Immunology, 1973, **24**, 655.

Cytotoxicity of Immune Guinea-pig Cells

II. THE MECHANISM OF MACROPHAGE CYTOTOXICITY

ANNE TEMPLE, G. LOEWI, P. DAVIES AND A. HOWARD

Divisions of Immunology and Cell Pathology, MRC Clinical Research Centre, Harrow, Middlesex HA1 3UJ; and MRC Rheumatism Unit, Canadian Red Cross Memorial Hospital, Taplow, Maidenhead, Berks.

(*Received 2nd August* 1972; *accepted for publication 26th September* 1972)

Summary. Macrophages from immune guinea-pigs, as well as macrophages from non-immune animals in the presence of specific antibody, were cytotoxic to chicken erythrocytes. Elution from macrophages produced a γ_2-globulin which enabled non-immune macrophages to form rosettes with chicken erythrocytes, to phagocytose, and to show cytotoxicity towards such cells. This antibody was target-cell specific; it also rendered spleen cells cytotoxic. Isoelectric focusing of antiserum gave a peak of cytophilic antibody at pH 6·8–7·6 which proved to be in the γ_2 region.

The significance of phagocytosis for the cytotoxic process was examined with the aid of cytochalasin B which inhibited macrophage–phagocytosis, but produced enhanced cytotoxicity in this system. At the same time there was greatly increased release of lysosomal enzymes from macrophages in the presence of target cells. It is considered that, although phagocytosis is of significance for macrophage cytotoxicity, target cell destruction can also occur by the liberation of lysosomal enzymes from the plasma membrane into the target cell when this is in close surface contact.

INTRODUCTION

In a review of the problems of graft destruction, Gorer, in 1961, described that in the case of peritoneal grafts, sensitized histiocytes may play the leading role in early graft rejection. Bennett, Old and Boyse (1964) described phagocytosis and destruction of tumour cells by peritoneal macrophages from mice in the presence of isoantibody. On the other hand, Granger and Weiser (1964) found that homograft target cells were destroyed after contact *in vitro* with immune peritoneal macrophages and that cell destruction apparently resulted from a non-phagocytic mechanism involving cell contact. Evans and Alexander (1970) have reported cooperation between immune spleen cells and normal macrophages in the destruction of lymphoma cells *in vitro*. They found that phagocytosis only occurred after 24 hours when the cells began to degenerate, and concluded that cell killing was not an intracellular event.

In a preceding paper (Loewi and Temple, 1972) we have reported specific cytotoxicity of guinea-pig peritoneal macrophages towards chicken erythrocytes (CRC) against which the cell donors had been immunized. Since, in this system, cellular events can readily be observed and cytotoxicity can be assessed by chromium (^{51}Cr) release, it appeared to be

suitable for a study of the mechanism of macrophage cytotoxicity *in vitro*, with the proviso that it involved a heterologous system. We describe here the involvement and characterization of a cytophilic antibody and the role of phagocytosis in this cytotoxic system.

MATERIALS AND METHODS

The methods for immunization, assessment of cytotoxicity and the preparation of cell suspensions have been described in a preceding paper (Loewi and Temple, 1972). In some experiments, where unimmunized effector cells were used, antiserum from guinea-pigs which had been immunized with CRC and Freund's complete adjuvant (FCA) was added, usually diluted 1:1000. This was in accordance with the method used in earlier work on passive cytotoxicity (McLennan, Loewi and Harding, 1970).

Phagocytosis

Six tubes, each containing $2{\cdot}5 \times 10^6$ immune peritoneal exudate cells (PE) and 5×10^6 chicken red cells (CRC) in 1 ml, were incubated at 37°. Non-immune PE cells were incubated in the presence of guinea-pig antiserum, diluted 1 in 1000. In order to assess the extent of phagocytosis, non-phagocytosed CRC, both free in the medium and adhering to PE cells, were counted in a haemocytometer. Counts of phagocytosed cells were obtained by preparing smears from centrifugates. The slides were stained by Leishman. At least 300 exudate cells were counted, enumerating phagocytosed and adherent CRC. The percentage phagocytosis and adherence was calculated.

Cytochalasin B (ICI) was added as a fine suspension in foetal calf serum to a final concentration of 10 μg/ml.

Enzyme assays

β-Glucuronidase (EC 3.2.1.31,-D-glucuronohydrolase) was assayed by the method of Talalay, Fishman and Huggins, (1946).

Acid protease was assayed by the method of Anson (1936); trichloracetic acid-soluble products were determined by measuring the optical density at 700 nm of the colour produced by reaction with Folin–Ciolcateau's reagent.

Leucine-2-naphthylamidase was assayed as described by Davies, Krakauer and Weissmann (1970).

Lactate dehydrogenase (EC 1.1.1.27 L-Lactate): NAD oxidoreductase was assayed by determining the rate of oxidation of 0·009 M reduced nicotinamide adenine dinucleotide at 340 nm in the presence of 0·0003 M pyruvate in phosphate buffer 0·05 M, pH 7·5 following the instructions issued with Biochemica Test Combination LDH UV-Test No. 15948 TLAC. To assay enzyme release, $2{\cdot}5 \times 10^6$ PE cells were incubated with an equal number of target cells at 37° for 5 hours. Samples were then centrifuged at 2000 rev/min for 10 minutes in an MSE Mistral 6L centrifuge. Supernatants were retained for enzyme assay. Triton X-100 and cytochalasin B were added to selected samples to final concentrations of 0·1 per cent v/v and 10 μg/ml respectively at the beginning of the incubation period.

Rosette test

One millilitre containing $2{\cdot}5 \times 10^6$ immune spleen, lymph node or PE cells was incubated at 4° overnight in the presence of $2{\cdot}5 \times 10^7$ CRC. Non-immune cells were in-

cubated in the presence of 1:1000 antiserum. The tubes were inverted gently until all the cells were in suspension, and a rosette count was performed in a haemocytometer. Cells with at least four attached CRC were considered to be rosettes. The test was performed in duplicate.

