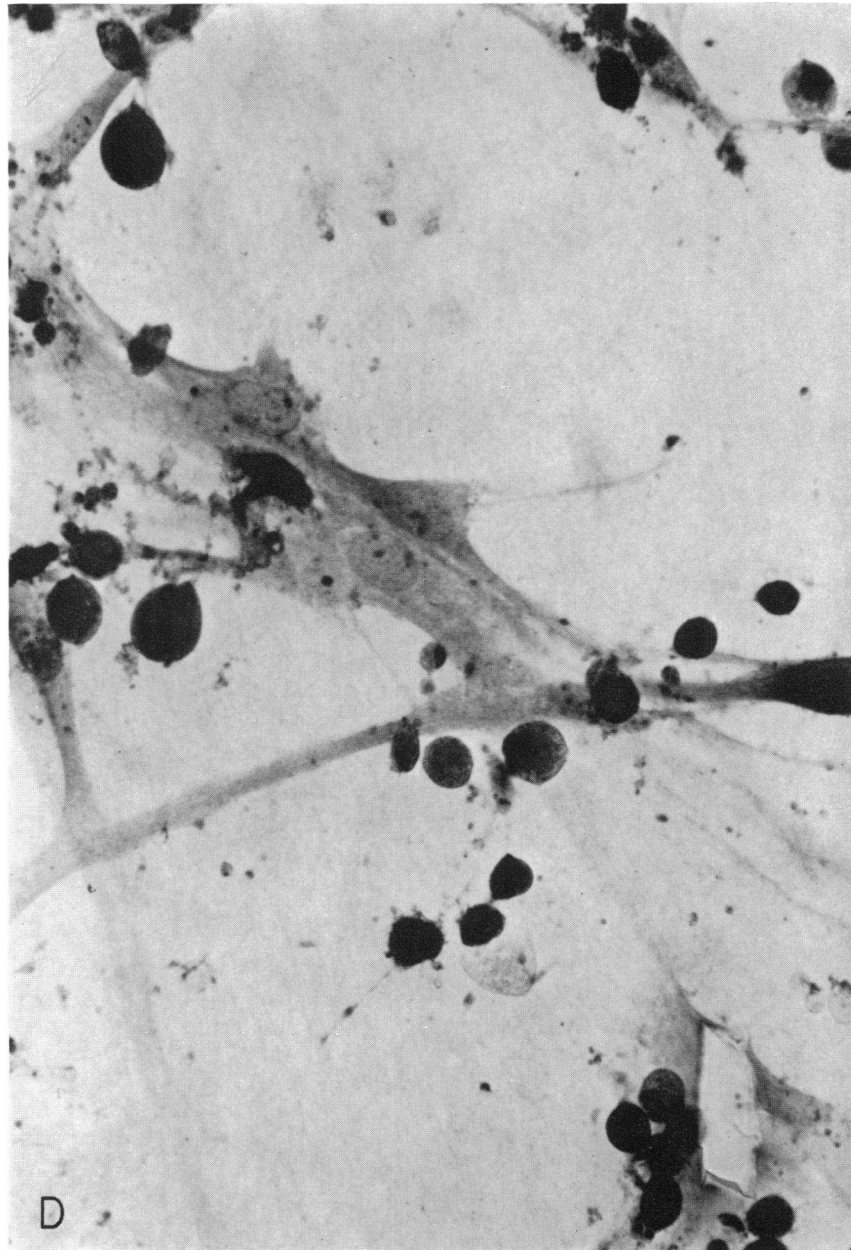


FIGURE 2D Human fetal muscle monolayer after 48 hr exposure to culture medium containing a dialyzed toxic supernate from leukocytes incubated with autochthonous muscle of a polymyositis patient (G. R.). The changes are similar to those shown in Fig. 2C. Masson trichrome stain. $\times 400$.

