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Macrophages induce antibody-dependent cytostasis but not lysis in guinea pig leukaemic cells

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Summary Guinea pig and mouse peritoneal macrophages formed antibody-dependent rosettes with guinea pig L_2C leukaemic cells, but were unable either to phagocytose the cells or to kill them extracellularly as judged by the retention of ^{51}Cr. Macrophages previously activated by BCG *in vivo* also failed to exhibit phagocytosis or cytoxicity towards the antibody-coated cells. These failures could not be attributed to deficient function of the macrophages nor to antigenic modulation of the L_2C cells. The antibodies involved were capable of mediating lysis by complement, and ADCC by human leukocytes.

However macrophages were cytostatic to antibody-coated L_2C cells in that uptake of ^3H-thymidine or ^3H-deoxycytidine was abruptly and in some cases completely inhibited upon cell contact being established. Antigenic modulation which had proceeded sufficiently to protect against lysis by complement did not protect against cytostasis. Syngeneic macrophages had greater cytostatic activity than did allogeneic or xenogeneic. Macrophage activation by BCG did not result in significantly increased cytostasis. A univalent antibody derivative Fab/c was also capable of mediating cytostatis by the macrophages.

Several reports that activated macrophages (mφ) are capable of mediating antibody-dependent cellular cytotoxicity (ADCC) towards lymphoid tumour cells (Alexander & Evans, 1971; Nathan *et al.*, 1979*a*, 1980; Berd & Mastrangelo, 1981; Koren *et al.*, 1981*a*) have prompted us to investigate their activity against neoplastic B lymphocytes of the guinea pig L_2C leukaemia. Previous findings from this laboratory have demonstrated the susceptibility of these cells *in vitro* to antibody-dependent cytotoxicity—both extracellular killing by human peripheral blood leukocytes (Stevenson & Elliott, 1978) and complement-mediated lysis (Gordon *et al.*, 1981).

There are at least three mechanisms by which a mφ can attack a tumour target cell. In phagocytosis, the mφ ingests the target, presumably degrading it once it is internalized (Bennett *et al.*, 1963). In cytotoxicity, the mφ lyses the target extracellularly, the mechanism possibly involving production of hydrogen peroxide (Nathan *et al.*, 1979*b*). Finally, a mφ in antibody-mediated contact with a tumour target cell can inhibit its proliferative activity (Pasternack *et al.*, 1978). Such cell-mediated cytostasis must be distinguished from population phenomena such as contact inhibition (Gyöngyossy *et al.*, 1979).

In the present study syngeneic, allogeneic and xenogeneic mφ were tested for their abilities to induce antibody-dependent cytotoxicity, cytostasis and phagocytosis of L_2C cells *in vitro*. Lysis was assessed by the release of ^{51}Cr, and cytostasis by inhibition of uptake of [^3H]-thymidine or [^3H]-deoxycytidine. Mφ populations were purified by density gradient centrifugation to reduce the possibility that any effects demonstrated were due to the presence of contaminating cells. In order to eliminate any artefacts arising from the use of heat-inactivated antiserum for sensitization, antibody-containing IgG or affinity-purified antibodies were used throughout. Xenogeneic anti-Id and the univalent antibody derivative Fab/c (Glennie & Stevenson, 1982) were used in some experiments to sensitize the L_2C cells in an attempt to assess the possible significance of any effects determined *in vitro* for immunotherapy *in vivo*.

Our results show that cytostasis occurred in L_2C cells following antibody-mediated contact with mφ, but neither phagocytosis nor cytotoxicity could be invoked even with the use of effector cells which had been activated by BCG *in vivo*. These results support the concept that antibody-dependent contact with the effector cell is a primary event, necessary but not sufficient for either phagocytosis or extracellular killing. A further requirement for phagocytosis, that the target cell be fully enveloped by antibody (Griffin *et al.*, 1976), also proved insufficient in our studies.

Materials and methods

Animals

New Zealand White rabbits, strain 2 and strain 13 guinea pigs and White Leghorn chickens were all bred on this site. Sheep and A strain mice were from Allington Farm, Porton, Wiltshire. Mature animals of either sex were used throughout.

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Preparation of macrophages

(i) Resident mφ were obtained by peritoneal lavage with PBS as the recovery medium. (ii) Induced mφ were harvested similarly 5 days after an i.p. injection of 15 ml liquid paraffin oil of density 0.86–0.89 g ml^{-1} (Evans Medical, Liverpool) for guinea pigs, and of 1 ml for mice; the yield was found to be maximal 5 days after injection of the eliciting agent. (iii) Activated mφ were recovered 10 days after an i.p. injection of Bacillus Calmette-Guérin (BCG, Glaxo; 2×10^7 viable organisms in 1 ml water for guinea pigs and 7×10^6 in 0.3 ml for mice) which had been followed after 5 days by an i.p. paraffin oil injection (volumes as above). It was found necessary to use oil with BCG as BCG alone induced insufficient numbers. Three washes in MEM (Minimum Essential Medium with Earle's salts and 20 mM HEPES; Flow Laboratories) at 100 g for 5 min removed excess oil from the cells after harvest.

In all cases oil-induced peritoneal mφ comprised 70–80% and BCG-activated and resident mφ 50–70% of the total cell populations. Characterization was by staining with May–Grünwald–Giemsa, staining for non-specific esterase activity (Yam *et al.*, 1971), and ingestion of India ink and latex particles (Cline & Lehrer, 1968). Chief contaminants were lymphocytes and erythrocytes, with $\sim$1% granulocytes.