Isoelectric focusing

Eight ml of pooled serum from guinea-pigs immunized 4 weeks previously with CRC in FCA was precipitated by $(NH_4)_2SO_4$ at pH 6·5, to a final concentration of 21·6 per cent. The precipitate was dissolved in 8 ml saline and dialysed against 0·01 M phosphate buffer at pH 9·0. Three millilitres were applied to an isoelectric focusing column of 1 per cent ampholines in a sucrose gradient, pH range 5·0–9·0, following the method of Vesterberg and Svensson (1966). The anode was at the bottom. Fractions were pooled, concentrated and dialysed against medium 199.

Elution and absorption of cytophilic antibody

PE cells from guinea-pigs immunized with CRC and FCA were washed three times, and resuspended at a concentration of $2{\cdot}5 \times 10^6$/ml, in medium 199. The cells were incubated, either at 37° for 18 hours, or at 56° for 30 minutes. After centrifugation at 270 ***g*** for 10 minutes, the supernatant was removed and passed through a Millipore sterilizing membrane. Concentrated eluates were prepared from 200×10^6 PE cells/ml. Control eluates were obtained from PE cells of unimmunized guinea-pigs, or others receiving adjuvant only. Eluates were used at a final dilution of 1:3 for cytotoxicity assay.

Absorption of eluates was performed by adding 0·3 ml packed CRC or human erythrocytes (HRC) to 5 ml eluate and rotating the mixture at room temperature for 1 hour, or at 4° for 18 hours.

Immunofluorescence

Erythrocytes were incubated with antibody at 4° for 18 hours. After washing, the cells were incubated for 30 minutes with rabbit anti-guinea-pig globulin at a dilution of 1:5, and then washed. They were resuspended in a 1:5 dilution of fluorescent goat anti-rabbit globulin (Nordic) for 30 minutes. Cells were washed twice and examined by incident ultraviolet light for fluorescence.

Cytophilic antibody from serum

Non-immune PE cells, after two washes, were incubated at 37° for 75 minutes in a 1:10 dilution of guinea-pig antiserum. They were then washed four times in ice-cold medium containing 3 per cent foetal calf serum (FCS). When serum fractions were used instead of whole serum, the incubation medium contained in addition 1 per cent methyl cellulose (viscosity 15 cps, Dow Chemical Company).

RESULTS

PHAGOCYTOSIS AND CYTOTOXICITY

The amount of phagocytosis of CRC by immune macrophages is shown in Table 1. At a ratio of one PE cell to two CRC, this had reached virtual completion by 3½ hours. Fig. 1 shows cytotoxicity at effector: target cell ratios varying between 50:1 and 10:1. At ratios of less than 5:1, significant cytotoxicity could not consistently be detected after 20

TABLE 1

PHAGOCYTOSIS OF CHICKEN ERYTHROCYTES BY IMMUNE PERITONEAL EXUDATE CELLS

Time (hours)	Per cent clearance	Per cent phagocytosis
1½	68±12	—
2½	73±20	55±14
3½	84±12	82±20
4½	90±11	—
5½	—	82±20

Clearance of CRC from cell suspensions, and phagocytosis of CRC by PE cells from guinea-pigs immunized with CRC in FCA. Results are means of five experiments±standard deviation. Ratio of two CRC to one PE cell.

hours' incubation. This shows that a greater effector:target cell ratio is needed for demonstration of cytotoxicity than for phagocytosis, using immune cells as the effector population.

In the passive system, in which normal PE cells, antiserum and target cells were used, cytotoxicity of 29 per cent was obtained at a 1:1 cell ratio (Table 2) while even at a ratio as low as 1:10, 10 per cent cytotoxicity could be obtained. The amount of phagocytosis was similar to that obtained with immune cells, reading 77 per cent at 5 hours. This demonstrates that target cells coated with antibody are both phagocytosed and destroyed with comparable efficiency by PE cells.

To determine the relationship of phagocytosis to cytotoxicity, cytochalasin B was used at a concentration of 10 μg/ml. Non-immune PE cells were incubated with CRC and 1:1000 antiserum. Fig. 2 shows that in the presence of cytochalasin B, CRC adhered to macrophages while phagocytosis was not observed. Quantification of adherence and

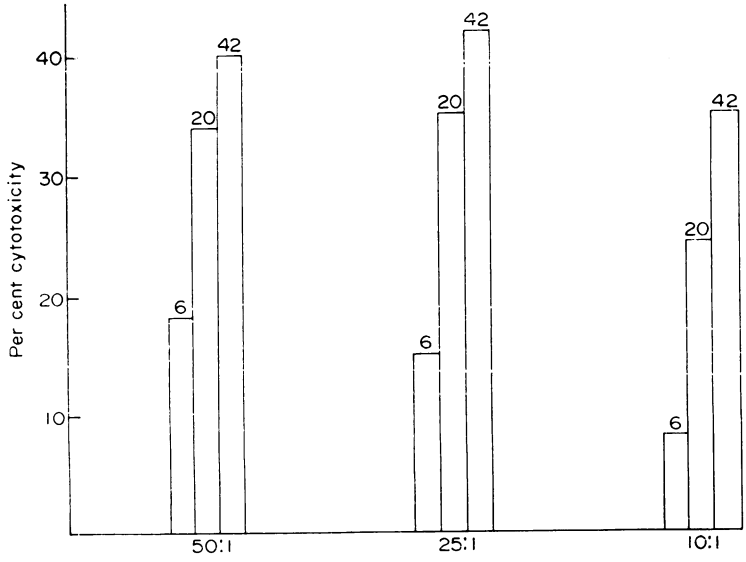

FIG. 1. Effect of incubation period on PE cell cytotoxicity. Immune PE cells incubated for 6, 20 and 42 hours with chicken-erythrocytes at ratios of 50, 25 and 10 effector cells to one target cell.

phagocytosis is shown in Table 3. Cytotoxicity obtained with rocking for 5 hours is shown in Fig. 8. PE cell cytotoxicity was increased in the presence of cytochalasin B. Accompanying this, there were striking increases in the release of two representative lysosomal enzymes, β-glucuronidase and acid protease, at a ratio of 10^5 effector to 10^5 target cells, from the cells into the medium (Table 8). In marked contrast only marginal increases in the release of non-lysosomal enzymes, exemplified by lactate dehydrogenase and leucine-2-naphthylamidase, occurred in the presence of cytochalasin B. Whereas cytotoxicity was favoured by a high effector: target cell ratio, the reverse was true of lysosomal enzyme release occurring in the presence of cytochalasin B. Incubation of 2×10^6 effector cells with 10^5 target cells in the presence of 10 μg/ml cytochalasin B resulted in only slight increase of lysosomal enzyme release into the medium. Non-immune macrophages in

TABLE 2

CYTOTOXICITY TOWARDS CHICKEN ERYTHROCYTES BY NON-IMMUNE PERITONEAL EXUDATE CELLS WITH ANTISERUM

Ratio effector: target cells	Per cent cytotoxicity	No. of experiments
50:1	34±12	7
25:1	46±10	4
10:1	37±13	8
5:1	37±4	5
1:1	29±11	8
1:10	10±6	6

Antiserum was used at a dilution of 10^{-3}.

the presence of cytochalasin B, but without antibody, caused cytotoxicity ranging from 0 to 7 per cent at 5 hours. The supernatant medium obtained from incubation of non-immune macrophages with antibody and cytochalasin B failed to show cytotoxicity for CRC.

