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Studies of lymphocytotoxins in infectious mononucleosis and systemic lupus erythematosus: evidence for immune complex-mediated cytotoxicity

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SUMMARY

The hypothesis that serum lymphocytotoxins are antigen–antibody complexes was examined. High molecular weight fractions from the sera of eighteen patients with infectious mononucleosis (IM), thirteen patients with systemic lupus erythematosus (SLE) and six healthy controls, were prepared by precipitation with polyethylene glycol 6000 (PEG). The lymphocytotoxic activity (LCA) of these PEG precipitates was significantly greater ($P<0{\cdot}01$) than that of the corresponding sera and a significant correlation ($r = 0{\cdot}66$, $P<0{\cdot}01$) was observed between the LCA of sera and the PEG precipitates. In contrast to the concentration of LCA in the PEG precipitates, the heterophil antibody titres of the precipitates from IM sera were significantly less ($P<0\ 05$) than serum titres.

Antisera raised against PEG precipitates from sera from nine patients with IM contained significant LCA. The nature of this LCA differed from that of the LCA in the original sera in temperature dependence and molecular size.

Antigen–antibody complexes in seven sera (four IM, three SLE) were dissociated at low pH (3·0) and fractionated by gel filtration at pH 3·0. The LCA of these fractions was compared with the LCA of equivalent fractions obtained by gel filtration at pH 7·2. The heterophil antibody present in sera from patients with IM and the cytotoxicity of anti-lymphocyte globulin (ALG) were used as 'antibody controls'. In this way it was shown that the LCA in patient sera, but not heterophil antibody or ALG cytotoxicity was significantly reduced ($P<0{\cdot}001$) by low pH gel filtration.

INTRODUCTION

Lymphocytotoxic activity (LCA) in the sera of patients with a variety of immunological and infectious diseases has been shown to be complement and temperature (i.e. maximum at 15°C) dependent (Mottironi & Terasaki, 1970; Terasaki, Mottironi & Barnett, 1970; Korsmeyer *et al.*, 1975). These lymphocytotoxins are of 19S or larger molecular size and indirect immunofluorescent staining suggests that IgM is the major antibody class involved (Winfield *et al.*, 1975a). Certain evidence suggests these 'cryolymphocytotoxins' to be an antigen–antibody complex: firstly, they are concentrated in cryoprecipitates of sera from patients with systemic lupus erythematosus (SLE) (Winfield *et al.*, 1975b; Zvaifler & Bluestein, 1976) and secondly, the LCA of sera from patients with certain viral diseases is destroyed by repeated freezing and thawing (Huang *et al.*, 1973). In this latter study cytotoxic activity could be restored in these deactivated sera by the addition of the appropriate virus.

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In this study the hypothesis that cryolymphocytotoxins are antigen–antibody complexes was examined. Immune complex-rich fractions were prepared from sera from patients with infectious mononucleosis (IM) and SLE by precipitation with polyethylene glycol (PEG) and the LCA of these fractions compared with that of the original sera. Immune complexes in seven sera (four IM, three SLE) were dissociated by low pH (3·0) and fractionated by G200 gel filtration at pH 3·0. The LCA of these fractions was compared with that observed in equivalent fractions obtained by G200 gel filtration at pH 7·2. The heterophil antibody present in sera from patients with IM was used as an 'antibody control' in all experiments. In this way it was shown that lymphocytotoxins, but not heterophil antibody, were concentrated in immune complex-rich serum fractions and in addition lymphocytotoxic activity, but not heterophil antibody, could be destroyed partially by low pH gel filtration.

PATIENTS AND METHODS

Patients. Sera from twenty patients with uncomplicated IM, twelve patients with SLE and six healthy controls were included in the study.

Methods. The method used to isolate lymphocytes and the performance of the lymphocytotoxin assay have been described in a previous paper (Charlesworth *et al.*, 1978).