Preparations of mφ of >95% purity were obtained with an *in situ* generated density gradient. Percoll (Pharmacia) at 1.130 g ml^{-1} was diluted with 0.15 M NaCl to give a starting density of 1.075 g ml^{-1}. This solution (6.2 ml) was mixed in 10 ml polycarbonate tubes with 0.8 ml peritoneal exudate cells at up to 5×10^7 ml^{-1} in PBS. The tubes, containing 7 ml of Percoll solution mixed with cells were centrifuged at 60,000 g for 9 min in a 20° 10 × 10 angle-head rotor. Dead cells remained at the top of the gradient, while contaminating lymphocytes with densities $\sim$1.090 g ml^{-1} were found towards the bottom. Typical densities of oil-induced guinea pig mφ were between 1.060 and 1.070. The gradient was calibrated with density marker beads (Pharmacia). Mφ recovered from the Percoll gradient after washing 3× in MEM (100 g for 5 min) retained a viability >95% as judged by the exclusion of trypan blue. Purified mφ populations gave normal distributions when analyzed for number against size on a fluorescence-activated cell sorter (FACS III, Becton-Dickinson).

Preparation of human effector cells

Venous blood from a normal donor was mixed 1:1 with PBS before layering onto an equal volume of Lymphoprep (Nyegaard) at 1.077 g ml^{-1}. Following centrifugation at 1000 *g* for 20 min, the interface cell layer was washed first in PBS then twice in MEM (100 *g* for 5 min) and was found to contain mainly lymphocytes with monocytes. Viability was judged to be >95% by the exclusion of trypan blue.

Preparation of target cells

Chicken red blood cells (CRBC) were obtained in heparin (20 units ml^{-1}, Weddel Pharmaceuticals) from wing vein bleeds. L_2C leukaemic cells were prepared as follows: Blood from strain 2 guinea pigs in the terminal stages of the disease was drawn by cardiac puncture into 0.2 volume 120 mM sodium citrate, pH 7.4. Contaminating red cells were removed after layering on Lymphoprep (Nyegaard) and centrifuging at 1000 *g* for 25 min. The cells which formed at the interface were washed first in PBS then twice in MEM (100 *g* for 5 min). L_2C cells comprised >95% of the total population and had a viability >95% as judged by the exclusion of trypan blue.

Preparation of antibodies

Rabbit antibodies to CRBC and L_2C cells were raised by injecting 3×10^8 cells emulsified in Freund's complete adjuvant (Difco, U.S.A.) to give a final volume of 1 ml per rabbit. Injections were given s.c. into the dorsa of the feet. An i.v. boost of 3×10^8 cells in aqueous medium followed after 6 weeks. One week later, the rabbits were exsanguinated and the serum collected. Rabbit IgG was prepared from the serum by sequential precipitation with 1.6 M $(NH_4)_2SO_4$, passage through DEAE-cellulose (Whatman DE52) equilibrated with 0.03 M phosphate buffer, pH 7.3, and gel filtration on Ultrogel AcA 34 (LKB) equilibrated with PBS.

Anti-Ia serum, directed towards the histocompatibility antigens Ia (2,4), was raised in strain 13 guinea pigs by immunization with normal strain 2 splenic and nodal lymphocytes (Schwartz *et al.*, 1976). The IgG was prepared as above.

Two rabbit antibodies directed against L_2C surface IgM were used: anti-Cλ, specific for the constant region of the λ chain, and anti-Id, specific for the idiotypic determinants. Anti-Cλ in the form of purified antibody was obtained from rabbit anti-guinea pig Fab$\gamma\lambda$ serum (Stevenson *et al.*, 1977*a*). Anti-Id in the form of total IgG was prepared in rabbits as previously described (Stevenson *et al.*, 1977*b*); antibodies directed against constant regions were removed by passage through two immunosorbent columns, one coupled with guinea pig IgM and the other with guinea pig serum globulins.

A univalent antibody fragment, Fab/c, was prepared from purified rabbit anti-Cλ as previously described (Glennie & Stevenson, 1982).

Sheep anti-rabbit IgG was obtained in purified form by elution from an immunosorbent column. A fluorescent conjugate of this antibody was also prepared, using fluorescein isothiocyanate (FITC).

Coupling of rabbit anti-L_2C IgG to Sephadex G-25 Superfine beads (Pharmacia) was achieved using cyanogen bromide (Porath *et al.*, 1967).

Culture medium

All assays were carried out in RPMI 1640 containing 25 mM HEPES buffer and L-glutamine (Gibco), supplemented with 20% heat-inactivated (56°C for 30 min) foetal calf serum (Froxfield, Hampshire), 100 units ml^{-1} Crystamycin (Glaxo), 50 units ml^{-1} Mycostatin (Squibb and Sons, Twickenham), 10 units ml^{-1} heparin (Weddel Pharmaceuticals) and 2 mM fresh L-glutamine (Gibco). This medium is referred to as RPMI-S.

Assessment of binding of effector to target cells and phagocytosis of target cells

Effector cells were incubated at 5×10^6 ml^{-1} in 2 ml RPMI-S with targets at 10^7 ml^{-1} in screwtop 5 ml bijoux bottles (Sterilin) for 2 h at 37°C. Rosette formation and phagocytosis were observed by viewing samples on a haemocytometer. Permanent records were made from cytospin preparations (Shandon) stained with May–Grünwald–Giemsa.

Cytotoxicity induced by complement

Assays to determine target cell lysis by complement were carried out as described previously (Gordon *et al.*, 1981) but with a 1:2 dilution of fresh serum in MEM as the complement source.

Assay of cellular cytotoxicity

Target cells (10^8) were washed in MEM (100 g for 5 min) before the pellet was resuspended in 200 μl sodium ^{51}chromate (CJS4 at 1 mCi ml^{-1} in PBS; Amersham International) and incubated at 37°C for 30 min. The cells were then washed 4 times in warm MEM and resuspended at 2.5×10^7 ml^{-1} for sensitization with antibody or incubation with normal IgG for 15 min. Antibody-coating was carried out at room temperature for rabbit anti-L_2C and guinea pig anti-Ia, which are resistant to antigenic modulation, and on ice for rabbit anti-Cλ and rabbit anti-Id, which are susceptible to antigenic modulation. Where antigenic modulation was specifically sought, sensitization was carried out at 37°C for 30 min. The final concentration of sensitizing antibody when in the form of total IgG was 400 $\mu g\,ml^{-1}$, while that for purified antibody was 40 $\mu g\,ml^{-1}$. Washing off excess antibody had no effect on the subsequent cytotoxicity and so was abandoned.