A comparison of the effect of cytochalasin B on PE cell and spleen cell cytotoxicity was obtained using the passive system with antibody dilution 1:1000, and a cell ratio of 50:1, as shown in Fig. 8. At 5 hours, PE cells cytotoxicity was increased by addition of cytochalasin B while spleen cell cytotoxicity was decreased. Estimation of lactic dehydrogenase as a measure of viability showed an increase of 18–23 per cent for PE cells, and no increase for spleen cells. At 19 hours, however, by trypan blue exclusion only 35 per cent of spleen cells were viable, compared to 60 per cent of PE cells.

ROSETTE FORMATION

Table 4 shows the distribution of rosette-forming cells from PE, spleen and lymph node from immunized and non-immunized guinea-pigs. PE cells gave similar results following immunization with CRC either in FCA or intravenously. Spleen cells formed fewer rosettes, but also showed no difference attributable to the method of sensitization. No significant numbers of rosettes were obtained with lymph node cells. Cytotoxicity of PE cells and spleen was similar following either method of immunization (Loewi and Temple, 1972).

CYTOPHILIC ANTIBODY ELUTED FROM PE CELLS

On incubation at either 37° for 18 hours, or 56° for 30 minutes, immune PE cells

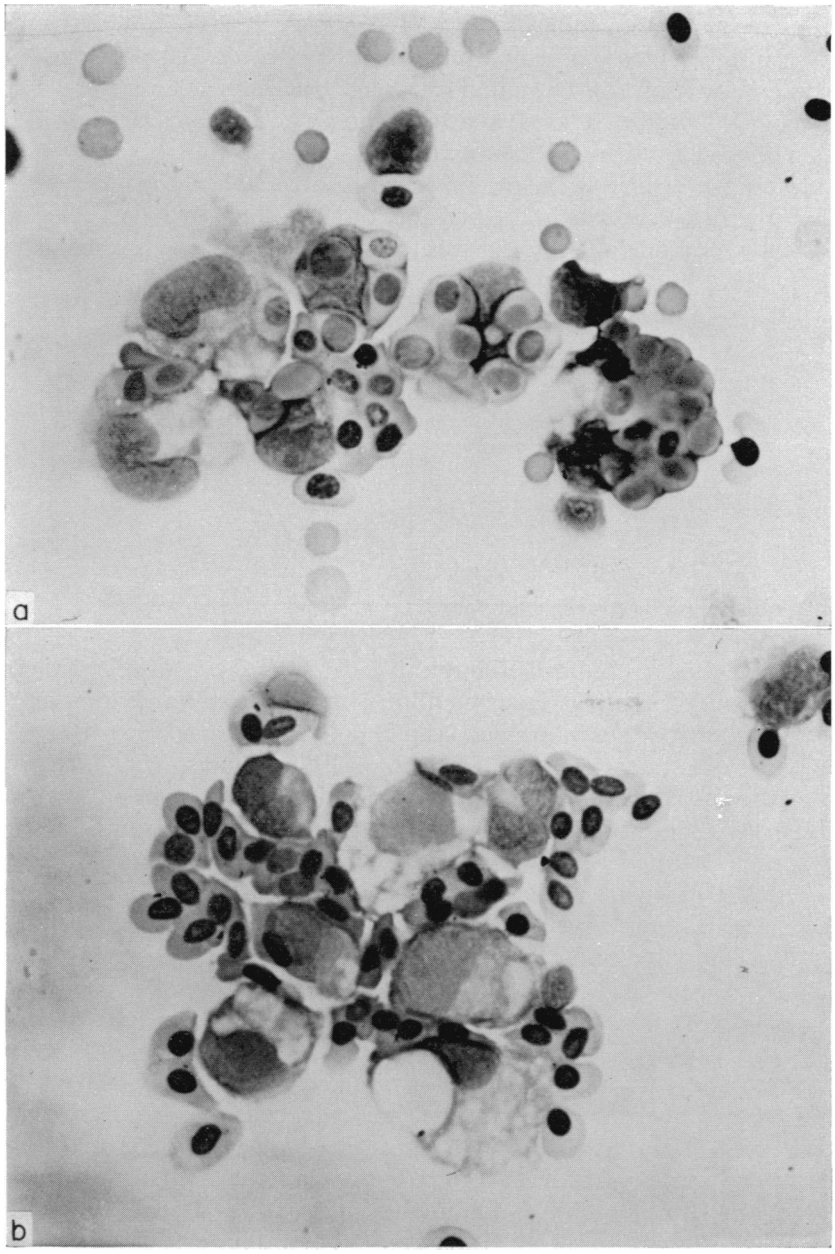

FIG. 2. Phagocytosis and adherence of chicken erythrocytes. (a) Phagocytosis of CRC by non-immune PE cells with antiserum diluted 1/1000. (b) Inhibition of phagocytosis by 10 μg cytochalasin B/ml.

TABLE 3

EFFECT OF CYTOCHALASIN B ON PHAGOCYTOSIS

	Per cent phagocytosis	Per cent adherence
Non-immune PE cells	95	1
+1:1000 antiserum	93	1
	65	33
	56	40
Non-immune PE cells	11	100
+1:1000 antiserum	2	95
+10 μg/ml cytochalasin B	0·3	31

Phagocytosis and adherence to non-immune PE cells of chicken erythrocytes with antiserum diluted 1/1000 ± cytochalasin B. Each number represents one experiment.

