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Quantitative and qualitative effects of cyclophosphamide administration on circulating polymorphonuclear leucocytes

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Summary. The effect of cyclophosphamide (CY) on the absolute numbers and function of polymorphonuclear leucocytes (PMN) surviving in the circulation following either a single dose (100 mg/kg, i.p.) or daily administration (20 mg/kg, i.p., for 5 days) was studied in the guinea-pig. The quantitative effect of CY on peripheral blood leucocytes was assessed by measuring the absolute numbers of neutrophils, lymphocytes, and monocytes daily for 5 days following the initial injection of CY. The qualitative effects of CY on PMN function were determined by measuring the ability of these cells to function as killer cells. The two functional assays employed were the PMN-mediated PHA-induced cellular cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) assays against chicken erythrocyte targets.

Both regimens of CY administration produced an equivalent degree of leukopenia 5 days after the initial injection with disproportionately severe neutropenia (<300 PMN/mm^3). However, neither regimen of CY administration produced a significant decrease in cytotoxic effector function as measured through a wide range of effector to target cell ratios, PHA concentrations, and antiserum dilutions. These findings have clinical relevance in that they demonstrate the dichotomy between the quantitative and qualitative effects of (CY) on PMNs in that CY administration can dramatically decrease the absolute numbers of circulating polymorphonuclear leucocytes while leaving intact certain effector cell functional capabilities of those PMN surviving in the circulation during drug administration.

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INTRODUCTION

Cyclophosphamide (CY) when administered in high doses to humans as well as animals consistently results in a severe circulating neutropenia (Dale, Fauci and Wolff, 1973; Gershwin, Goetzl and Steinberg, 1974; Balow, Hurley and Fauci, 1975). However, the effect of this drug on the functional capabilities of the polymorphonuclear leucocytes (PMN) surviving in the circulation during drug administration has not been well delineated. A variety of *in vitro* studies have been employed to assess the functional capabilities (both bactericidal and inflammatory) of PMN (Klebanoff, 1975; Stossel, 1975; Miller, 1975). Recently, several assays have been described which are indicative of the ability of a cell to function as a cytotoxic effector cell. Two of these assays are the phytohaemagglutinin (PHA) induced cellular cytotoxicity and the antibody-dependent cellular cytotoxicity (ADCC) against chicken red blood cell

(CRBC) targets (Perlmann and Holm, 1969). In both human and animal studies, various populations of peripheral blood leucocytes including PMN have been shown to possess these non-specific cytotoxic effector cell capacities (Perlmann and Holm, 1969).

The present study was undertaken to further delineate the relative effect of various regimens of CY administration on the absolute numbers of circulating PMN, and on certain PMN-mediated effector functions as measured by the PHA-induced cellular cytotoxicity and ADCC assays.

MATERIALS AND METHODS

Animals

Inbred strain 13 guinea-pigs (350–500 g) obtained from the Division of Research Services, National Institutes of Health, Bethesda, Maryland were used throughout the study.

Drug administration

Guinea-pigs were treated with either daily intraperitoneal (i.p.) injections of saline or CY (20 mg/kg) (Cytoxan®, Mead Johnson Laboratories, Evansville, Indiana) for 5 consecutive days or were given a single i.p. injection of saline or CY (100 mg/kg). The 20 mg/kg dose is equivalent to approximately 150 mg/m^2 body surface area (Freireich, Gehan, Rall, Schmidt and Skipper, 1966). On various days following treatment, peripheral blood (PB) was obtained for study.

Preparation of PMN

Peripheral blood was obtained from guinea-pigs bled by intracardiac puncture into syringes containing acid–citrate anticoagulant. Aliquots were taken for total leucocyte counts which were measured in a Coulter Counter (Coulter Electronics, Incorporated, Hialeah, Florida) and for differential counts done by counting 200 cells on Wright stained blood smears.

Mononuclear cells were separated from whole blood by the Hypaque–Ficoll method and PMN were separated from red blood cells by dextran sedimentation and hypotonic lysis (Böyum, 1968). The PMN were washed once with phosphate-buffered saline (PBS) and twice with Eagle's minimal essential medium (MEM-S) (Grand Island Biological Company, Grand Island, New York). These cells, containing >95 per cent PMN and <5 per cent mononuclear cells, were used for the cytotoxicity assays. The final cell suspension was >95 per cent viable as measured by the trypan blue dye exclusion test.

