Cell-mediated immunity to myoglobin in polymyositis

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SUMMARY Using the leucocyte migration inhibition (LIF) test we looked for evidence of cell-mediated hypersensitivity against myoglobin in 8 patients with polymyositis (PM) or dermatomyositis (DM). The migration index for PM-DM patients was $47.5 \pm 17\%$, while in the controls the index was $86 \pm 12\%$ ($p < 0.001$). The presence of serum antibodies against myoglobin was also investigated by passive haemagglutination (PH) and counterimmunoelectrophoresis (CIE). By PH the reciprocal titre of antimyoglobin antibodies was 150 ± 28 in PM-DM compared with 16.2 ± 15.9 and 8.7 ± 7.7 in control patients with diseases ($p < 0.01$) and negative in normal persons. CIE showed antimyoglobin antibodies in 3 PM-DM patients and in none of controls. Cell-mediated immunity against myoglobin may be implicated in the pathogenesis of PM. The pathological significance of antimyoglobin antibodies remains to be determined.

Polymyositis (PM) is an inflammatory muscle disease that affects mainly proximal muscular groups; when the skin is affected it is called dermatomyositis (DM). The aetiology is unknown and the pathogenesis obscure; however, some immunological features have been described. These include direct mononuclear cytotoxicity to muscle fibres^{1,2} and lymphotoxin-mediated cytotoxicity to fetal muscle when PM-DM mononuclear cells have been previously cultured with autologous muscle extract.³ Furthermore, when patients' mononuclear cells are stimulated in culture with muscle tissue they undergo blastogenesis.^{2,4}

Several autoantibodies have been found in PM-DM patients, including anti-PM-1,^{5,6} the Jo-1 antibody system,⁷ antimyosin,⁸ and antimuscle antibodies.^{9–12} In addition deposits of immunoglobulins and complement have been reported^{13,14} in the vessel walls of involved muscles. By passive haemagglutination Nishikai and Homma¹⁵ found antimyoglobin antibodies in patients with PM-DM, while in control groups and in patients with other myopathies such antibodies were either absent or in very low titre. To our knowledge no specific muscle antigen has been implicated in cell-mediated hypersensitivity to muscle in PM-DM patients. Myoglobin is a major

component of striated muscle and is not found in smooth muscle. We therefore sought evidence of delayed hypersensitivity and specific antibodies directed against myoglobin in patients with PM-DM.

Materials and methods

Patients. Eight patients with PM-DM according to the criteria proposed by Bohan and Peter¹⁶ were studied. Three had polymyositis, 4 typical dermatomyositis, and one childhood dermatomyositis. None had either associated neoplasia or overlap syndrome. Two patients were considered active, as defined by high serum enzyme levels, muscle tenderness, mild fever in the absence of infection, and high sedimentation rate (Wintrobe). Other conditions producing high serum enzyme levels were ruled out. Seven patients were receiving prednisone (5 to 60 mg per day); 2 were also receiving azathioprine (100 and 150 mg daily), and one patient was untreated at the time of the study. The normal control group comprised 8 healthy individuals. Control disease groups were 8 patients with systemic lupus erythematosus (SLE) who had 4 or more ARA criteria¹⁷ and 8 with classic or definite rheumatoid arthritis (RA) according to the ARA criteria.¹⁸ These disease groups were included only in the serological studies.

Preparation of patients' cells. Peripheral blood was obtained by venepuncture with heparinised syringes and left to sediment spontaneously for 90 minutes. The

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leucocytes in the supernatant plasma were washed thrice in Hanks's balanced salt solution (HBSS). After the last washing the cells were resuspended in 199 medium (Difco, Detroit, Michigan) and adjusted to a concentration of 20×10^6 per ml. Viability always exceeded 95% by trypan blue exclusion.

