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A possible role for polymorphonuclear leucocytes in the defence against recrudescent herpes simplex virus infection in man

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Summary. We have used a ^{51}Cr release assay to demonstrate that human polymorphonuclear leucocytes (PMNL) can damage herpes simplex infected target cells sensitized with antiviral antibody. Effective sensitizing antibodies were found in both serum and saliva of all those persons tested who were subject to recurrent cold sores. PMNL were much less effective as killer cells than peripheral blood mononuclear cells, but as they are the predominant inflammatory cell within the HSV1 lesion they may be, quantitatively, more important. The cytotoxic effects of both PMNL and mononuclear cells were significantly reduced by prostaglandin E1 as well as by several drugs that were tested. It is suggested that antibody dependent PMNL-mediated cytotoxicity may play a role in the human host defences against recrudescent herpes simplex infection.

INTRODUCTION

Herpes simplex virus can spread from one infected cell to another via intercellular bridges (Stoker, 1958). During this mode of spread the virus is not exposed to the extracellular fluid and transmission is therefore not inhibited by any neutralizing antibody or complement that may be present (Lodmell, Niwa, Hagashi & Notkins, 1973). Virus release into the extracellular fluid occurs at a fairly late stage of the cellular infection; but, in contrast, it has been shown *in vitro* that specific viral antigens can be detected on the membranes of infected cells within 3 h of infection (Shore, Cromeans & Romano, 1976; Pfizenmaier, Starzinski-Powitz, Rollinghoff, Falke & Wagner, 1977). These antigenic determinants render the cell susceptible to lysis by mechanisms involving antibody dependent cell-mediated cytotoxicity (ADCC). The results of experiments *in vitro* suggest that early damage to infected cells can occur in this way at a critical stage before infectious virus has been transmitted across the intercellular bridges (Shore *et al.*, 1976). This phenomenon of antibody dependent cell-mediated cytolysis is a very efficient system *in vitro* and could therefore be an important host defence mechanism in preventing or minimizing recrudescent clinical infections with this, or other, viruses (Shore, Nahmias, Starr, Wood & McFarlin, 1974; Rager-Zisman & Allison, 1976). We have previously used a line of human amnion cells (HAE 70) acutely infected with HSV1 as the target cells in this system and have shown that, in man, the principal mononuclear cell involved is the 'null' cell (Russell & Kaiser, 1976). This has since been confirmed by Shore, Melewicz & Gordon (1977).

Although a role for polymorphonuclear leucocytes (PMNL) is not normally mentioned in reviewing the host defences against viral infection (Allison, 1972; Merigan, 1974), our preliminary studies showed

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that they too functioned as effectors in ADCC using the above herpes simplex infected target cells (Russell & Essery, 1977). As the inflammatory cells infiltrating an HSV lesion are predominantly PMNL (McSorley, Shapiro, Brownstein & Hsu, 1974; Hill, Field & Blyth, 1975), these cells are potentially available for a defensive role *in vivo*.

In the present study we have compared the relative efficiency of the lymphocyte and PMNL effectors *in vitro* and assessed factors which may, *in vivo*, modulate these responses.

SUBJECTS AND METHODS

Subjects

Blood was obtained from healthy volunteers, some of whom were selected because of a history of frequent herpes labialis. Eight patients with chronic myeloid leukaemia also agreed to provide blood for this study. Two standard, high titre, HSV antisera obtained from patients with recurrent disease were used for most experiments, but, where indicated, the subject's own serum was also used.

Effector cell preparation

Ten ml of heparinized whole blood were mixed with 2·5 ml of warmed plasma gel (Lab. Roger Beilon, Neuilly, France) and allowed to sediment for 30 min at 37°. The supernatant was removed and layered onto Ficoll Hypaque (Harris & Ukaejiofo, 1969). The mononuclear cells were removed from the interface and the pellet containing PMNL and red cells was then used, either without further purification or after lysis of the RBC. After initial comparative studies with 0·9% ammonium chloride, a hypotonic saline solution containing ethylene diamine tetracetic acid (Severson, Greazel & Thompson, 1974) was used to obtain RBC-free PMNL preparations. The pellet was mixed with 10 ml of the hypotonic saline. 1 ml of neutralizing solution was then added and the cells were washed with TC199. This technique usually achieved a purity of over 97% PMNL and all preparations were over 95% pure. Virtually all the cells were shown to be viable when tested by trypan blue.

Some PMNL preparations of 95–97% purity were subjected to a single repeat sedimentation through Ficoll Hypaque to selectively increase the proportion of PMNL present. The activity of these cells was then compared with that of the initial preparation.