TABLE 4

ROSETTE-FORMING CELLS FROM IMMUNE GUINEA-PIGS

Immunization route	PE cells per cent RFC ± S.D.	No. of animals	Spleen per cent RFC ± S.D.	No. of animals	L.N. per cent RFC ± S.D.	No. of animals
CRC+FCA	29·0 ± 8·0	6	3·2 ± 2·1	5	1·1 ± 1·0	3
CRC I/V × 2	21·0 ± 4·6	3	3·1 ± 2·5	3	0·2 ± 0·3	2
Nil	0·6 ± 0·2	3	0·1 ± 0·1	3		

Percentage rosette-forming cells from PE, spleen and lymph node of animals immunized with chicken erythrocytes.

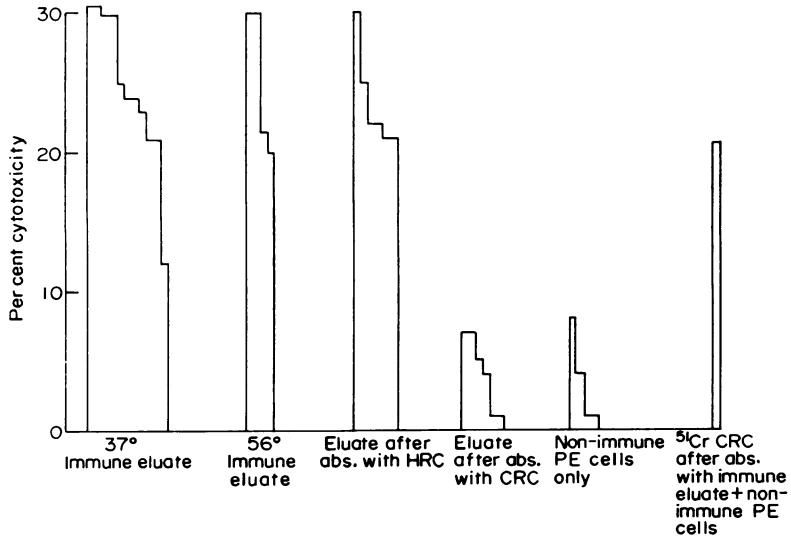

FIG. 3. Cytotoxicity of eluate from immune PE cells in the presence of non-immune peritoneal cells. Steps on histogram blocks are levels of cytotoxicity obtained with individual eluates. The last block shows cytotoxicity of CRC which had been coated with immune eluate prior to incubation with non-immune PE cells.

TABLE 5

EFFECT OF ELUATE ON PHAGOCYTOSIS AND ROSETTES

	Per cent phagocytosis			
	1½ hours	2½ hours	4 hours	Per cent rosettes
CRC coated with eluate	35	39	43	25
HRC coated with eluate	0·3	0·1	0·1	0

Effect of non-immune PE cells on chicken or human erythrocytes which had been exposed at 4° to eluate from immune PE cells.

released a factor which rendered cells from unimmunized animals cytotoxic to CRC. Results are shown in Fig. 3. The activity of eluates obtained at 56° was similar to that obtained at 37°, demonstrating that the activity was not caused by antibody formation by contaminating lymphocytes during incubation. Eluate caused no cytotoxicity without addition of effector cells.

Absorption by CRC removed all cytotoxic activity, whereas absorption with HRC did not. CRC which had been incubated with eluate and then labelled with ^{51}Cr, gave 21 per cent cytotoxicity on exposure to non-immune PE cells.

After eluate absorption and washing, CRC or HRC were exposed to non-immune PE cells at 37° or 4° to observe phagocytosis and rosette formation. Results are shown in Table 5. Phagocytosis of CRC occurred, but few HRC were phagocytosed within 4 hours. Twenty-five per cent of PE cells formed rosettes with CRC, but none with HRC.

Immunofluorescence, shown in Fig. 5, demonstrates that guinea-pig globulin was present on CRC following treatment with eluate but was not present on HRC similarly treated. Immune electrophoresis, performed on concentrated eluate demonstrated a precipitation band in the γ_2-globulin region (Fig. 4).

The specificity of the cytophilic antibody for effector cells was examined by comparing normal guinea-pig spleen, lymph node and peripheral blood lymphocytes with PE cells. Blood lymphocytes were obtained by ficoll gradient separation, following the method of Harris and Ukaejiofo (1969). Results, in Table 6, show that the eluate is inactive with lymph node cells, and has very little activity with blood lymphocytes, whereas spleen cells showed cytotoxicity comparable to that shown by PE cells.

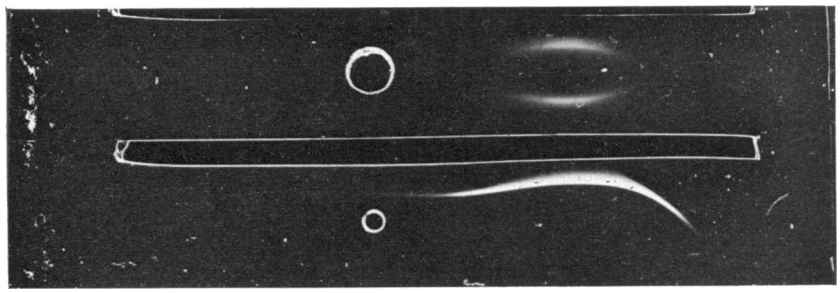

FIG. 4. Characterization of eluate from immune PE cells immunoelectrophoresis of (a) normal guinea-pig serum (lower) and immune eluate (upper) developed with rabbit anti-guinea-pig globulin, showing a line in the γ_2-globulin region.

TABLE 6

CYTOTOXICITY OF LYMPHOID AND PE CELLS WITH ELUATE FROM PE CELLS

Cell type	Number of effector cells	Per cent cytotoxicity with immune eluate	Per cent cytotoxicity with control eluate
Peripheral blood lymphocytes	$2{\cdot}5\times10^6$	4, 2	1, 0
Lymph node	5×10^6	1, 1	1
	$2{\cdot}5\times10^6$	0, 0	0
Spleen	5×10^6	43, 42, 32	4
	$2{\cdot}5\times10^6$	38, 13, 12	2
PE	5×10^6	30, 21, 18	2, 0
	$2{\cdot}5\times10^6$	36	0

Each number under heading of cytotoxicity represents results obtained with cells from one animal.