Analysis of LCA in immune complex-rich serum fractions. Five millilitres of 3% (w/v) polyethylene glycol (PEG) (molecular weight 6000 D Sigma) in borate buffer, pH 8·3 (0·1 M boric acid, 0·025 M disodium tetraborate, 0·07 M NaCl) was added to 1 ml serum from the thirty-two patients and six healthy controls. This mixture was incubated for 90 min at 4°C and then centrifuged at 1500 *g* for 15 min. The resultant precipitate was washed with a further 10 ml 3% PEG and recentrifuged. The precipitate was redissolved in 1 ml of phosphate buffered saline (PBS) pH 7·2 and fractionated by gel filtration on G200 sephadex (Pharmacia) in PBS (column size 3 × 80 cm). In twelve sera the supernatant from the first centrifugation was also saved. This supernatant was diluted with 40 ml PBS, pH 7·2, and reconcentrated to approximately 1 ml by membrane filtration (Diaflow XM 50, retains molecules > 50,000 D).

The molecular size and temperature dependence of LCA found in redissolved PEG precipitates were compared with those found in the original sera. Molecular size was determined by fractionation of 1-ml aliquots on G200 sephadex. Twenty-five-millilitre column fractions were collected and reconcentrated to approximately 1 ml by membrane filtratioin. The IgM, IgG and IgA concentrations in the sera and PEG precipitates were measured by single radial immunod ffusion (Mancini Carbonara & Heremans, 1965). In six IM patients the heterophil antibody titre of whole serum, PEG supernatant and PEG precipitate was tested by use of the monospot slide test (Ortho, USA). 0·1-ml aliquots of these samples were then included in the same lymphocytotoxin assay.

Preparation of antisera to immune complex-rich fractions of IM sera. Antisera to the redissolved PEG precipitates from nine acute IM sera and a pool of normal human sera (NHS) were prepared in ten rats by the intramuscular injection of 0·1-ml samples in 0·05 ml Freunds' complete adjuvant (FCA) (Commonwealth Serum Laboratories, Australia). Three injections were given at ten-day intervals. Fourteen days after the last injection the animals were anaesthetized and bled. The LCA of these antisera, and rabbit antisera, against human immunoglobulin and whole human serum (Dako, Denmark), was determined against two pools of normal lymphocytes.

G200 gel filtration of lymphocytotoxic sera at pH 3·0 and pH 7·2. One millilitre of serum from four patients with acute IM and three patients with SLE was diluted with 4 ml PBS, pH 7·2 and fractionated by gel filtration on G200 Sephadex in PBS (column size 3 × 80 cm). A second 1 ml sample of each of the sera was diluted with 4 ml of 0·2 M glycine 0·15 M NaCl, HCl buffer, pH 3·0 and the pH of the diluted sample adjusted to 3·0 with 1 M HCl. This diluted specimen was then fractionated on G200 Sephadex in glycine HCl, pH 3·0 (column size 3 × 80 cm). Twenty-five millilitre fractions from both columns (i.e. pH 7·2 and pH 3·0) were collected and concentrated to 2 ml by dialysis against 20% PEG (w/v) (4000, Ajax Chemicals, Australia) in PBS, pH 7·2, with 0·05% phenol red as a pH indicator. At the end of the concentration step, samples were dialysed against Hanks' balanced salt solution pH 7·2, for 6 hr; 0·1-ml aliquots of fractions were then tested for LCA against normal lymphocytes. Those fractions with greater than 50% lymphocyte killing were titrated to obtain a LD 50 titre. The heterophil antibody titre of the concentrated fractions of the IM sera was also determined. One millilitre of anti-lymphocyte globulin (ALG) was added to 1 ml NHS and fractionated as above. This was used as a positive control for LCA.