Unless other wise stated, targets were diluted in RPMI-S to 2×10^5 ml^{-1}; effectors were at 2×10^7 ml^{-1} in RPMI-S, giving a maximum effector to target (E:T) ratio of 100:1. Effector cells (100 μl) were dispensed into wells of microtitre plates (Sterilin U-well) and 100 μl of targets that had been subjected to different treatments were then added. The E:T ratio was varied while maintaining a constant target cell number of 2×10^4. The microtitre plates were sealed (Dynatech) and incubated at 37°C in 5% CO_2 for 4 h, then centrifuged at 150 g for 10 min (MSE Coolspin) before harvest of 125 μl of supernatant for counting in a γ-counter (LKB Wallac Rackgamma II).

Percentage cytotoxicity was equated with specific ^{51}Cr-release calculated as follows:

$$\frac{\text{counts released from antibody-coated targets by effectors} - \text{spontaneous release from antibody-coated targets}}{\text{counts released by NP40} - \text{spontaneous release from antibody-coated targets}} \times 100\%$$

Assay for cytostasis

Mφ were washed by suspension in MEM, centrifuged at 100 g for 5 min, resuspended in RPMI-S at 2.5×10^6 ml^{-1}, and dispensed into the wells of microtitre plates (Sterilin U-well). L_2C target cells were washed similarly and resuspended in RPMI-S at 2.5×10^7 ml^{-1}. Sensitization procedures were as already described for the cellular cytotoxicity assay. Following dilution to 2.5×10^6 ml^{-1} in RPMI-S, target cells were added to the mφ. The E:T ratio was varied while maintaining a constant total volume of 200 μl and total cell number of 5×10^5 in each well.

The microtitre plates were left for 1 h at the same temperature as that which was used for target cell sensitization, to allow antibody-mediated contact between effector and target cells. 10 μl [^{3}H]-thymidine (TRK 120) or [^{3}H]-deoxycytidine (TRK 211) (Amersham International) both at 200 $\mu Ci\,ml^{-1}$ in MEM were then added to each well, and the microtitre plates were sealed (Dynatech) before incubation at 37°C with 5% CO_2 for 5 h.

The cells were then harvested (Titertek) with distilled water onto filter discs which were dried (37°C for 30 min) before being pressed out into scintillation counter insert vials (Sterilin). Liquid Scintillation Cocktain T (Hopkins & Williams) was added to each vial in 200 μl aliquots and the uptake of [^{3}H]-nucleoside by the cells during the incubation was measured in a β-counter (LKB Wallac Rackbeta).

To determine accurately the number of counts taken up by the mφ when mixed with L_2C cells at various E:T ratios, correction factors based on the uptake of [^{3}H]-thymidine by 5×10^5 mφ alone were employed. Uptake of [^{3}H]-thymidine by mφ when rosetting antibody-coated irradiated L_2C cells (2000 rads X-rays; M.E.L. LINAC) was also measured.

Cytostasis was determined as the percentage inhibition of [^{3}H]-thymidine- or [^{3}H]-deoxycytidine-uptake by L_2C cells in antibody-mediated contact with mφ when compared to the uptake by these cells in the presence of the same number of mφ and the same concentration of normal IgG.

Percentage inhibition was calculated as follows:

$$\frac{X-Y}{X} \times 100\%$$

where X is: Counts taken up by L_2C in the presence of mφ and normal IgG

− Counts taken up by mφ alone.

and Y is: Counts taken up by antibody-coated L_2C in the presence of mφ

$$- \left[\begin{array}{ll} \text{Counts taken up} & \text{Counts taken} \\ \text{by m}\varphi\text{ in} & \text{up by} \\ \text{antibody-mediated} & -\text{irradiated} \\ \text{contact with} & L_2C \\ \text{irradiated } L_2C & \end{array} \right]$$

This formula allows for the fact that L_2C cells take up some 20% more [^{3}H]-nucleoside when in the presence of mφ and normal IgG than when cultured alone.

Results

Cell contact

Syngeneic, allogeneic and xenogeneic macrophages formed antibody-dependent rosettes with L_2C cells. The mφ was always found at the centre of the rosette, even when high E:T ratios were used. Encircling L_2C cells numbered up to 6. Activation by BCG *in vivo* and induction by paraffin oil made no observable difference to the ability of the mφ to form rosettes *in vitro*. Very few interactions and no rosettes were observed between mφ and target cells in the presence of normal IgG.

All the antibodies tested were capable of mediating rosette formation, and contact was not noticeably inhibited if the target cells were first allowed to undergo antigenic modulation. For example, indirect immunofluorescence with FITC-sheep anti-rabbit IgG showed that the majority of bound rabbit anti-Cλ was cleared from the surface of an L_2C cell at 37°C within 15 min. Nevertheless, this antibody was still able to mediate rosette formation even after sensitization of the L_2C cells at 37°C for 30 min. This indicates that cellular interaction is dependent only on a very small quantity of antibody being present, and perhaps highly localized, on the surface of the target cell.

Phagocytosis

No mφ population was capable of phagocytosing L_2C cells sensitized with allogeneic (guinea pig strain 13) or xenogeneic (rabbit) antibodies. The same result was obtained whether the sensitization with antibody proceeded at 0°C, room temperature, or 37°C. However 95% of BCG-activated guinea pig mφ phagocytosed CRBC sensitized with rabbit anti-CRBC IgG, indicating that the mφ are capable of phagocytosing a nucleated target cell.