Assay

The target cells were prepared as previously described (Russell, Percy & Kovithavongs, 1975). They were grown in Roux bottles, and 48 h before use, one bottle was infected with 4×10^5 plaque forming units stock virus. The monolayers were harvested by trypsinization, after the medium and dead cells had been decanted. The viability of these remaining cells was generally greater than 95%. Both infected and uninfected control cells were labelled with ^{51}Cr according to the method of Perlmann & Perlmann (1970). 1 ml of effector cell suspension ($2{\cdot}5 \times 10^6$/ml) was added to 25 μl of ^{51}Cr labelled target cells together with 100 μl of heat inactivated antibody-containing serum, or a control, inactive, foetal calf serum. All tests were carried out in plastic tubes (12 $\times$ 75 mm). The effector to target cell ratio was initially varied to determine the optimum value for both PMNL and mononuclear cell mediated killing. Most subsequent studies were carried out with ratios of 100:1. The mixed suspension was incubated for 3 h at 37°, and the reaction stopped by adding 1 ml of ice cold Hanks balanced salt solution. The suspension was then centrifuged for 8 min at 1200 rev/min, the supernatant removed and both the supernatant and pellet were counted. The total amount of ^{51}Cr releasable was determined for each run by three times freezing and thawing suspensions containing only labelled target cells. The percentage of ^{51}Cr released in test samples was corrected by subtraction of the background release and relating the result as a percentage of the total releasable chromium.

The antibody titre of the serum used in each test was the highest dilution that induced significant ^{51}Cr release (Russell & Kaiser, 1976), and was assessed separately for both mononuclear and PMNL effector cells. In some experiments fresh guinea-pig complement was substituted for the effector cells and the titre of complement-dependent antibody determined. Fresh guinea-pig complement was also added to the standard suspension containing effector cells, antibody and targets in order to assess whether there were C3 and Fc receptors on these effector cells that could act synergistically to increase the amount of target cell damage.

Saliva was obtained from subjects with anti-HSV antibody in the serum. It was centrifuged at room temperature to remove mucus, but was subject to no further purification. In some experiments it was substituted for serum in the above assays.

Drugs

The effector cells were incubated with a number of agents prior to use in the above assays. These were aminophylline, isoproterenol, hydrocortisone, levamisole, prostaglandins, A2 and E2 and propanolol. PMNL are added to a solution of the drug in medium so that there was no increase in the final incubation volume. The mixture was incubated for 15 min at 37° and then, without further washing, added to the antiserum and target cell suspension. It was maintained at 37° for a further 3 h as previously described.

RESULTS

The three techniques of PMNL separation generally provided cells with equivalent activity (Table 1). Removal of red blood cells was less reliable if the hypotonic step was omitted and in some cases erythrocytes appeared to interfere with the PMNL–target cell interaction. We found no evidence that 0·9% ammonium chloride interfered with the function of the PMNL in this system in contrast to the reported effect on mononuclear cells (Yust, Smith, Wonderlick & Mann, 1976). Hypotonic buffered saline was used in all the experiments cited below.

Mononuclear cells and PMNL from fifty-three subjects were tested using an effector to target cell ratio of 100:1. In all cases, the mononuclear cells were considerably more effective, cell for cell, in killing target cells than PMNL. Fig. 1 illustrates this relationship using cells from twenty-seven different subjects and a single sensitizing antiserum. The correlation coefficient between the mononuclear cell activity and the specific ^{51}Cr release induced by PMNL was 0·66 ($P < 0·01$). The effect of varying the attacker to target cell ratio was assessed with PMNL and mononuclear cells from several subjects. The optimal specific release with both cell types was obtained with a ratio of 200:1. The specific ^{51}Cr release was much higher than that by PMNL. Parallel dose-response curves showed the PMNL in a ratio of 200:1 had an effect equivalent to mononuclear cells