RESULTS

Analysis of LCA in immune complex-rich serum fractions

The redissolved PEG precipitates from the thirty-two patient sera contained 8·2±7·5% IgM (mean± s.d.), 1·3±1·9% IgG and 1·0±2·4% IgA compared to the corresponding whole sera. The LCA of these precipitates however, was significantly greater ($P<0·01$) than that of the sera (Fig. 1) and a significant correlation ($r = 0·66$, $P<0·01$) was observed between the LCA of sera and PEG precipitates.

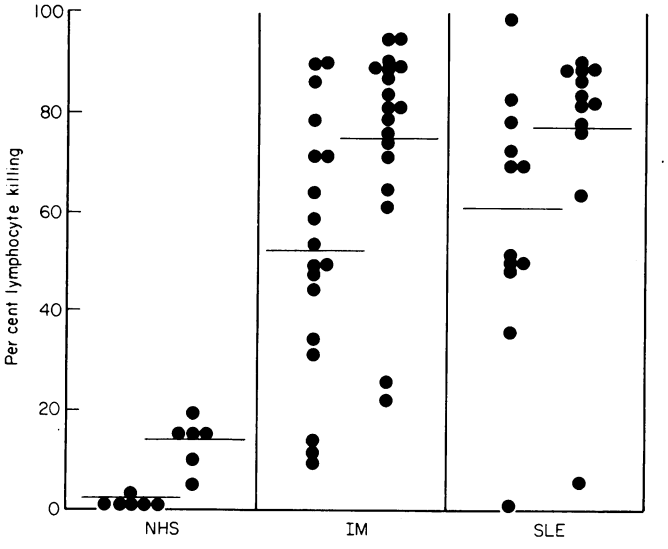

FIG. 1. The LCA observed in sera and PEG precipitates of six healthy controls, eighteen patients with IM and twelve patients with SLE. The mean is shown as a horizontal line.

TABLE 1. The LCA and heterophil antibody titres of IM sera and their PEG precipitates and supernatants

		PEG	
	Serum	Precipitate	Supernatant
LCA	24·7±7·13*	26·4±18·7	8·4±7·9†
Heterophil antibody titre	260±220	3·5±5·1‡	180±118

* Mean±s.d.
† Significantly less than serum ($P < 0.001$).
‡ Significantly less than serum ($P < 0.05$).

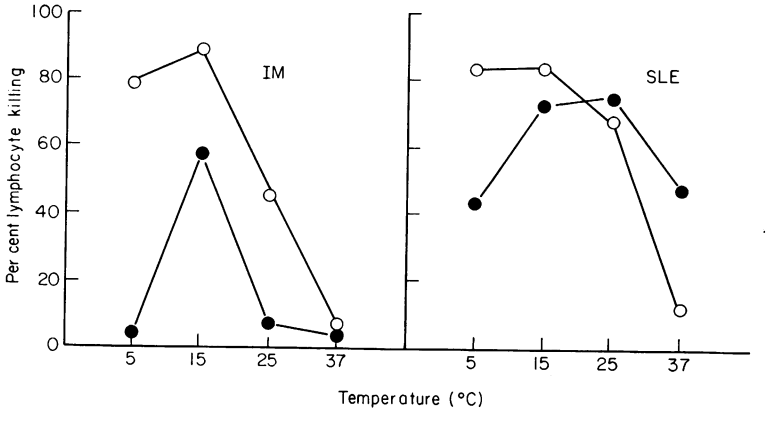

FIG. 2. The LCA of sera (●—●) and PEG precipitates (○—○) from one patient with IM and one with SLE, performed at various incubation temperatures.

In contrast the heterophil antibody titres of the PEG precipitates from IM sera were significantly less ($P<0{\cdot}05$) than the titres of the original sera (Table 1).

Although slight, a definite level of lymphocytotoxicity was observed with 3% PEG solution alone ($14\pm1\%$ killing). It was therefore necessary to dilute the reconcentrated PEG supernatants with Hanks' balanced salt solution before testing for LCA. The LCA of these samples and the corresponding whole sera was thus determined at a 1 in 10 dilution. The results are shown in Table 1. The LCA of the PEG supernatants was significantly less ($P<0{\cdot}001$) than the LCA of the sera or PEG precipitates. However, the heterophil antibody titres of the supernatants were not different from the serum titres (Table 1).