Cytotoxicity (ADCC)

No mφ population tested—syngeneic, allogeneic or xenogeneic—was able to kill antibody-coated L_2C cells as judged by release of ^{51}Cr in assays of up to 8 h duration. The antibodies used had a range of origins and specificities: xenogeneic (rabbit) anti-whole cell, anti-Cλ, anti-Id; and allogeneic (guine pig strain 13) anti-Ia. Activation with BCG *in vivo* also failed to render the mφ cytotoxic towards antibody-coated tumour cells *in vitro*. Figure 1 shows a typical attempt to kill antibody-coated L_2C cells by incubation with mφ. Cytotoxicity is at a very low level when compared to the percentage of specific ^{51}Cr-release observed when human leukocytes were used as effectors. The latter is likely to represent predominantly killing by K cells among the peripheral lymphoid population (MacLennan *et al.*, 1969). and confirms that the anti-whole cell IgG used to try to obtain a cytotoxic effect with mφ was capable of mediating cellular killing of L_2C cells. The antibody could also initiate complement-dependent lysis of L_2C cells (Figure 2). Antibody-coated L_2C cells excluded trypan blue after incubation with all mφ populations for 8 h. Antibody-coated L_2C cells treated with 0.1 mM cycloheximide were also resistant to macrophage-dependent cytotoxicity.

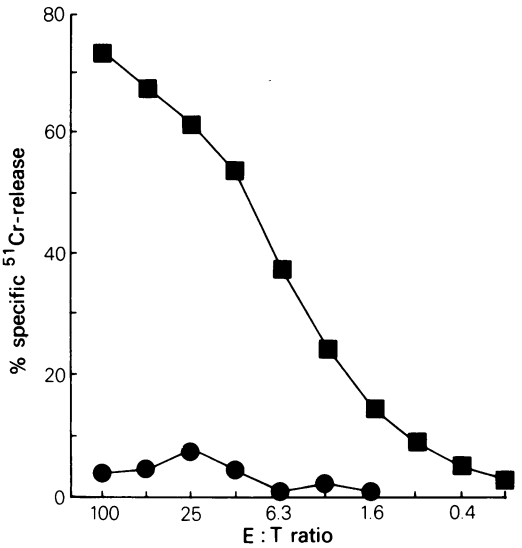

Figure 1 Cellular killing of L_2C cells by human peripheral blood leukocytes (■) and BCG-activated strain 2 guinea pig mφ (●), mediated by rabbit anti-L_2C IgG at 400 μg ml^{-1} in a 4 h incubation at 37°C. Points represent means of duplicate determinations which had a range of up to 5%.

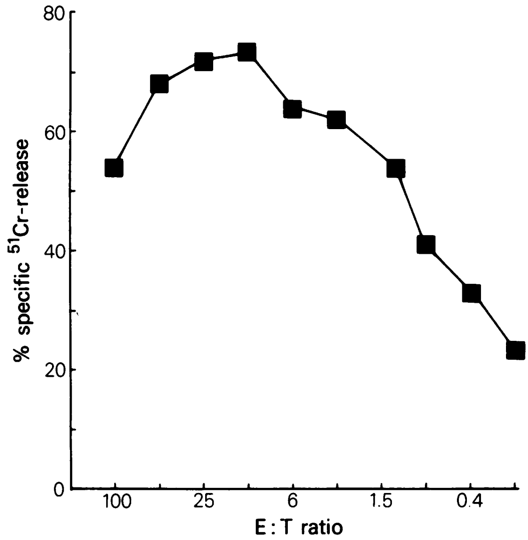

Figure 3 Extracellular killing of CRBC by oil-induced strain 2 guinea pig mφ, mediated by rabbit anti-CRBC IgG at 400 μg ml^{-1} in a 4 h incubation at 37°C. Points represent means of triplicate determinations which had a range of <5%.

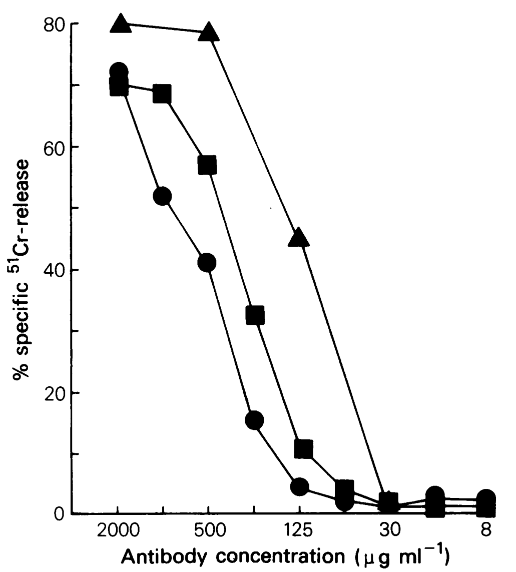

Figure 2 Complement-dependent killing of 10^5 ^{51}Cr-labelled L_2C cells, mediated by rabbit anti-L_2C IgG at the concentrations shown. Complement sources were: rabbit (▲), strain 2 guinea pig (■), and strain 13 guinea pig (●). Fresh sera were all diluted 1:2 with MEM. The assay was carried out at 37°C for 30 min. Points represent means of triplicate determinations which had a range of <5%.

In contrast to their behaviour towards L_2C cells, guinea pig mφ were capable of performing ADCC with nucleated erythrocytes (Figure 3). Oil-induced guinea pig mφ formed rosettes with antibody-coated CRBC cells in a similar manner to those which were formed with L_2C cells. A small proportion (5%) of these mφ phagocytosed the CRBC target cells, but only extracellular killing was measured in the 4 h cytotoxicity assay: our experience and that of Sanderson & Thomas (1978) indicates that there is no measurable release of ^{51}Cr from phagocytosed target cells during this period. Release of ^{51}Cr was somewhat inhibited at high E:T ratios, perhaps due to those effector cells which phagocytosed the antibody-coated CRBC depleting the target cell population available for extracellular killing. Phagocytosis appears to be a relatively rapid event compared to extracellular killing, which in our system required 4 h to reach a plateau. Cytotoxicity towards CRBC was induced by small concentrations of sensitizing antibody: 70% specific ^{51}Cr-release was obtained at an E:T ratio of 10:1 with 100 μg ml^{-1} of antibody-containing IgG. The number of target cells used in the cytotoxicity assays depicted was 2×10^4, but similar results were obtained within the range 7×10^3 to 10^5. Control preparations in which antibody and/or effector cells were absent revealed no specific ^{51}Cr-release.