TABLE 7

COMPARISON OF CYTOTOXICITY WITH CYTOPHILIC AND FREE ANTIBODY

Immunization		5×10^6 PE cells coated 1:10 antiserum (per cent)	2×10^6 PE cells +antiserum 10^{-3} (per cent)
Time	Type of immunization		
1 week	i.v. × 2	21	37
3 weeks	i.v. × 2	25 ± 13	54 ± 5
1 week	FCA	42 ± 8	29 ± 1
3 weeks	FCA	42 ± 13	67 ± 5
	nil	1	0

PE cells previously exposed to antiserum were incubated with target cells at 50:1 ratio, PE cells in the presence of antiserum were at a ratio of 20:1. Results are means of four separate antisera.

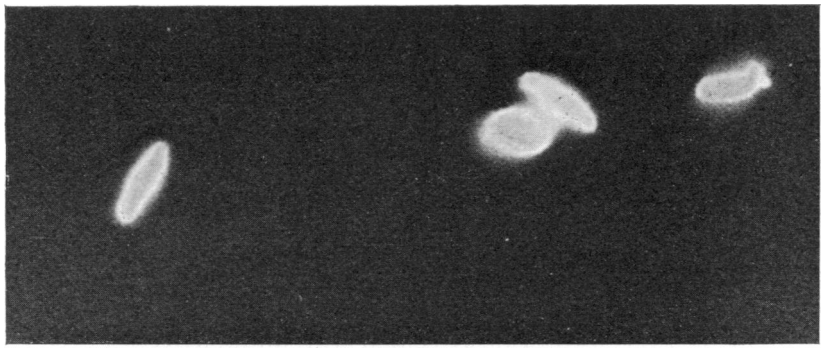

FIG. 5. Chicken erythrocytes coated with eluate, followed by rabbit anti-guinea-pig globulin, and stained with goat anti-rabbit globulin F.I.T.C.

Serum cytophilic antibody

Immunization of guinea-pigs by CRC with FCA or by the intravenous route gave rise to sera which were cytotoxic to CRC in the presence of non-immune PE cells. To assess cytophilic antibody, PE cells were treated with antiserum prior to addition of CRC for

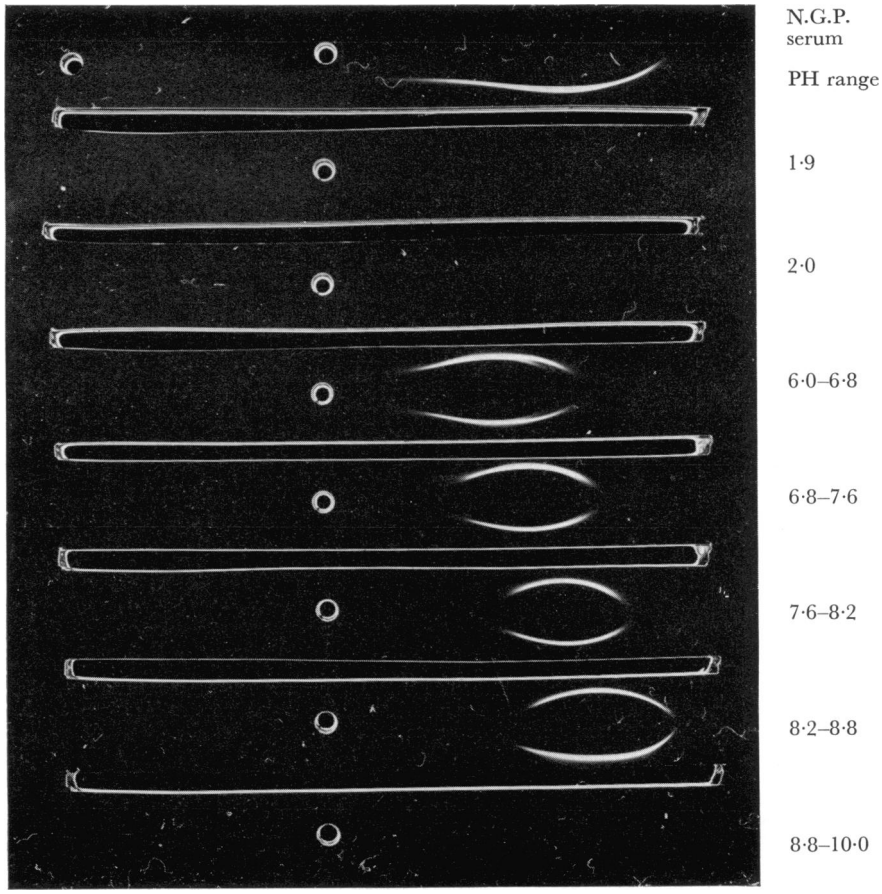

FIG. 6. Immunoelectrophoresis of fractions from an isoelectric focusing column.

cytoxicity assay. Results, shown in Table 7 demonstrate that PE cells coated with antisera from FCA-immunized animals showed greater cytotoxity than those treated with antisera from intravenously immunized animals. Cytotoxic titres for cytophilic antibody were similar whether sera were obtained 1 or 3 weeks after immunization. Table 7 shows cytotoxicity levels obtained when immune sera were added to the incubation mixture instead of employing PE cells previously coated with antibody. This, even at a dilution of 10^{-3}, and employing fewer effector cells, gave greater cytotoxicity than the cytophilic system.