Myoglobin isolation and purification. Myoglobin was isolated by a modification of Singer's method.¹⁹ Human muscle fragments were obtained within 2 hours of death and stored at -70°C . The fragments were minced and homogenised, and, except where noted, the pH was maintained at 7.0 during the procedure. The unpigmented proteins were precipitated with 0.5 M basic acetate. The supernatant was treated with phosphate mixture (Na_3PO_4 160 mg, Na_2HPO_4 40 mg, for each 20 g of muscle), and dialysed against ammonium sulphate solution in progressive concentrations from 40 to 100% for 8 to 24 hours. After the last dialysis the metamyoglobin was precipitated at the bottom of the dialysis bag. To remove the sulphate from the myoglobin a further 24-hour dialysis against phosphate buffered saline (PBS) pH 7.4, 0.01 M was performed. The myoglobin extract was purified by Sephadex G-50 gel filtration (Pharmacia Fine Chemicals, Uppsala, Sweden) in a 2.6×60 column (Pharmacia) at an elution speed of 60 ml per hour, and 3 ml samples were collected in glass tubes with an LKB-8300 Uvikord II fraction collector. Myoglobin purity was assessed by cellulose acetate gel electrophoresis in a Beckman 110 densitometer. Further characterisation of myoglobin was in a Carl-Zeiss PMQ-II spectrophotometer. Protein concentration was determined by Lowry's method.²⁰ The myoglobin sample was sterilised by Millipore filtration with 22 μm pore size (Millipore Corp., Bedford, MA).

Leucocyte migration inhibition (LIF) test. The LIF test was done according to modified Bendixen and Sjøborg's method.²¹ Briefly, Mackness type migration chambers (Falcon Plastics, Oxnard, CA) were filled with either 199 medium alone or 199 medium containing 1% myoglobin. The cells were taken up in capillary tubes (Propper Manufacturing Co. Inc., Long Island City, NY) and centrifuged at 200g for 15 min. Portions of the capillaries containing the cell pellets were placed in the chambers. Experiments were done either with PM-DM cells or with healthy donor cells. The chambers were incubated for 48 hours at 37°C . Each assay was done in quadruplicate, and results were included only when reproduced at least 3 times. The migration areas were measured by projection on paper, the shaded areas being cut out and weighed in a precision balance. The migration index was defined as:

$$\% \text{Migration} = \frac{\text{mean area of migration of cells plus antigen}}{\text{mean area of migration of cells alone}} \times 100.$$

Absorption of sera. Sera were heat-inactivated at 56°C for 30 min and then in order to remove heterophile antibodies were absorbed for one hour with sheep red blood cells (SRBC) at room temperature.

Passive haemagglutination (PH). SRBC were coated with human myoglobin by the technique described by Nishikai and Homma.¹⁵ Myoglobin-coated tanned SRBC (My-SRBC) were resuspended at 5% in HBSS. The PH assays were done in Microtiter U-plate microwells (Cooke Engineering, Alexandria, VA) containing 0.2 ml of 0.5% heat-inactivated rabbit serum in HBSS, 0.2 ml of either patient or control serum in dilutions from 1:10 to 1:160, and 0.2 ml of My-SRBC suspension. The U-plates were first incubated for 60 min at 37°C and then overnight. After 18 hours' incubation the haemagglutination in each microwell was recorded.

Counterimmunoelectrophoresis (CIE). This was done in a Hyland unit (Hyland, Costa Mesa, CA) by the method of Gocke and Howe.²² Plastic 10×10 plates with 2% agarose (Sigma Chemical Co., St Louis, MO) prepared in barbital buffer 0.05 M, pH 7.2 were used. The running time was 45 minutes at 30 mA. The myoglobin was placed in the cathode well at a concentration of 5.5 mg/ml.

Statistical analysis was by Student's *t* test.

Results

ANTIGEN

After Sephadex G-50 gel filtration of human myoglobin (Fig. 1) the cellulose acetate gel electrophoresis showed that it was pure myoglobin with beta electrophoretic migration pattern. Spectrophotometric characterisation showed 2 peaks at 502 and 624 nm, confirming its nature (Fig. 2). The protein concentration in the sample was 5.5 mg/ml (5.5 g/l).

LIF TEST

When incubated with 199 medium alone the PM-DM leucocyte migration area was taken as 100% and did not differ from that of the control leucocytes incubated in the same way. When PM-DM cells were incubated in presence of myoglobin the migration index was $47.5 \pm 17\%$ (range 10–63%), significantly less than the control index when incubated with myoglobin, which was $86 \pm 12\%$ ($p < 0.001$). Fig. 3 shows the individual migration indices.

PASSIVE HAEMAGGLUTINATION

All the PM-DM patients had antibodies to myoglobin, their mean reciprocal titre being 150 ± 28 (range 80 to 160). Values for the disease controls were 16.2 ± 15.9 for RA and 8.7 ± 7.7 for SLE, both differences being significant ($p < 0.01$). Healthy control sera were all negative (Fig. 4).