The suggestion that little or no overall ^{51}Cr-release in cytotoxicity assays involving tumour target and mφ effector cells reflects uptake by mφ of ^{51}Cr released by other cells was discounted in an experiment where the ^{51}Cr-rich supernatant from a CRBC cytotoxicity assay was incubated for 4 h with a fresh population of oil-induced guinea pig mφ. No uptake of ^{51}Cr-labelled debris occurred.

Cytostasis

All mφ populations were capable of inducing cytostasis in antibody-coated L_2C cells as measured by inhibition of uptake of [^{3}H]-thymidine or [^{3}H]-deoxycytidine; this is in contrast to the very low levels of cytotoxicity expressed as judged by release of ^{51}Cr. Figure 4 shows values for cytostasis and cytotoxicity typically obtained. Only very small quantities of sensitizing antibody were required. For example, cytostasis mediated by a purified antibody, rabbit anti-Cλ, was maximal even at 0.7 μg ml^{-1}, a concentration at which lysis by syngeneic complement could not be invoked (see Figure 7b).

Figure 5 shows the cytostatic activity of syngeneic mφ. Uptake of [^{3}H]-thymidine was completely inhibited at E:T ratios above 10:1. The figure also shows that the resident peritoneal population was capable of causing a cytostatic effect. Activation of mφ *in vivo* with BCG did not result in significantly increased cytostasis.

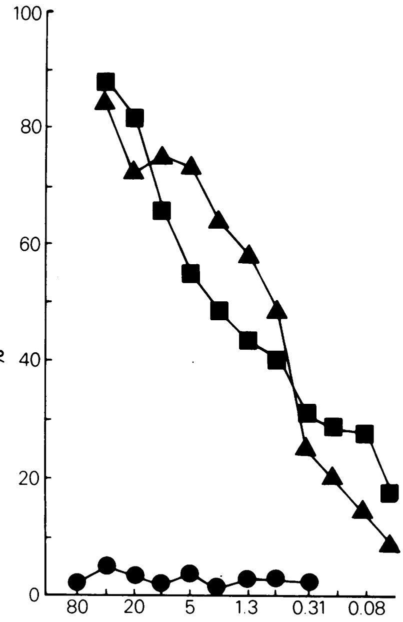

Figure 4 Cytostatic (% inhibition of uptake of ^{3}H-thymidine (■); or ^{3}H-deoxycytidine (▲)); and cytotoxic (% specific ^{51}Cr-release (●)) effects of oil-induced strain 13 guinea pig mφ on separate populations of L_2C cells sensitized with rabbit anti-L_2C IgG at 400 μg ml^{-1}. Both assays were performed at 37°C for 5 h. Points represent means of triplicate determinations which had ranges of up to 10% in the cytostasis assay and <5% in the cytotoxicity assay.

In Figure 6 the cytostatic activities of syngeneic, allogeneic and xenogeneic mφ are compared to any effect resulting from the interaction of target cells with inert "effectors". Syngeneic mφ were more cytostatic than allogeneic or xenogeneic towards L_2C cells. In a control experiment Sephadex G-25

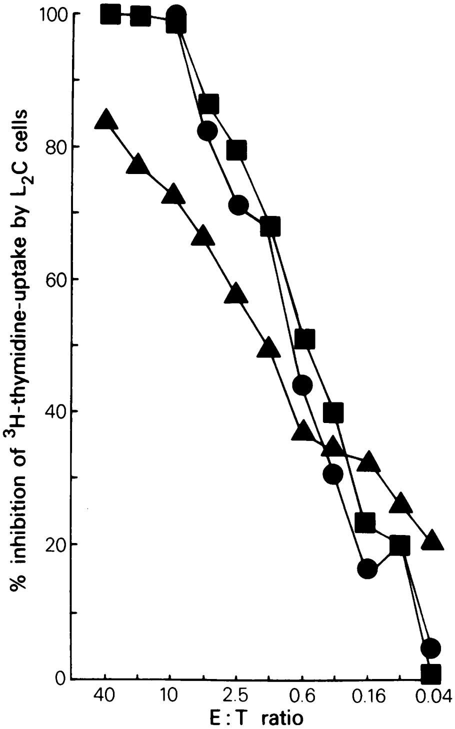

Figure 5 Cytostatic effect (% inhibition of ^{3}H-thymidine-uptake) mediated by strain 2 guinea pig mφ: resident population (▲), oil-induced (■), and BCG-activated (●) on L_2C cells sensitized with rabbit anti-L_2C IgG at 400 μg ml^{-1}. The assay was carried out at 37°C for 5 h. Points represent means of triplicate determinations which had a range of up to 10%.

Superfine beads (average diameter 25 μm), with rabbit anti-L_2C IgG coupled to their surfaces, were used to mimic mφ: these beads formed rosettes with unsensitized L_2C cells just as mφ had done with antibody-coated cells. G-25 beads with normal IgG coupled to their surface did not form rosettes with L_2C cells and caused no inhibition of [^{3}H]-thymidine-uptake. Rosettes formed by antibody-coated beads were associated with a small reduction in uptake, up to 20% of that caused by the mφ.

Figure 6 also shows that mixed agglutination with CRBC yielded a very small reduction in uptake. It is apparent that little of the inhibition of thymidine-uptake observed in macrophage-dependent cytostasis can be attributed to simple diffusion or metabolic effects associated with inert bodies interacting with target cell surfaces.