TABLE III
Results of Cytotoxicity Assays Performed on Supernate from Control Subjects

Subject	Diagnosis	cpm ¹⁴ C in untreated monolayers	cpm ¹⁴ C in monolayers exposed to supernates			% Change ¹⁴ C amino acid uptake compared with untreated monolayers		
			Leukocytes alone	Muscle alone	Leukocytes +muscle	Leukocytes alone	Muscle alone	Leukocytes +muscle
B. F.	Tubal ligation	6,850	5,766	6,400	6,522	-16	-7	-5
D. L.	Tubal ligation	6,850	6,498	10,767	6,333	-5	+57	-8
W. M.	Tubal ligation	6,850	7,403	6,761	7,546	+8	-1	+10
B. K.	Tubal ligation	8,841	8,577	7,429	7,515	-3	-16	-15
R. S.	Cholecystitis	10,484	8,781	N. D.	9,572	-16	N. D.	-9
J. B.	Cholecystitis	4,857	4,762	6,410	3,982	-2	+32	-18
E. S.	Leiomyomata	7,541	8,821	6,787	6,185	+17	-10	-18
G. B.	Polyarteritis Nodosa	1,846	1,938	1,601	3,323	+5	-13	+80
B. R.	Muscular dystrophy	3,001	3,752	4,122	3,014	+25	+37	0

N. D., not done.

The supernates after incubation of muscle alone from two normal controls, D. L. and J. B. (Table II) and three polymyositis patients, S. L., W. A., and K. B. (Table III), showed stimulation of incorporation of ¹⁴C-labeled amino acids at a significant level, as did the supernate from K. B. of muscle and leukocytes incubated together. This activity, which was nondialyzable, was not further evaluated.

Effect of tumor tissue on leukocytes of patient with polymyositis and carcinoma of breast. The availability of both normal breast tissue and tumor tissue from patient A. P. who had a carcinoma of the breast permitted application of the techniques used for muscle

to seek evidence for delayed hypersensitivity to the tumor as well as muscle in this patient (Table VI). The proportion of wet weight of normal breast tissue and tumor to leukocytes was identical to that used in the studies of muscle. As shown, there was no evidence for production of lymphotoxin by leukocytes incubated with breast tissue or tumor. However, the addition of autologous muscle or of PHA to the leukocytes lead to the production of cytotoxic activity.

Effect of corticosteroid on cytotoxic activity. In view of the evidence that lymphotoxin-derived cytotoxicity, released by lymphocytes on antigenic stimulation (27, 30) or upon stimulation by PHA (27, 28,

TABLE IV
Results of Cytotoxicity Assays Performed on Supernates from Polymyositis Patients

Subject	Type of polymyositis	cpm ¹⁴ C in untreated monolayers	cpm ¹⁴ C in monolayers exposed to supernates			% Change amino acid- ¹⁴ C uptake compared with untreated monolayers		
			Leukocytes alone	Muscle alone	Leukocytes +muscle	Leukocytes alone	Muscle alone	Leukocytes +muscle
B. C.	I	4,857	4,459	—	2,911	-8	N. D.	-40*
S. L.	II	8,906	10,686	17,456	4,722	+20	+96	-47*
K. L.	II	8,906	9,175	6,403	2,136	+3	-18	-76*
W. A.	I	5,705	6,106	8,853	3,305	+7	+53	-42*
C. F.	II	12,150	3,278	366	3,159	-73*	-97*	-74*
G. R.	II	12,150	2,552	3,645	851	-79*	-70*	-93*
K. B.†	II	3,232	3,715	4,551	4,556	+13	+41	+41
A. P.	IV	4,407	3,922	4,400	1,899	-12	0	-57*
B. G.	I	4,407	3,729	2,736	2,154	-15	-28	-50*
P. R.	II	6,595	5,961	4,890	2,711	-10	-26	-59*
D. M.§	III	6,423	7,075	7,306	4,169	+10	+14	-35*

N. D., not done.

* Values significant at 95% confidence level or greater.

† Clinically inactive dermatomyositis.

§ Cytotoxicity tested against L-929 fibroblasts only.

TABLE V
Correlation of Muscle Pathology with Cytotoxicity of Supernates

Subject	Supernate cytotoxicity			Pathologic change					
	Leukocytes alone	Muscle alone	Leukocytes + muscle	Degeneration*	Cellular infiltration	Atrophy	Regeneration	Fibrosis	None
B. C.	—	N. D.	+	√√√√	√√√√	√√	√√	—	—
S. L.	—	—	+	—	—	—	—	—	√
K. L.	—	—	+	—	√	—	—	—	—
W. A.	—	—	+	√√√	√	—	—	—	—
C. F.	+	+	+	√√	√√√√	√√	—	√	—
G. R.	+	+	+	√√	√√√	√√	—	—	—
K. B.	—	—	—	√	—	√√√	—	√√√	—
A. P.	—	—	+	√‡	√	—	—	—	—
B. G.	—	±	+	√√√	√	√√√	√√√	—	—
P. R.	—	±	+	√√	√	—	√	—	—
D. M.§	—	—	+	√√	√√	√√	√	√	—

N. D., not done.

* Includes necrosis, altered staining characteristics, and loss of striations. In B. C., W. A., C. F., and G. R., necrosis was all significant histologic feature.