Cytotoxicity assays

PHA-induced cellular cytotoxicity assays were performed by a slight modification (Kirchner and Blaese, 1973) of a previously described method (Perlmann, Perlmann and Holm, 1969) and the ADCC assay was performed by a method previously described for mononuclear cells (Perlmann and Perlmann, 1970). Both assays employed measurement of the release of isotope from radioactive chromium ^{51}Cr-labelled chicken red blood cell (CRBC) targets. For the ADCC assay, rabbit anti-CRBC antiserum was used. It was obtained by hyperimmunizing rabbits (three subcutaneous injections over a 3-week period) with 0·3 ml of packed CRBC mixed in an emulsion with 2 ml of Freund's complete adjuvant. Two weeks after the last injection, animals were bled and serum was prepared, heat-inactivated at 56° for 60 min, and stored in 2-ml aliquots at −20°. Ten-fold dilutions of rabbit anti-CRBC antiserum from 10^{-2} to 10^{-4} were used in the ADCC assay as indicated. For the PHA-induced cellular cytotoxicity assay, PHA was added to cultures to obtain final concentrations of 1, 10, and 100 μg/ml. The effector (E) to target (T) cell ratios employed were 1:20–100:1 as indicated. Cultures done at 1:1 E:T ratio had 1×10^6 effector cells and 1×10^6 labelled CRBC targets for a total of 2×10^6 cells in culture. As the E:T ratio was varied greater or less than 1:1, the total number of effector cells in culture was kept at 1×10^6 in order to preserve consistent culture conditions. Cultures were performed in $1 \times 7{\cdot}5$ cm plastic tubes. Into each culture was added 1 ml of effector cells in MEM-S with 10 per cent foetal calf serum (FCS), varying concentrations of PHA or anti-CRBC antiserum in 0·1 ml of MEM-S, or 0·1 ml of MEM-S alone as control, and varying concentrations of ^{51}Cr-labelled CRBC depending on the E:T ratio. Cultures were incubated at 37° in 5 per cent CO_2 in air at 100 per cent humidity. The PHA-induced cellular cytotoxicity and ADCC assays were terminated after 40 h and 24 h respectively. Tubes containing a total volume of 1·2 ml were spun at 1000 *g* for 5 min at 4° and 0·6 ml of the supernate was pipetted into a separate tube and the remaining 0·6 ml containing the pellet were counted separately in an automatic gamma counter (Series 1185, Nuclear Chicago Corporation, Des Plaines, Illinois). The percentage of ^{51}Cr released from the CRBC targets

into the supernate was determined as follows (Kirchner and Blaese, 1973):

$$\text{percentage } {}^{51}\text{Cr release} = \frac{\text{supernatant c.p.m.} \times 2}{\text{supernatant c.p.m.} + \text{pellet c.p.m.}} \times 100.$$

The degree of cytotoxicity was expressed as the percentage ^{51}Cr release in the presence of PHA or anti-CRBC antiserum minus the percentage ^{51}Cr release in the absence of PHA or anti-CRBC antiserum.

RESULTS

Effects of administration of a single dose or repeated doses of CY on numbers of PB leucocytes

The effect of a single dose of CY on PB leucocytes was studied in animals which received a single i.p. injection of either saline or CY (100 mg/kg). Animals (n = 6) were studied daily for five consecutive days following injection. The mean (±s.e.m.) total leucocyte count per mm^3 measured at each point in time (0, 1, 2, 3, 4 and 5 days following injection) was 8200 (±320), 4340 (±380), 3240 (±640), 2000 (±210) and 1795 (±152), respectively. The effect of a single injection of CY on the absolute numbers of neutrophils, lymphocytes, and monocytes measured serially for 5 days following the injection is shown in Fig. 1.