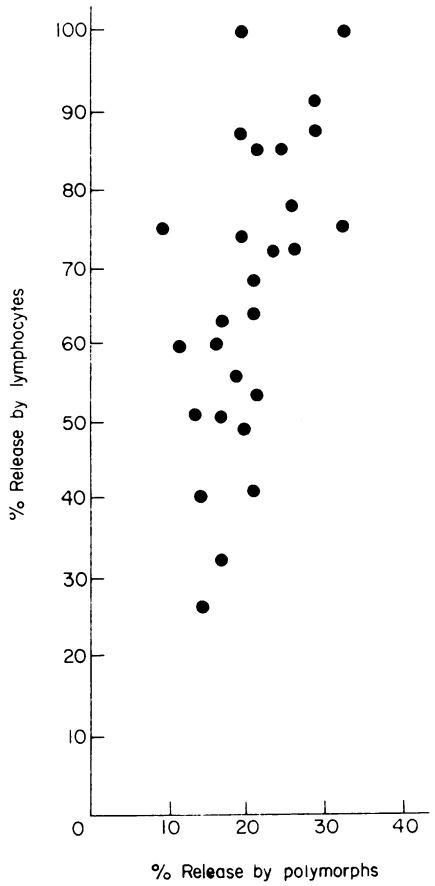

Figure 1. ^{51}Cr specific release from HSV-1 infected target cells by equal numbers of mononuclear cells and polymorphonuclear leucocytes from the same subject in the presence of the same serum specimen.

Table 1. Comparison of % ^{51}Cr release from antibody sensitized, HSV infected target cells by polymorphonuclear leucocytes

Cells used	Technique of PMNL separation		
	Plasma gel alone	Hypotonic saline	0·9% Ammonium chloride
PMNL 1	19·5±2·1	28·6±2·9	27·7±3·2
PMNL 2	25·8±1·6	36·6±2·8	28·8±2·4
PMNL 3	29·7±3·1	29·8±3·1	34·0±2·8
PMNL 4	18·3±2·0	29·5±1·9	31·2±3·4

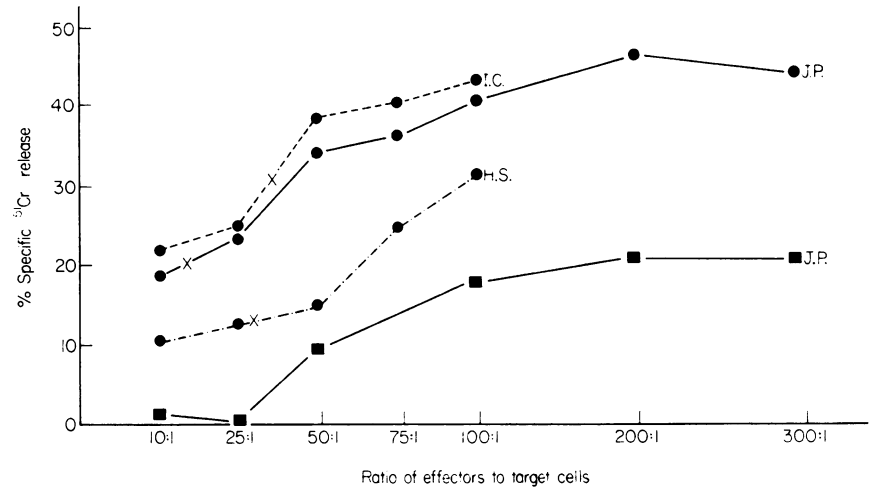

Figure 2. To compare the relative activities of equal numbers of polymorphonuclear leucocytes (■) and mononuclear effector cells (●) against antibody sensitized HSV1 infected target cells. The full dose–response curve for polymorphonuclear leucocytes is shown for only JP. The '×' on the dose–response curve for mononuclear cells marks the ^{51}Cr release by polymorphonuclear leucocytes from the same patient used in a ratio of 200:1.

used in a ratio of between 20 and 30:1 (Fig. 2). The increased efficacy of mononuclear cells in comparison to PMNL was also reflected in the difference in antibody titres seen when either mononuclear or PMNL effector cells were used with the same antiserum (Fig. 3).

Some PMNL preparations were recycled once through Ficoll Hypaque when the resultant purity was increased to 99% PMNL. The reduction in lymphocyte contamination did not reduce the effector cell activity of these preparations.

Target cells were incubated with antibody and fresh complement, both with and without PMNL. The sensitivity of the complement mediated release