‡ Markedly edematous and distorted muscle spindles.

§ Cytotoxicity tested against L-929 fibroblasts only.

31), is inhibitable by corticosteroids (34), the effect of corticosteroid on the cytotoxic activity of the supernates from the polymyositis patients was assayed. Fig. 2E demonstrates the sparing of muscle cells in vitro when methyl prednisolone was added to a sample of

the toxic polymyositis supernate which produced the histologic changes seen in Fig. 2D.

While it was noted in preliminary experiments that methylprednisolone was effective in suppressing the cytotoxicity of lymphotoxin in vitro, a reduction in the capacity of human fetal muscle monolayers to incorporate ¹⁴C-labeled amino acids into protein was observed at concentrations of corticosteroid comparable to those reported to suppress lymphotoxin activity. To quantitate this effect, a group of six muscle monolayers were incubated with labeled amino acids in RPMI medium (Grand Island Biological Co.) containing 0.05 μg/ml of methyl prednisolone, an approximate equivalent of the normal plasma cortisol level. In parallel, another

TABLE VI
Results of Cytotoxicity Assays of Supernates after Incubation of Breast Tumor and Muscle Tissue with Autochthonous Leukocytes*

Test supernate‡	% Decrease in incorporation amino acid- ¹⁴ C
Leukocytes	—14
	—8
Muscle	+1
	0
	0
Leukocytes + normal breast tissue	—19
	+2
Leukocytes + breast carcinoma tissue	—23
	—6
	—7
Leukocytes + muscle	—58
	—56
Leukocytes + PHA	—95
	—96
	—91

* Control monolayers mean ±2 SD = 4407±1264. Decrease of ≥26% represents 95% confidence level.

‡ After 48 hr incubation.

TABLE VII
Effect of Methyl Prednisolone on Human Fetal Muscle Monolayers

Methyl prednisolone	Mean cpm amino acid- ¹⁴ C incorporation*	Statistical significance
μg/ml	cpm	
0.05	15,447	P < 0.01‡
5	12,926	

* Mean of six determinations in each case. Cultures performed in RPMI-1640 medium with 10% inactivated fetal calf serum.

‡ Student's *t* for the comparison of two independent samples with 11 degrees of freedom was calculated.