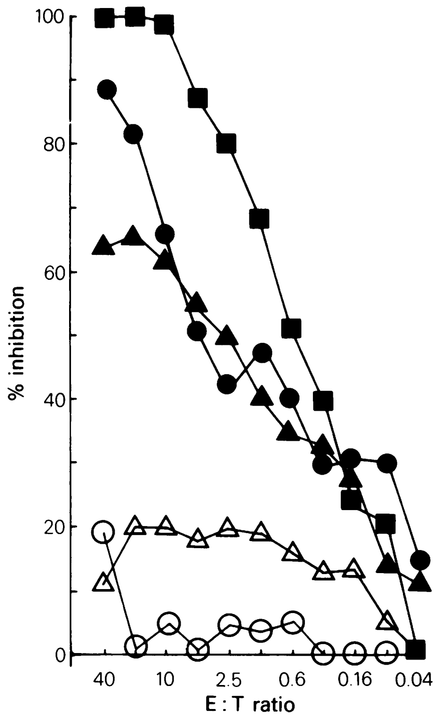

Figure 6 Cytostatic effect (% inhibition of ^{3}H-thymidine-uptake) mediated by oil-induced mφ: strain 2 guinea pig (■), strain 13 guinea pig (●), and mouse (▲) on L_2C cells sensitized with rabbit anti-L_2C IgG at 400 μg ml^{-1} compared to controls. The assay was carried out at 37°C for 5 h. The controls were: Sephadex G-25 Superfine beads with rabbit anti-L_2C IgG coupled to their surfaces (△); and CRBC sensitized with rabbit anti-CRBC IgG at 400 μg ml^{-1}. and incubated at 37°C for 5 h in the presence of purified sheep anti-rabbit IgG at 50 μg ml^{-1} with L_2C cells sensitized with rabbit anti-L_2C IgG at 400 μg ml^{-1} (○). Sephadex beads with normal rabbit IgG coupled to their surfaces gave no effect. CRBC took up trace amounts of [^{3}H]-thymidine. Points represent means of triplicate determinations which had a range of up to 10%.

In a further control experiment no inhibition of [^{3}H]-thymidine-uptake was observed when fresh L_2C cells were exposed to supernatants from cultures of mφ, cultures of mφ and L_2C cells in the presence of normal IgG, cultures of mφ and antibody-coated L_2C cells or cultures of mφ and antibody-coated irradiated L_2C cells.

Cytostatisis exhibited by syngeneic mφ was not susceptible to antigenic modulation by the L_2C target cells (Figure 7*a*). Taken in conjunction with the morphological observations described above, it would appear that once a mφ was in antibody-mediated contact with an L_2C leukaemic cell, cytostasis followed. Figure 7*b* shows that the residual surface-bound antibody following antigenic modulation, caused by carrying out sensitization at 37°C, is insufficient to mediate lysis of the target cell by complement. At 40 μg ml^{-1}, the concentration of purified rabbit anti-Cλ used in cytostasis assays, modulation has rendered the L_2C cells completely resistant to lysis by syngeneic complement, even though they are still susceptible to macrophage-dependent cytostasis (Figure 7*a*).

A rabbit anti-Id was also able to mediate cytostasis (Figure 8). Again activation of syngeneic mφ by BCG *in vivo* did not enhance their cytostatic activity *in vitro*. A small decrease in cytostasis occurred at high E:T ratios when L_2C cells were

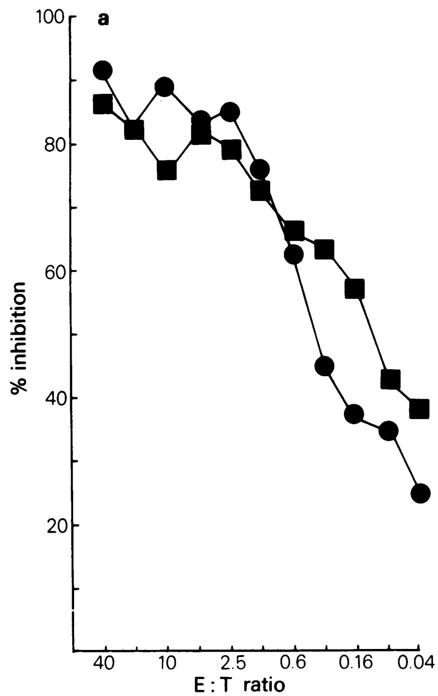

Figure 7a Cytostatic effect (% inhibition of ^{3}H-thymidine-uptake) mediated by oil-induced strain 2 guinea pig mφ on L_2C cells sensitized with purified rabbit anti-Cλ at 0°C (■) and at 37°C (●) for 30 min at 40 μg ml^{-1}. The cytostasis assay was carried out at 37°C for 5 h. Points represent means of triplicate determinations which had a range of < 10%.

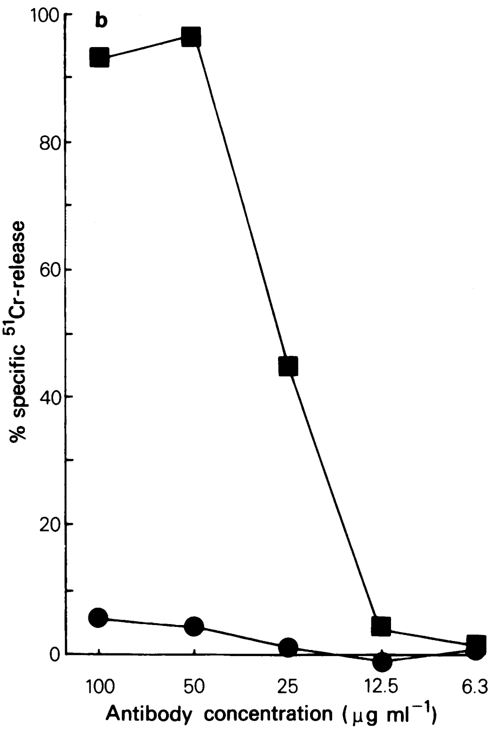

Figure 7b Lysis (specific ^{51}Cr-release) by strain 2 guinea pig complement (fresh serum diluted 1:2 with MEM) of L_2C cells sensitized with purified rabbit anti-Cλ, at 0°C (■) or 37°C (●), for 30 min at the concentrations shown. The assay was carried out at 37°C for 30 min. Points represent means of triplicate determinations which had a range of <5%.