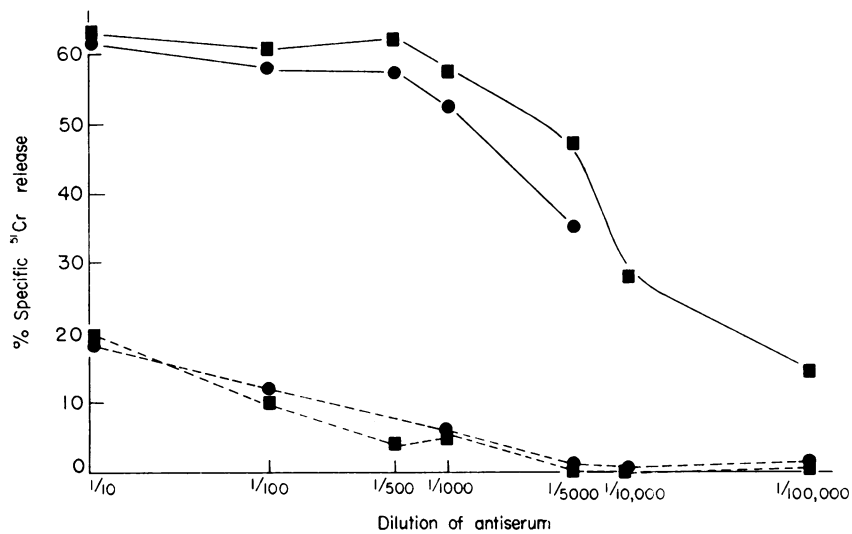

Figure 3. To illustrate the relative sensitivity of antibody dependent cell-mediated immune lysis when mononuclear cells or polymorphonuclear leucocytes were used. (■, subject P.R.; ●, subject T.O.) The same antiserum was present throughout. The effector to target ratio for mononuclear cells and polymorphonuclear leucocytes (- - -) was 100:1 to 200:1 respectively.

Table 2. A comparison of ^{51}Cr release from HSV1 infected cells in the presence of antibody, and: (i) *either* fresh complement *or* PMNL; (ii) *both* fresh complement *and* PMNL

Cells	Serum	Fresh complement	% ^{51}Cr release* from target cells: HSV1 infected	Non-infected
PMNL	—	—	6·7±2·8	6·3
PMNL	PD 1/100	—	19·3†±2·7	8·3
—	PD 1/100	+	23·2‡±3·0	7·0
PMNL	PD 1/100	+	19·8±1·7	6·8
PMNL	PD 1/250	—	15·7±2·6	6·7
—	PD 1/250	+	19·8±2·4	8·2
PMNL	PD 1/250	+	16·2±2·3	8·3
PMNL	PD 1/500	—	4·2±1·9	4·6
—	PD 1/500	+	6·9±1·9	7·5
+	PD 1/500	+	7·2±1·7	7·6
PMNL	GE 1/100	—	26·6±1·8	6·4
—	GE 1/100	+	17·9±2·2	7·5
PMNL	GE 1/100	+	22·4±2·4	7·3
—	GE 1/100	—	3·6±2·0	4·9
—	—	+	7·5±1·8	7·9

PMNL—Polymorphonuclear leucocytes.
—, Omitted.
* Background not subtracted.
† Antibody dependent PMNL-mediated release.
‡ Antibody dependent complement-mediated release.

Table 3. To illustrate the effect of salivary antibodies in mediating ^{51}Cr release from infected targets in the presence of either mononuclear cells or polymorphonuclear leucocytes

Subject	% Specific ^{51}Cr release from HSV1 infected target cells by: Serum† and polymorphonuclear leucocytes	Serum† and mononuclear cells	Saliva‡ alone	Saliva‡ and polymorphonuclear leucocytes	Saliva‡ and mononuclear cells
E.B.	13·2	39·7	−3·8	6·1	14·2
I.S.	28·3	58·5	−2·3	13·1	15·2
A.S.	32·4	52·8	−0·7	8·7	50·2
J.C.*	−2·4	3·8	−0·6	0·8	0·3
J.S.*	−0·5	6·4	−3·3	−2·6	−0·9

* No history of HSV infections.
† Used in a 1:100 dilution.
‡ Used in a 1:2 dilution.

Table 4. The effect of drugs on the % ^{51}Cr release from antibody sensitized, HSV infected cells using PMNL polymorphonuclear leucocytes effector cells

Cell source	Drug used (concentration)*	% Specific ^{51}Cr release (without drug)	% Specific ^{51}Cr release (with drug)
Sm	Hydrocortisone (3×10^{-4} M)	13·6±2·8†	7·8±2·0
	Prostaglandin A_2 (10^{-4} M)	13·6±2·8	1·4±1·3
Ch	Prostaglandin E_2 (10^{-5} M)	30·5±3·1	17·4±2·8
Ci	Levamisole (10^{-3} M)	19·2±3·2	10·3±2·1
Ba	Isoproterenol (4×10^{-7} M)	37·5±3·0	24·5±2·6
	Aminophylline (10^{-5} M)	37·5±3·0	27·5±2·4
Te	Hydrocortisone (3×10^{-4} M)	15·5±2·5	6·5±2·1

* Minimal effective concentration shown.
†± Standard error of the mean.

was similar to the antibody dependent PMNL mediated system (Table 2). No increase in PMNL mediated ^{51}Cr release was seen if the targets were initially incubated with fresh complement in addition to antibody (Table 2). This remained true even when sublytic concentrations of serum were used.