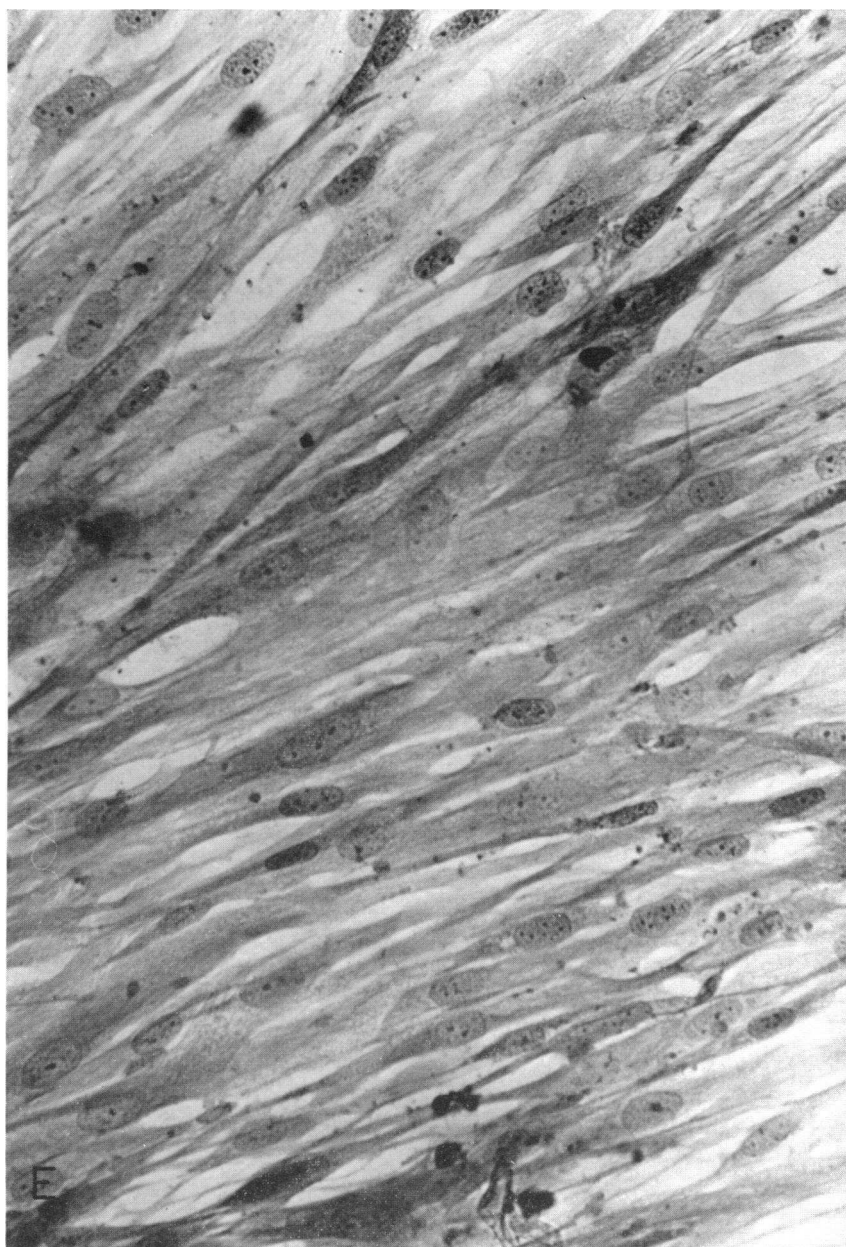


FIGURE 2E Fetal muscle monolayer after 48 hr exposure to culture medium containing dialyzed toxic supernate from leukocytes incubated with autochthonous muscle of a polymyositis patient (G. R.) in the presence of 5 μ g/ml methyl prednisolone. The cells appear essentially normal. Masson trichrome stain. $\times 400$.

group of six muscle monolayers were similarly incubated in medium containing 5 μ g/ml methyl prednisolone. The results are given in Table VII. The mean depression in 14 C-labeled amino acid incorporation at the higher dosage level was 16%. This figure was applied as a correction factor to the cytotoxicity measurements given in Table VIII. The values given in

Table VIII, therefore, reflect the decrease in cytotoxicity of toxic supernates upon addition of methyl prednisolone as indicated by increased 14 C-labeled amino acid incorporation. In the last column, appropriate correction has been made for the catabolic action of this steroid, as measured in the control experiments cited.

All polymyositis supernates tested with the exception

TABLE VIII
Inhibition of Cytotoxicity by Methyl Prednisolone

Cytotoxic supernate	cpm amino acid- ¹⁴ C incorporation with methyl prednisolone		% Increase in amino acid- ¹⁴ C incorporation	
	Control (0.05 µg/ml)	Treated (5 µg/ml)	Uncorrected*	Corrected†
	cpm		%	
Lymphotoxin preparation§	986	1,465	49	65
B. C.	1,821	2,211	21	37
K. L.	751	1,004	34	50
S. L.	1,705	2,466	45	61
G. R.	5,023	7,975	56	72
A. P.	996	1,414	42	58
B. G.	2,226	8,629	288	304

* All values but that of B. C. significant at 95% confidence level.

† Corrected for depression of amino acid-¹⁴C incorporation in control monolayers treated with methyl prednisolone at 5 µg/ml level (see Table VIII). All values significant at 95% confidence level.

§ Purified by 60% NH₄SO₄ precipitation, G-200 chromatography, and extensive dialysis against RPMI-1640.

of that of B. C. showed a highly significant increase in incorporation of ¹⁴C-labeled amino acids as a result of incubation with methyl prednisolone both before and after correction for the catabolic effect. This observation is consistent with the known effect of corticosteroids on lymphotoxin activity (34).