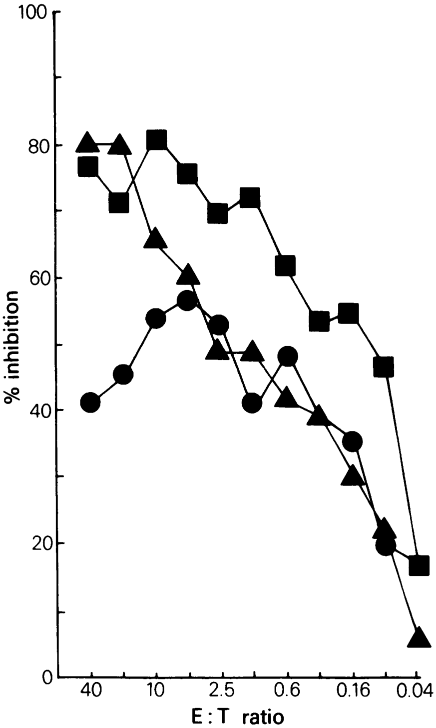

Figure 8 Cytostatic effect (% inhibition of ^{3}H-thymidine-uptake) mediated by oil-induced (■●) and BCG-activated (▲) strain 2 guinea pig mφ on L_2C cells sensitized at 0°C (■▲) and at 37°C (●) for 30 min with rabbit anti-Id at 200 μg ml^{-1}. The assay was carried out at 37°C for 5 h. Points represent means of triplicate determinations which had a range of up to 10%.

sensitized at 37°C. The reason for this prozone effect under these conditions is not clear.

A univalent antibody derivative, Fab/c prepared from purified rabbit anti-Cλ, was also capable of mediating cytostasis. Figure 9 shows the effect of syngeneic mφ on L_2C cells sensitized with the Fab/c derivative at 40 μg ml^{-1}. The degree of cytostasis, judged by the percentage inhibition of [^{3}H]-thymidine-uptake by the target cells, compares favourably with that obtained with the whole antibody (Figure 7*a*). As expected, no difference in percentage inhibition was obtained when the L_2C cells were sensitized at 37°C, as Fab/c is not susceptible to antigenic modulation (Glennie & Stevenson, 1982).

Discussion

The data presented show that binding of mφ to antibody-coated leukaemic cells was not sufficient in itself to invoke cytotoxicity. This was the case even when the macrophages had been activated by BCG *in vivo*. Similar findings have been reported by Cabilly & Gallily (1981) using syngeneic murine embryonic fibroblasts as target cells. These observations are in contrast to other reports (Alexander & Evans, 1971; Nathan *et al.*, 1979*a*, 1980; Berd & Mastrangelo, 1981; Koren *et al.*, 1981*a*) in which antibody-mediated contact with activated mφ led to lysis of lymphoid tumour cells from established cell lines. The reason for lack of cytotoxicity in L_2C cells is unclear. It may be that aneuploid cellular targets from lines cultured *in vitro* are much more susceptible to this form of attack than are the diploid L_2C cells, maintained wholly by passage *in vivo* (Nadel, 1977), or than are the cultures of embryonic fibroblasts employed by Cabilly & Gallily (1981). However, experience with a wide range of cell targets will be necessary to decide this point. Cellular repair mechanisms, such as might be involved in resistance to complement-mediated lysis (Schlager *et al.*, 1979), may be

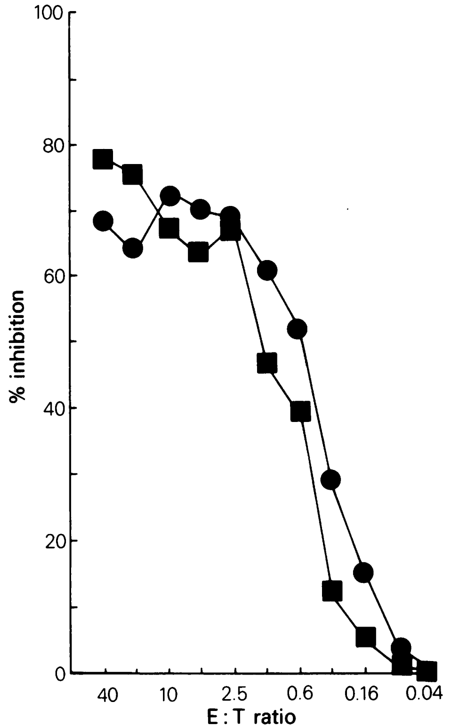

Figure 9 Cytostatic effect (% inhibition of ^{3}H-thymidine-uptake) mediated by oil-induced strain 2 guinea pig mφ on L_2C cells sensitized with rabbit anti-Cλ Fab/c at 0°C (■) and at 37°C (●) for 30 min at 40 μg ml^{-1}. The assay was carried out at 37°C for 5 h. Points represent means of triplicate determinations which had a range of < 10%.

relevant here. Lack of cytotoxicity towards cycloheximide-treated L_2C cells demonstrates that if repair mechanisms are responsible, they do not depend on *de novo* protein synthesis. It is extremely unlikely that all the antibodies used were of the wrong isotype to induce macrophage-dependent cytotoxicity, particularly as they could all mediate rosette formation with mφ. No isotypes are recognised in rabbit IgG (Nisonoff *et al.*, 1975) so it is highly improbable that our failure to observe phagocytosis is due to chance occurrence of a non-opsonising isotype in all our preparations.