Salivary antibodies were able to sensitize targets to both mononuclear and PMNL damage. The amount of ^{51}Cr release was much less than that seen with equivalent dilutions of serum (Table 3).

PMNL from seven of the eight patients tested with chronic myeloid leukaemia were effective in damaging the antibody sensitized target cells. The cells from one subject demonstrated a blastic crisis and were ineffective.

Effect of drugs

Levamisole when used in concentrations of 10^{-4} or less had no effect on the PMNL-mediated ^{51}Cr release from sensitized target cells and when used in higher doses, this release was reduced. The other drugs used significantly reduced the ^{51}Cr released. Some results illustrating the minimum effective concentrations of drug are shown in Table 4; they will be reported in detail elsewhere (Russell, Davis & Miller, 1977).

DISCUSSION

We have previously suggested that polymorphonuclear leucocytes (PMNL) can act as effector cells in the antibody dependent cell-mediated cytotoxicity (ADCC) system (Russell & Essery, 1977). The purity of the PMNL preparations used could be increased from 95 to 99% without any loss of effector activity, thus demonstrating that the target cell damage was not due to the small numbers of mononuclear cells that may be irregularly present. PMNL are known to have receptors for the Fc fragment of IgG (Zighelboim, Gale & Kedar, 1976), but while this is necessary, is not in itself sufficient to ensure that they can act as effector cells in ADCC.

Like macrophages and other mononuclear cells, they have been shown to be effective with some targets and not others (Nelson, Bundy, Pitchon, Blaese & Strober, 1976; Wardley, Babiuk & Rouse, 1976; Zighelboim *et al.*, 1976). Wardley *et al.* have shown that bovine PMNL are active against antibody sensitized bovine kidney cells infected with a bovine herpes virus—infectious bovine rhinotracheitis (Wardley *et al.*, 1976). In this animal system, PMNL are the most active cell and in contrast to our own system, non-adherent lymphocytes were not effective at all. The reasons for the marked variability in the type of effector cells active under different circumstances have not been adequately explained.

PMNL have receptors for C3b (Eden, Miller & Nussenzweig, 1973) and the Fc and C3b receptors have been shown *in vitro* to cooperate in opsonization and phagocytosis of the sensitized RBC (Mantovani, 1975). The addition of fresh complement to the antibody containing suspension of PMNL and infected target cells did not demonstrate any evidence of synergy between Fc and C3 receptors. Both PMNL and fresh complement would induce significant ^{51}Cr release from antibody sensitized target cells, but the systems were of approximately equal sensitivity. When the antibody titre was progressively reduced, no increased sensitivity was obtained by the addition of *both* fresh complement

and PMNL (Table 2). Saliva contained antibodies that were effective in sensitizing the target cells to damage by both PMNL and mononuclear cells. We have not yet determined whether this relates to the small amounts of IgG present or whether IgA itself can act as a sensitizing antibody (see Table 3).

Many subjects, otherwise entirely healthy, are susceptible to recurrent 'cold sores', i.e. circumoral infections with herpes simplex virus. Cell mediated immunity to viral antigens appears to be a factor of major importance in preventing widespread dissemination of these lesions—but the factors underlying resistance to the initiation of the local infection are still controversial (Wilton, Ivanyi & Lehner, 1972; Rasmussen, Jordan, Stevens & Merigan, 1974; Russell *et al.*, 1975). It is paradoxical that active virus may often be present around the lips even in susceptible subjects without any significant lesion developing. Hill & Blyth (1976) have suggested that these susceptible subjects may have continual, rather than intermittent, virus activation and continual centrifugal spread along the trigeminal nerve. Local host factors would then become of prime importance in inhibiting virus multiplication in the skin to form a discernible lesion. We would suggest that although it has been previously ignored in antiviral defences, the PMNL would be readily available and in cooperation with plasma or salivary antibodies could therefore function in inhibiting virus spread. We have shown that PGE1 and some agents that increase intracellular cAMP levels are able to reduce the described antibody-dependent cell-mediated damage to targets and we would agree with the suggestion (Hill & Blyth, 1976) that such factors may indeed play a role here.

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