Column chromatography of cytotoxic supernates. Three supernates were available in sufficient quantity for examination by G-200 chromatography. Since previous work has established the approximate molecular weight of purified lymphotoxin (28), it was possible to compare the elution volumes of the cytotoxic factor in polymyositis supernates with those of a preparation of lymphotoxin. Fig. 3 compares the elution characteristics of purified lymphotoxin, prepared as described above, and the

three concentrated toxic supernates obtained from B. C., C. F., and G. R. Cytotoxic activity was in all three instances localized to an eluted volume similar to that of the lymphotoxin preparation. This was slightly less than the elution volume of albumin but subsequent to the elution of IgG. The cytotoxic supernates showed a wider spread of activity than that of the lymphotoxin preparation. However, the latter preparation had been taken from a central fraction of the active eluates during its chromatographic purification whereas there was no prior chromatography of the polymyositis supernates. A spread of activity on chromatography of lymphotoxin has been previously reported (28). A control preparation consisting of medium containing 10% fetal calf serum was concentrated fivefold and applied to the G-200 column as described for the polymyositis muscle-lymphocyte supernates. No one of the eluted fractions revealed cytotoxicity in the lymphotoxin assay.

DISCUSSION

The association of polymyositis with other connective tissue diseases such as systemic lupus erythematosus (4-6), the presence of autoantibodies (1, 2, 4, 6-8), and the recent report of fluorescent antibody staining of immunoglobulin and C3 or in the vessels in some polymyositis patients (9), have suggested a humoral immune mechanism in this disease. In the few reported cases associated with carcinomas (22-24), anti-tissue antibodies have also been identified. However, no direct evidence in support of a humoral mechanism for muscle injury has been forthcoming. On the other hand, the presence of lymphoid infiltrates in the animal models studied (10-12, 14, 15) and the recent work of Currie and coworkers (19-21) showing damage to human fetal muscle cells in vitro by lymphoid cells from patients with polymyositis favor a cellular rather than humoral mechanism. In the latter investigations, however, no attempt was made to identify the basis of the target cell injury noted in terms of lymphokines (17) which would specifically establish the occurrence of a cellular immune response. Moreover, control lymphocytes were not obtained from patients with inflammatory diseases in which proliferating lymphocytes, potentially capable of liberating lymphotoxin, circulate in the peripheral blood (35). Furthermore, allogeneic differences between the muscle assayed and the test lymphocytes utilized could, in the period of incubation required to demonstrate target cell damage, have resulted in primary immunization and the production of lymphokines (36, 37). In the work of Currie and coworkers (19) and that of Kakulas (13), both of which demonstrate target cell damage by lymphocytes layered directly upon target cells, the additional possibility of cell-to-cell mediated injury through contact lysis (38-41) has not been excluded.

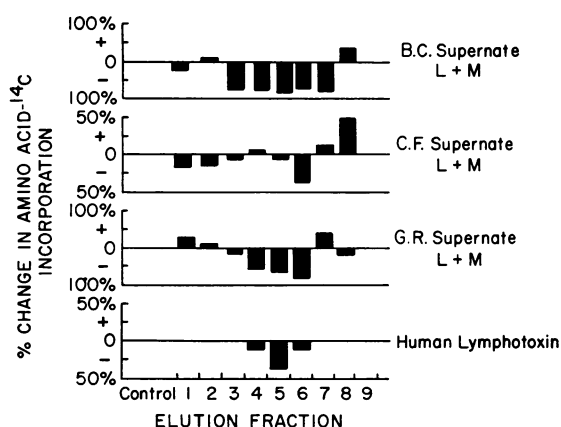


FIGURE 3 Cytotoxicity of eluted fractions of toxic supernates from polymyositis patients compared with that of purified lymphotoxin.

In the present experiments, the liberation of a cytotoxic agent by lymphocytes of polymyositis patients has been demonstrated. This occurred when such lymphoid cells were exposed to autochthonous muscle, thereby excluding allogeneic differences and the mechanism of contactual lysis. It has further been shown that these supernates contain cytotoxic activity which has the characteristics of lymphotoxin (27, 28) a mediator of delayed hypersensitivity. It has also been demonstrated that these supernates as well as lymphotoxin induce cellular damage in human muscle grown *in vitro*, and that the activity of these supernates, like that of lymphotoxin, is inhibitable by corticosteroids. Finally, it has been shown that both have a similar elution volume on G-200 Sephadex chromatography. These findings, in a heterogeneous population of patients with active polymyositis, strongly suggest that delayed hypersensitivity mediators play a role in the muscle injury of polymyositis. The presence of other lymphokines in the supernates was not specifically investigated, but from the work of others it appears likely that a spectrum of lymphokines was present and that these may contribute in some way to the expression of the disease. However, lymphotoxin by its characteristics (27, 28, 42) appears to be the most likely agent among these factors to be associated with the tissue injury observed in polymyositis. It has been shown by Granger et al. (27, 28, 30) that although the release of lymphotoxin requires the action of specifically sensitized lymphocytes, once released this agent has the capacity to induce cellular injury in a wide variety of target cell lines. There is a great deal of variability in the susceptibility of the various target cell lines studied, and it is of interest that in the studies reported here muscle tissue, in the form of human fetal muscle grown in monolayers, was found to be more sensitive to lymphotoxin than the mouse L-929 fibroblast cell line, which is the standard cell line utilized for lymphotoxin assay.