The maximum LCA (per cent lymphocyte killing and LD_{50} titre) for the redissolved PEG precipitates and the untreated sera from both diseases studied, was recovered in the exclusion peak of a G200 Sephadex gel filtration (PBS, pH 7·2). In addition, the optimum temperature for lymphocyte killing in both the sera and PEG precipitates was 15°C (see Fig. 2). However at 4°C the LCA of the PEG precipitates was significantly higher ($P<0{\cdot}05$) than the LCA of the sera.

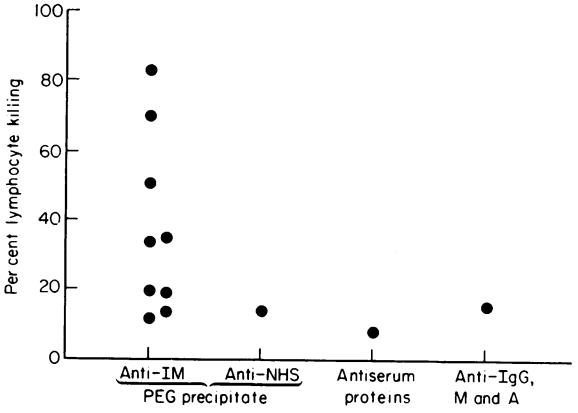

FIG. 3. The LCA of antisera prepared against (a) the PEG precipitates of nine patients with IM, (b) the PEG precipitates of a pool of normal sera, (c) human serum proteins and (d) human immunoglobulin classes G, M and A.

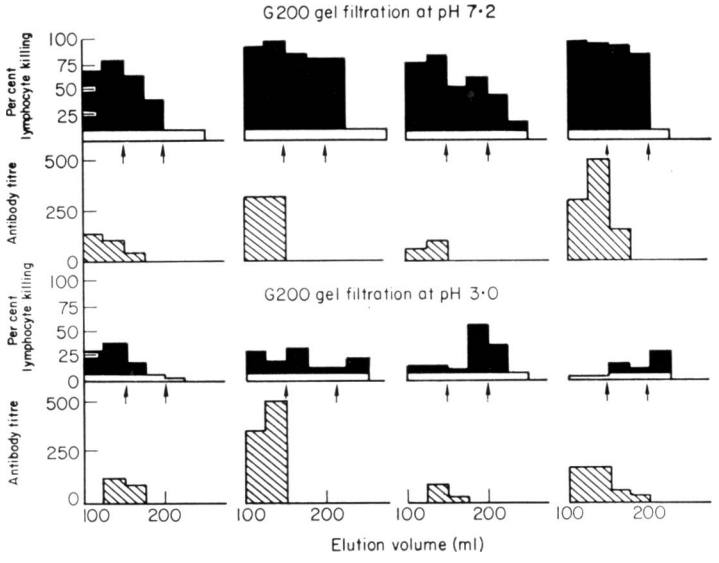

FIG. 4. The LCA and heterophil antibody titre of serum fractions from four patients with IM separated by G200 sephadex gel filtration at pH 7·2 and pH 3·0. Arrows indicate the first and second elution peaks.