Gamma-globulin from a pool of antiserum from five guinea-pigs, 28 days after immunization by CRC with FCA, was fractionated using isoelectric focusing (IEF). Fractions from various peaks were estimated for protein concentration, immunoelectro-

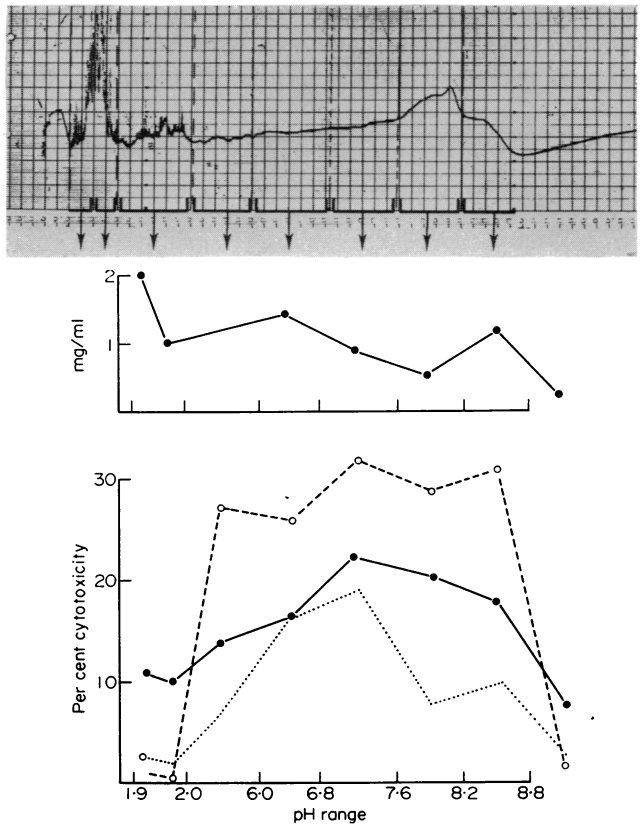

FIG. 7. Cytotoxicity and rosette formation of fractions obtained by isoelectric focusing of antiserum. Upper: Elution pattern from isoelectric focusing column in terms of optical density. Centre: Protein concentration of pooled fractions estimated by the Lowry method. Lower: Cytotoxicity (○—○) caused by 5×10^6 non-immune PE cells incubated with chicken erythrocytes in the presence of 1 in 10,000 dilution of isoelectric focusing fractions. Cytotoxicity (●—●) and rosette formation (· · · · ·) caused by 5×10^6 non-immune PE cells coated with 1 in 2 dilution of isoelectric focusing fractions.

TABLE 8

EFFECT OF CYTOCHALASIN B ON ENZYME RELEASE

	Enzyme release (per cent of total released by 0·1 per cent v/v Triton X-100)	
	Control	Cytochalasin B (10 μg/ml)
β-Glucuronidase	16·0 ± 2·7	65·6 ± 7·6
Acid protease	15·7 ± 1·8	35·1 ± 4·0
Lactate dehydrogenase	17·7 ± 2·0	23·4 ± 1·5
Leucine-2-naphthylamidase	5·9 ± 1·2	13·0 ± 13·1

The release of lysosomal (β-glucuronidase and acid protease) and non-lysosomal (lactate dehydrogenase and leucine-2-naphthylamidase) enzymes from PE cells by cytochalasin B. Values are expressed as percentage of enzyme activity released by Triton X-100. 100 per cent represents 162·1 nm phenolphthalein/10^6 cells/hour, 1077 nm tyrosin/10^6 cells/hour, 177·6 m units/10^6 cells and 1968 nm naphthylamine/10^6 cells/hour for β-glucuronidase, acid protease, lactate dehydrogenase and leucine-2-naphthylamidase respectively. Each value represents the mean ± standard deviation of three different samples.

phoresis was performed and cytotoxicity was assayed using non-immune PE cells. Fractions were either coated on PE cells prior to cytotoxicity assay or incubated with PE cells and CRC. Results are shown in Figs 6 and 7. Cytotoxicity was not dependent on protein concentration as such.

Cytophilic antibody associated with cytotoxicity was obtained from IEF fractions.

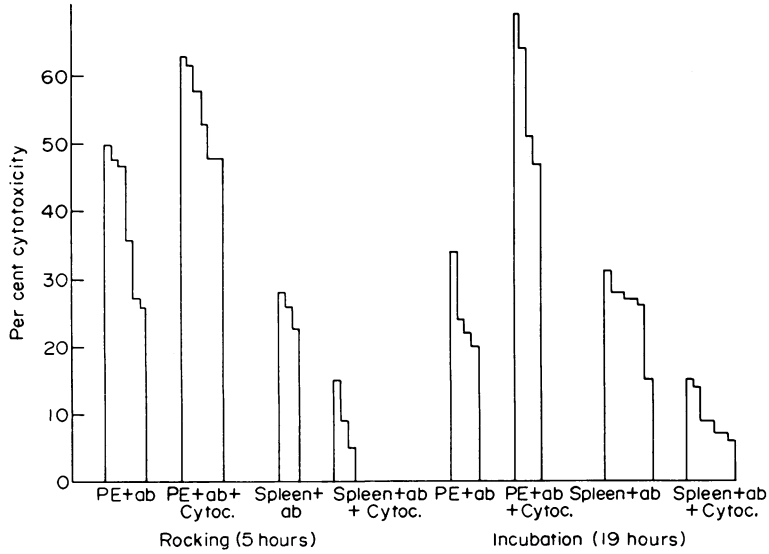

FIG. 8. Effect of cytochalasin B on spleen and PE cytotoxicity. Non-immune spleen and PE cells with antiserum diluted 1/1000 ± cytochalasin B at a concentration of 10 μg/ml. Steps on histogram block show cytotoxicity obtained in individual experiments. Results with and without rocking are shown. Effector cell to target cell ratio 50:1.

Maximal cytoxicity coincided with the fractions taken at pH 6·8–7·6. This was shown on immunoelectrophoresis (Fig. 6) to be a γ_2-globulin of relatively fast mobility. When the fractions were incubated with the cell mixture, however, cytotoxicity occurred even at a dilution of 10^{-4}. At 1:100, cytotoxicity of 50 per cent was demonstrated, using IEF fractions of pH 2·0–8·8.