Recent work indicates that delayed hypersensitivity mediators may produce changes in cell membranes and cell metabolism apart from morphologic lysis or cell death (43, 44). This is of particular interest in view of the frequent disparity observed in polymyositis patients between muscle weakness and electromyographic changes on the one hand and the amount of necrosis found histologically on the other.

The release of lymphotoxin by muscle incubated alone occurred solely in the two specimens with prominent cellular infiltration. In these two instances, the degree of cytotoxicity evoked in all of the supernates examined, precluded detection of an additional increment of cytotoxicity when peripheral blood leukocytes were added to the muscle pieces. This observation indicates that tissue foci of lymphoid cells, when suitably stimulated, can release lymphotoxin, and suggests that this agent may be released

in sufficient excess to induce muscle injury at sites which are remote from the area of infiltration. With regard to the cytotoxicity generated by leukocytes alone, this occurred in the same two patients whose muscle alone was active. It appears likely that this resulted from the release of antigen or of antigen-triggered cells into the circulation. The specificity of the release of lymphotoxin by lymphoid cells is indicated in the results reported here (a) by the production of significant cytotoxicity in 7 of the polymyositis patients only upon combination of leukocytes and muscle, (b) by the failure of normal breast tissue and breast tumor to stimulate release of this agent from lymphocytes which were capable of producing lymphotoxin on exposure to autologous muscle, and (c) by the correlation of the presence of leukocytic infiltration in muscle pieces with the release *in vitro* of lymphotoxin by these pieces when incubated alone.

Further studies are indicated to define the nature of the antigen i.e. material present in muscle, its localization within muscle tissue, and its presence or absence in normal homologous muscle as well as other human tissues. In this respect, the studies of Currie and coworkers (19-21) have demonstrated that normal human skeletal muscle can serve as target tissue for direct cell-to-cell injury by lymphocytes from polymyositis patients. In another study, Saunders, Knowles, and Currie (45) found that lymphocytes from polymyositis patients were stimulated more frequently than normal lymphocytes on exposure to homologous muscle homogenates. Whether the source of stimulation in these experiments is relevant to the stimulation by autochthonous muscle described here remains to be determined. Reports of a variety of virus-like particles (46-52) in the endothelial cells of muscle of polymyositis patients have appeared recently. These particles have been regarded as possible triggering agents for initiation of this disease. If this were so, these agents, which have had the appearance of myxoviruses (46, 47), picornaviruses (48), or coxsackie viruses (49) might serve as antigens in a cellular immune response which could bring about the release of lymphotoxin. The possibility that an exogenous agent such as a virus might be involved in an immunological response in polymyositis is raised by the fact that in the present experiments circulating lymphocytes were stimulated by autochthonous muscle.

Although the current study has demonstrated the release of lymphotoxin from patient leukocytes on exposure to muscle and the capacity of this agent to damage muscle, it should be kept in mind that there may be other mechanisms of muscle injury in the conditions which make up the clinical spectrum of polymyositis. The role of contactual lysis mediated by direct aggressor-target cell interaction through IgM (38-41) has not been excluded, and as suggested by the work of Williams and

Engel (9) and by observations reported in several cases of polymyositis occurring with malignancy (22-24), humoral responses may at times occur. However, the direct contribution of serum antibodies with specificity and cytotoxicity for muscle of polymyositis patients remains to be established. Finally, it should be pointed out that the local environment of an inflammatory reaction includes a variety of lysosomal enzymes, vasoactive amines, and other agents which may also contribute significantly to muscle injury.

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