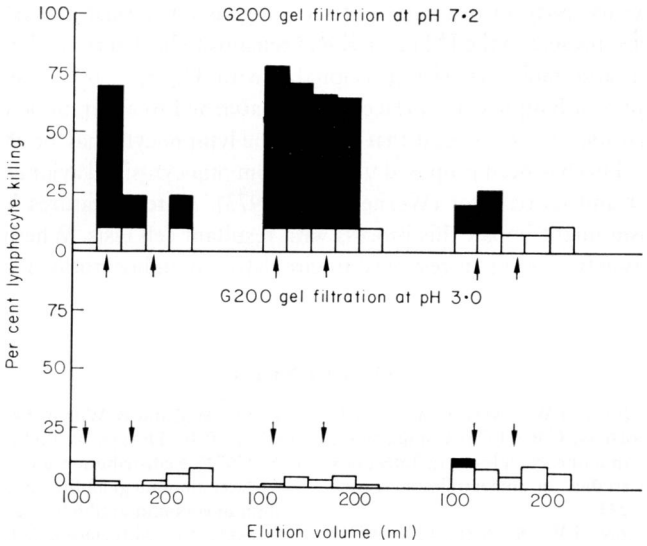

FIG. 5. The LCA of serum fractions from three patients with SLE separated by G200 sephadex gel filtration at pH 7·2 and pH 3·0. Arrows indicate the first and second elution peaks.

Antisera against the immune complex-rich serum fractions

The antisera raised against the redissolved PEG precipitates from IM sera showed LCA (see Fig. 3). The LCA of these antisera was significantly higher ($P<0·01$) than that observed with antisera against human immunoglobulin or whole human serum. The nature of the LCA of the antisera differed from that of the IM sera in the following ways: (a) there was less temperature-dependence, and (b) maximal killing was recovered in the second elution peaks from fractionation on G200 Sephadex in PBS, pH 7·2 with little activity in the exclusion peak.

Gel filtration of LCA at pH 7·2 and pH 3·0

The LCA of four IM sera separated by gel filtration at pH 7·2 and 3·0 are shown in Fig. 4. Similarly the LCA of three SLE sera separated by gel filtration at pH 7·2 aud 3·0 are shown in Fig. 5. In both diseases the percentage killing of serum fractions separated at pH 3·0 was significantly less ($P<0·001$) than that of the fractions from the same sera separated at pH 7·2. In contrast the heterophil antibody titres of the IM serum fractions were not significantly different.

DISCUSSION

Polyethylene glycol 6000 has been used to precipitate differentially antigen–antibody complexes without protein denaturation (Harrington, Fenton & Pert, 1971). The LCA in the sera of patients with IM and SLE was shown to be completely precipitable with 3% PEG whereas heterophil antibody and IgM were only slightly precipitated. This suggests that such LCA results from the action of immune complexes or aggregated antibody. The loss of LCA by gel filtration at low pH, without a significant effect on heterophil antibody in the IM sera or LCA in ALG, would support the hypothesis that lymphocytotoxins are antigen–antibody complexes.

The presence of LCA in antisera raised against the PEG precipitates from IM sera, but not normal sera, suggests that there are soluble lymphocyte surface antigens or, at least antigens immunologically similar to lymphocyte surface antigens, present in the IM sera. The nature of the LCA of these antisera was similar to that observed in ALG and it is suggested that the LCA of the antisera is due to a 7S antibody. A similar observation has been made on sera from patients with SLE (Klippel, Bluestein & Zvaifler, 1979): in this study, antisera against serum cryoglobulins were shown in an antibody-dependent,

cell-mediated cytotoxicity assay to recognize surface antigens on normal and SLE lymphocytes. The nature of these antigens, present in the IM or SLE sera remains to be determined but they may be present as soluble antigens of large molecular size (precipitable with 3% PEG 6000), as lymphocyte antigen–antibody complexes or as a lymphocyte surface receptor attached to an immune complex.

Observations *in vivo* and *in vitro* suggest that at 37°C the lymphocyte may be able to process lymphocytotoxins effectively. This has been proposed to result from pinocytosis (Taylor *et al.*, 1971) or shedding of the cytotoxic agent and its receptor (Wernet *et al.*, 1973). At temperatures less than 37°C, altered lymphocyte metabolism might impair this process with resultant cell lysis. Whether more subtle alterations in lymphocyte function occur in response to circulating lymphocytotoxins at 37°C remains to be examined.

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