DISCUSSION

A phagocytic mechanism suggests itself as the most readily available explanation for macrophage cytotoxicity, and, as we have described, in a heterologous system phagocytosis is indeed observed. Bennett (1965) who used a mouse tumour system, suggested that tumour cell suppression might be the result of phagocytosis by macrophages. Old, Boyse, Bennett and Lilly (1963) had shown that mouse tumour cells became adherent to peritoneal macrophages from immunized donors, and some phagocytosis took place, resulting in apparent destruction of the target cells. Granger and Weiser (1964) also described specific adherence and destruction on *in vitro* contact between immune peritoneal macrophages and mouse homograft fibroblast target cells, using a plaque technique to determine cell destruction. They failed, however, to find any cytological evidence that target cell injury resulted from phagocytosis by immune cells. Evans and Alexander (1970, 1971) found, in an *in vitro* system, that added macrophages were necessary for the destruction of

lymphoma cells by syngeneic immune spleen cells. Nevertheless, they reported that phagocytosis only occurred after 24 hours when the target cells began to degenerate, suggesting that phagocytosis was not an essential step. Using a heterologous system which allows quantitative observations of phagocytosis and rosette formation as well as quantification of cytotoxicity to be made, we found all three phenomena, so that it was essential to dissociate phagocytosis from cytotoxicity, to discover whether or not cytotoxic target cell destruction was merely incidental to phagocytosis. Addition of cytochalasin B allowed such a differentiation, for although phagocytosis could be inhibited, cytotoxicity was undiminished. We have provided evidence that cytotoxicity was not a consequence of the presence of cytochalasin B and our results do not suggest that the lysosomal enzymes which were lost from macrophages into the incubation medium made a major contribution to chromium release from the target cells, since target cells incubated in such medium failed to release chromium. Cytochalasin B has been shown to interfere with several processes associated with the activity of contractile microfilaments, a group of actin-containing filamentous proteins, found in the peripheral cytoplasm of many cells (Wessels *et al.*, 1971). It is not clear at present whether cytochalasin B is able to interact directly with these subcellular components. Amongst cellular functions inhibited are phagocytosis by macrophages (Allison, Davies and De Petris, 1971; Malawista, Gee and Bensch, 1971) and polymorphonuclear leucocytes (Davies, Estensen and Quie, 1971; Davies, Allison, Fox, Polyzonis and Haswell, 1972). Phagocytosis *in vitro* by these cells is normally associated with a selective loss of small amounts of lysosomal enzymes (Weissmann, Dukor and Zurier, 1971; Henson, 1971; Hawkins, 1971, 1972). Despite the inhibition of phagocytosis by cytochalasin B it enhances the selective release of polymorphonuclear leucocyte lysosomal enzymes into the extracellular medium (Davies *et al.*, 1972). Lysosomal enzymes cannot be completely solubilized (Baccino, Rita, Zuretti, 1971) so that if their release by cytochalasin B occurs by exocytosis, their concentration at the plasma membrane would be expected to increase, thereby increasing its potential for target cell lysis. The increase in ^{51}Cr release seen in the presence of cytochalasin B has at least two alternative explanations. First, it may result from the increased lytic capacity of the plasma membrane discussed above. Secondly, it could be the consequence of the inhibition of the phagocytic capacity of the macrophages; damaged target cells would normally be accumulated within macrophage secondary lysosomes and ^{51}Cr sequestered by the acid glycolipid component of these organelles (Dingle and Barrett, 1969). In the absence of phagocytosis, ^{51}Cr would be more readily released into the supernatant resulting in the increased cytotoxicity observed in our experiments.

We conclude that macrophages are able to mediate cytotoxicity by making contact with target cells, an event which is presumed to be followed by the transmission of enzymes causing a breach in the integrity of the target cell, thus leading to disintegration and release of the chromium label. Such a mechanism would serve to explain the observations of non-phagocytic macrophage killing by Evans and Alexander (1970, 1971) and Granger and Weiser (1964), while not denying that phagocytosis may serve as part of a cytotoxic mechanism in other systems, including the model which we have studied.

The specificity of macrophage cytotoxicity was explicable in our system by the finding of specific cytophilic antibody directed against the target cells. We were able to elute this from the PE cells of immunized animals and absorb it specifically to chicken erythrocytes, thus showing its cytophilic nature as well as specificity for antigen. A fraction in the γ_2-region of immune sera similarly provided a specific cytophilic antibody, which gave

cytotoxicity. However, when all isoelectric focusing fractions from pH 2·0 to pH 8·8 were added to the incubation mixture instead of being selectively coated on macrophages, the resulting cytotoxicity in the presence of normal macrophages was considerably greater, suggesting that antibody which was not necessarily cytophilic for macrophages was also instrumental in rendering such cells cytotoxic for target cells. Phagocytosis and the ability to form rosettes were similarly mediated by this type of antibody, whether eluted from immune macrophages or obtained from immune serum.

Old *et al.* (1963) reported that peritoneal macrophages from immunized donors phagocytosed and destroyed tumour cells *in vitro*, but that this capacity could be removed by simply washing the peritoneal cells. However, since such cells were still able to inhibit tumour growth *in vivo*, these experiments cannot be readily interpreted. Granger and Weiser (1964) were unable to render normal peritoneal macrophages cytotoxic for target cells *in vitro* by adding iso-antiserum. On the other hand, the same authors (1966) have reported that heating of immune macrophages at 56° produced a specific target cell agglutinin which, when a source of complement was added, killed a high proportion of cells. They concluded that cell adherence was a passive phenomenon depending on adherent antibody, whilst subsequent target cell destruction, which could be inhibited by actinomycin D or chloramphenicol, depended on biosynthetic activity. Evans' and Alexander's (1970) work on immunity to syngeneic murine lymphoma similarly indicates the possibility that antibody may 'arm' macrophages, since normal syngeneic macrophages could be rendered cytotoxic following incubation with immune spleen cells.

The cytophilic antibody which we have described, fulfils the criterion of conferring specific rosette formation on macrophages while at the same time inducing cytotoxicity. In view of the findings of Nelson and Mildenhall (1968) and of other work reviewed by Nelson (1969) on the requirement for immunization with Freund's complete adjuvant for cytophilic antibody formation, we were surprised to find this in the sera of animals immunized by the intravenous route; however, Berken and Benacerraf (1966) report the finding of cytophilic antibody in sera of some guinea-pigs immunized with sheep erythrocytes intravenously. In agreement with other authors on guinea-pig cytophilic antibodies, we have found the peak of cytophilic activity in the γ_2-globulin region, both for serum and eluate from macrophages.

Lastly, it must be noted that in spite of the evidence we have adduced for cytotoxic macrophage activity in some systems, there is clear-cut evidence for the cytotoxic effectiveness of lymphocytes from peritoneal exudates in others. Berke, Sullivan and Amos (1972) found that purified lymphocytes from peritoneal exudates of BALB/c mice immune to EL4 leucosis destroyed EL4 cells more effectively than lymphoid cells taken from other sources. This activity was enhanced by removal of macrophages. It should be emphasised that all these are *in vitro* models, and a definitive appraisal of the role of different systems must include the consideration of their *in vivo* efficacy.