It is not clear what requirements exist for expression of cytotoxicity by mφ, additional to sensitization of the target cell with antibody of a suitable class and activation of the effector cells. L_2C and similar lymphoblastic cells may not be susceptible to the ADCC activity of mφ under any circumstances, even though they can succumb to lysis mediated by K cells among human peripheral blood leukocytes. Alternatively, macrophage-mediated cytotoxicity may be possible given a further signal (Cabilly & Gallily, 1981), which in most other systems appears to follow directly from antibody-mediated cellular contact with activated macrophages (Yamazaki *et al.*, 1976; Adams & Marino, 1981). A three-step model for lysis of tumour cells by cytotoxic T lymphocytes involving cellular contact, a Ca^{2+}-dependent programming for lysis and then the lytic event (Gately & Martz, 1981), may be relevant to ADCC by mφ and other effector cells. The primary event of cellular contact would be mediated by antibody, while the second step, that of programming for lysis, requires investigation.

Regardless of the pertaining E:T ratio, antibody-mediated rosette formation resulted in a characteristic arrangement with the L_2C cells surrounding the mφ. The factors dictating this pattern remain obscure. It was unlikely to be due to polar accumulation of antigen-antibody complexes on the surface of the L_2C cells as a similar pattern resulted when anti-Ia, which is not susceptible to antibody-induced redistribution (Gordon & Stevenson, 1981), was used to sensitize the target cells.

Antibody-coated murine lymphoma cells from the line L5178Y have been reported to be phagocytosed by mφ (Evans, 1971). In common with other investigators (Nathan *et al.*, 1979*a*, 1980; Berd & Mastrangelo, 1981; Koren *et al.*, 1981*a*), we have not observed phagocytosis of the lymphoid tumour cells. Even when L_2C cells were sensitized with antibodies which were not susceptible to surface redistribution, phagocytosis did not occur. The latter observation rules out the possibility that escape was due to capping of the antigen-antibody complexes on the target cell surface, which leaves inadequate antibody cover for opsonization (Griffin *et al.*, 1976). Evasion of phagocytosis may be due to possible defence mechanisms of the L_2C cells or to the inability of the mφ to recognize a second signal. The relative sizes of the two cell types—15 μm diameter for L_2C cells and typically 23–28 μm diameter for guinea pig mφ—may also be important here. The functional capacity of the mφ for phagocytosis was clearly demonstrated towards sensitized CRBC. When CRBC were sensitized with rabbit IgG, BCG-activated guinea pig mφ phagocytosed them more avidly than did oil-induced guinea pig mφ. This finding is in contrast to the reports of other investigators. Koren *et al.*, 1981*b*, observed greater antibody-dependent phagocytosis by thioglycollate-induced than by BCG-activated mouse mφ of trinitrophenyl-modified CRBC sensitized with rabbit antiserum. Nathan & Terry (1977) have also reported decreased capacity for phagocytosis of a wide range of particulate targets by BCG-activated mouse mφ. The reason for such differences is not clear.

Antibody-dependent binding of L_2C cells induced cytostasis, reflected by an abrupt and profound inhibition of thymidine- or deoxycytidine-uptake. Activation of the mφ by BCG did not enhance their potential for cytostasis. However the precise nature of "activation", and the possibility that components in the oil used for induction have some activating potential, make this whole aspect difficult to evaluate. We could not relate data obtained in cytostasis assays to actual cell numbers *in vitro* as L_2C cells do not survive in culture for a sufficient period. Calculation of the percentage inhibition of [^{3}H]-thymidine-uptake by target cells took into account the uptake by both free and rosetted mφ, allowing us to investigate cytostasis at relatively high E:T ratios where the contribution of mφ to the counts measured became significant. In contrast to other reports (Keller, 1973; Krahenbuhl *et al.*, 1976; Bandlow & Gröner, 1979; Campbell *et al.*, 1980; Matsunaga *et al.*, 1980; Hogg & Balkwill, 1981), the cytostasis was entirely antibody-dependent, so that the measured inhibition of [^{3}H]-thymidine-uptake is extremely unlikely to have been due to competition from cold thymidine secreted by mφ (Evans & Booth, 1976; Stadecker & Unanue, 1979).

Furthermore, supernatants from cultures of mφ with antibody-coated irradiated L_2C cells caused no inhibition of [^{3}H]-thymidine-uptake by fresh L_2C cells. This is particularly important as antibody-coated irradiated L_2C cells would be expected to stimulate any putative secretion of thymidine by the mφ, but would be unable to take up and incorporate much of the free nucleoside, which should thus appear in the supernatant of such cultures. No such thymidine-secretion was demonstrated in our system.

Control cultures lacking antibody also showed clearly that cytostasis cannot be ascribed to any crowding phenomenon such as contact inhibition (Gyöngyossy *et al.*, 1979). In fact uptake of [^{3}H]-thymidine by L_2C cells in the presence of mφ was some 20% greater than when the L_2C cells were cultured alone under the same conditions. Similar findings have caused concern (Evans, 1979; Nelson, 1981), but we interpret this phenomenon as a probable feeder-layer effect, with the counts taken up by L_2C cells cultured without mφ reflecting sub-optimal conditions. Finally the inhibition of thymidine-uptake was seen to require an active contribution from the mφ, because little inhibition followed the antibody-mediated binding of inert beads or CRBC to the target cell surfaces.

It is not clear what the significance of our finding of antibody-mediated cytostasis would be for survival and proliferation of the tumour *in vivo*. It could of course be of considerable importance, particularly as we have shown that cytostasis can be induced by extremely small concentrations of specific antibody and is not readily susceptible to antigenic modulation. It is interesting that both xenogeneic anti-Id and the univalent antibody fragment Fab/c (Glennie & Stevenson, 1982) were capable of mediating cytostasis in L_2C cells by syngeneic macrophages *in vitro*. Thus macrophage-mediated cytostasis could well represent another major factor to be evaluated together with complement-mediated killing, extracellular killing and phagocytosis when considering antibody-dependent defence mechanisms against tumour cells.

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