Fig. 1 Sephadex G-50 gel filtration chromatography of human myoglobin. The elution speed was 60 ml per hour, myoglobin eluted from fractions 15 through 23 (3 ml fractions were collected). The graph was plotted in an LKB spectrophotometer fraction analyser.

Fig. 2 Spectrophotometric characterisation of human myoglobin in 0.01 M PBS, pH 7.4. The characterisation was done from 400 to 700 nm. The absorption spectrum (502 and 624 nm) is characteristic of myoglobin.

Fig. 3 LIF test to myoglobin. The differences between PM-DM patients (left side) who had a migration index from 10 to 63 % are evident when compared with normal persons who had a range from 60 to 100 % (see text).

In order to define the antimyoglobin specificity found by PH, absorption studies were done as follows: sera were incubated for 30 min at 37°C with 1 % myoglobin solution in 0.01 M PBS. All sera tested in this way became negative for antimyoglobin activity by PH. A further attempt to define this specificity was by indirect immunofluorescence with mouse muscle as substrate. By this method all sera showed 2 staining patterns: both sarcolemmal and sarcoplasmatic. When sera were previously absorbed with My-SRBC, the sarcoplasmatic staining disappeared and sarcolemmal remained positive (data not shown).

Counterimmunoelectrophoresis was positive in only 3 of the 8 patients, 2 of them with active disease. All the controls were negative.

- 10 Curtis A C, Heckamann J H, Wheeler A H. Study of the auto-immune reaction in dermatomyositis. *JAMA* 1961; **178**: 571-3.
- 11 Copeman P, Alexander S. Dermatomyositis. Adenocarcinoma of male breast. Detection of antibodies to neoplasm in serum. *Proc R Soc Med* 1967; **60**: 183-4.
- 12 Alexander S, Forman L. Dermatomyositis and carcinoma. A case report and immunological investigation. *Br J Dermatol* 1968; **80**: 86-9.
- 13 Tan E M, Reid R T, Pearson C M. Autoallergic diseases with serum IgG reactive with vascular structures. *Arthritis Rheum* 1970; **13**: 353.
- 14 Whitaker J N, Engel W K. Vascular deposits of immunoglobulin and complement in idiopathic inflammatory myopathy. *N Engl J Med* 1972; **286**: 333-8.
- 15 Nishikai M, Homma M. Circulating antibody against human myoglobin in polymyositis. *JAMA* 1977; **237**: 1842-4.
- 16 Bohan A, Peter J B. Polymyositis and dermatomyositis. *N Engl J Med* 1975; **292**: 344-7, 403-7.
- 17 Cohen A S, Reynolds W E, Franklin E C, *et al*. Preliminary criteria for the classification of systemic lupus erythematosus. *Bull Rheum Dis* 1971; **21**: 643-8.
- 18 Ropes N W, Bennett G A, Cobb S, Jacox R, Jessar R A. Proposed diagnostic criteria for rheumatoid arthritis. *Bull Rheum Dis* 1956; **7**: 121-4.
- 19 Singer K, Angelopolus R, Ramont B. Studies of human myoglobin. I. Myoglobin in sickle cell disease. *Blood* 1955; **10**: 979-86.
- 20 Lowry H O, Rosenbrough N J, Farr A L, Randall R J. Protein measurement with the folin-phenol reagent. *J Biol Chem* 1951; **193**: 265-75.
- 21 Bendixen G, Sjøberg M. A leukocyte migration technique for *in vitro* detection of cellular (delayed type) hypersensitivity in man. *Dan Med Bull* 1969; **16**: 1-6.
- 22 Gocke D J, Howe C. Rapid detection of Australia antigen by counterimmunoelectrophoresis. *J Immunol* 1970; **104**: 1031-2.
- 23 Haas D C. Absence of cell-mediated cytotoxicity to muscle cultures in polymyositis. *J Rheumatol* 1980; **7**: 671-6.
- 24 Rocklin R E. Products of activated lymphocytes: leukocyte inhibitory factor (LIF) distinct from migration inhibitory factor (MIF). *J Immunol* 1974; **112**: 1461-6.
- 25 Spitler L, Huber H, Fudenberg H H. Inhibition of capillary migration by antigen-antibody complexes. *J Immunol* 1969; **102**: 404-11.
- 26 Hooper K E, Geczy C L, Davies W A. A mechanism of migration inhibition in delayed-type hypersensitivity reactions. I. Fibrin deposition on the surface of elicited peritoneal macrophages *in vivo*. *J Immunol* 1981; **126**: 1052-8.