REFERENCES

Allison, A. C., Davies, P. and De Petris, S. (1971). 'Role of contractile microfilaments in macrophage movement and endocytosis.' *Nature: New Biology*, **232**, 153.

Anson, M. L. (1936). 'The estimation of cathepsin with haemoglobin and the partial purification of cathepsin.' *J. gen. Physiol.*, **20**, 565.

Baccino, F. M., Rita, G. A. and Zuretti, M. F. (1971). 'Studies on the structure-bound sedimentability of some rat liver lysosome hydrolases.' *Biochem. J.*, **122**, 363.

Bennett, B. (1965). 'Phagocytosis of mouse tumour cells *in vitro* by various homologous and heterologous cells.' *J. Immunol.*, **95**, 80.

BENNETT, B., OLD, L. J. and BOYSE, E. A. (1964). 'The phagocytosis of tumour cells *in vitro*.' *Transplantation*, **2**, 183.

BERKE, G., SULLIVAN, K. A. and AMOS, B. (1972). 'Rejection of ascites tumor allografts. I. Isolation, characterization, and *in vitro* reactivity of peritoneal lymphoid effector cells from BALB/c mice immune to EL4 leukosis.' *J. exp. Med.*, **135**, 1334.

BERKEN, A. and BENACERRAF, B. (1966). 'Properties of antibodies cytophilic for macrophages.' *J. exp. Med.*, **123**, 119.

DAVIES, P., KRAKAUER, K. and WEISSMANN, G. (1970). 'Subcellular distribution of neutral protease and peptidases in rabbit polymorphonuclear leukocytes.' *Nature (Lond.)*, **228**, 761.

DAVIES, P., ALLISON, A. C., FOX, R. I., POLYZONIS, M. and HASWELL, A. D. (1972). 'The exocytosis of polymorphonuclear leukocyte lysosomal enzymes induced by cytochalasin B.' *Biochem. J.*, **128**, 78P.

DAVIES, A. T., ESTENSEN, R. and QUIE, P. G. (1971). 'Cytochalasin B. III. Inhibition of human polymorphonuclear leukocyte phagocytosis.' *Proc. Soc. exp. Biol. (N.Y.)*, **137**, 161.

DINGLE, J. T. and BARRETT, A. J. (1969). 'Uptake of biologically active substances by lysosomes.' *Proc. roy. Soc. B.*, **173**, 85.

EVANS, R. and ALEXANDER, P. (1970). 'Co-operation of immune lymphoid cells with macrophages in tumour immunity.' *Nature (Lond.)*, **228**, 620.

EVANS, R. and ALEXANDER, P. (1971). 'Rendering macrophages specifically cytotoxic by a factor released from immune lymphoid cells.' *Transplantation*, **12**, 227.

Gorer, P. A. (1961). 'The antigenic structure of tumours.' *Advanc. Immunol.*, **1**, 345.

GRANGER, G. A. and WEISER, R. S. (1964). 'Homograft target cells: Specific destruction *in vitro* by contact interaction with immune macrophages.' *Science*, **145**, 1427.

GRANGER, G. A. and WEISER, R. S. (1966). 'Homograft target cells: Contact destruction *in vitro* by immune macrophages.' *Science*, **151**, 97.

HARRIS, R. and UKAEJIOFO, E. O. (1969). 'Rapid preparation of lymphocytes for tissue-typing.' *Lancet*, **ii**, 327.

HAWKINS, D. (1971). 'Biopolymer membrane: A model system for the study of the neutrophilic leukocyte response to immune complexes.' *J. Immunol.*, **107**, 344.

HAWKINS, D. J. (1972). 'Neutrophilic leukocytes in immunological reactions: evidence for the selective release of lysosomal constituents.' *J. Immunol.*, **108**, 310.

HENSON, P. (1971). 'The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and complement on non-phagocytosable surfaces or phagocytosable particles.' *J. Immunol.*, **107**, 1535.

LOEWI, G. and TEMPLE, A. (1972). 'Cytoxicity of immune guinea pig cells. I. Investigation of a correlation with delayed hypersensitivity and a comparison of cytotoxicity of spleen, lymph node and peritoneal exudate cells.' *Immunology*, **23**, 559.

MACLENNAN, I. C. M., LOEWI, G. and HARDING, B. (1970). 'The role of immunoglobulins in lymphocyte-mediated cell damage, *in vitro*. I. Comparison of the effects of target cell specific antibody and normal serum factors on cellular damage by immune and non-immune lymphocytes.' *Immunology*, **18**, 397.

MALAWISTA, S. E., GEE, J. B. L. and BENSCH, K. G. (1971). 'Cytochalasin B reversibly inhibits phagocytosis: functional, metabolic and ultrastructural effects in human blood leukocytes and rabbit alveolar macrophages.' *Yale J. Biol. Med.*, **44**, 286.

NELSON, D. S. (1969). *Macrophages and Immunity*, p. 63. North-Holland, London.

NELSON, D. S. and MILDENHALL, (1968). 'Studies on cytophilic antibodies. II. The production by guinea pigs of macrophage cytophilic antibodies to sheep erythrocytes and human serum albumin: relationship to the production of other antibodies and the development of delayed-type hypersensitivity'. *Aust. J. exp. Biol. med. Sci.*, **46**, 33.

OLD, L. J., BOYSE, E. A., BENNETT, B. and LILLY, F. (1963) 'Peritoneal cells as an immune population in transplantation studies.' In: *Cell-Bound Antibodies* (Ed. by B. Amos and H. Koprowski) Wistar Institute Press, Philadelphia.

TALALAY, P., FISHMAN, W. H. and HUGGINS, C. (1946). 'Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity.' *J. biol. Chem.*, **166**f, 757.

VESTERBERG, O. and SVENSSON, H. (1966). 'Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins.' *Acta chem. scand.*, **20**, 820.

WEISSMAN, G., DUKOR, P. and ZURIER, R. B. (1971). 'Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles.' *J. exp. Med.*, **134**, 149.

WESSELS, N. K., SPOONER, B. S., ASH, J. F., BRADLEY, M. O., LUDUENA, M. A., TAYLOR, E. L., WRENN, J. T. and YAMADA, K. M. (1971). 'Microfilaments in cellular and developmental processes.' *Science*, **171**, 135.