In addition to the above regimen, the effect of daily administration of CY on PB leucocytes was studied in animals which received either saline or CY (20 mg/kg, i.p., daily for 5 consecutive days). The mean (±s.e.m.) total leucocyte count per mm^3 measured 24 h after the last dose of CY or saline for each group of animals (n = 6) receiving either saline or CY (20 mg/kg) was 8200 (±295) and 1895 (±89), respectively. The effect of this regimen of CY on the absolute numbers of neutrophils, lymphocytes, and monocytes is shown in Fig. 2.

It is noteworthy that almost identical degrees of leukopenia were achieved 5 days after initiation of

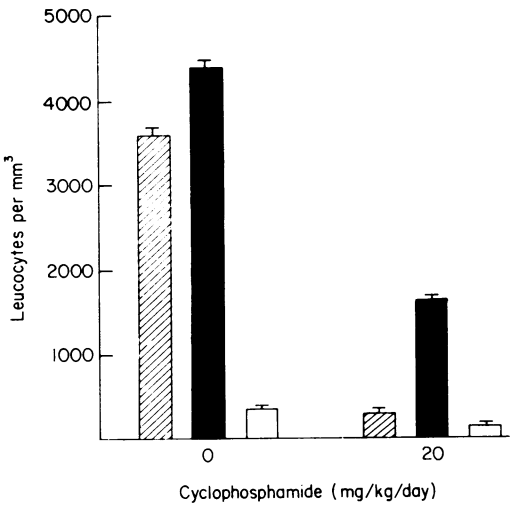

Figure 2. The effects of daily administration of cyclophosphamide (20 mg/kg, intraperitoneally, daily for 5 consecutive days) on the absolute numbers of peripheral blood neutrophils (hatched columns), lymphocytes (solid columns) and monocytes (open columns) measured 5 days following the initial injection of cyclophosphamide.

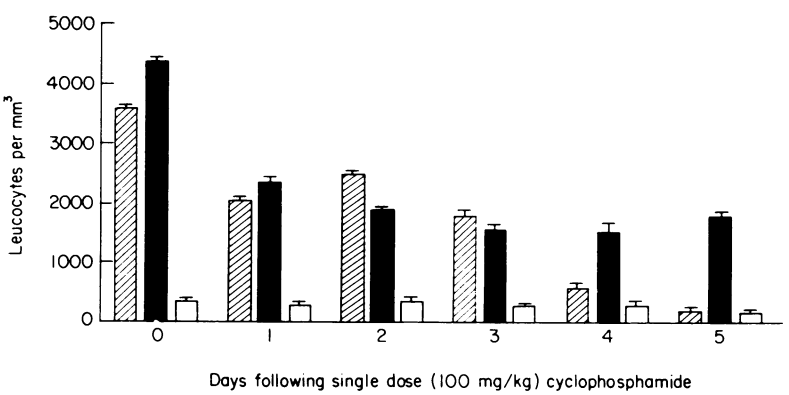

Figure 1. The effects of a single intraperitoneal injection of cyclophosphamide (100 mg/kg) on the absolute numbers of peripheral blood neutrophils (hatched columns), lymphocytes (solid columns) and monocytes (open columns), measured serially for 5 days following injection.

treatment by the administration of CY as either 20 mg/kg daily for 5 days or as the same total dosage (100 mg/kg) in a single injection. Although no morbidity or mortality was observed in any of the CY-treated groups during this relatively short-term study period, it was clear that lymphocytopenia was achieved at the expense of a disproportionately severe neutropenia (Figs 1 and 2). These studies were extended to the 5th day following injection because previous observations in this laboratory revealed that the point of maximal neutropenia occurred at this time with both regimens of CY administration.

Effect of CY on PMN-mediated PHA-induced cellular cytotoxicity and ADCC

In experiments designed to determine the effects of CY on PMN function *in vitro*, PMN from each CY treatment group were compared with PMNs from saline-treated (control) groups for their ability to mediate PHA-induced cellular cytotoxicity and ADCC. The effect of a single intraperitoneal injection of either saline or CY (100 mg/kg) on PMN-mediated ADCC was assayed at E:T ratios ranging from 1:20 to 100:1 and antiserum dilutions from 10^{-2} to 10^{-4}, and on PMN-mediated PHA-induced cellular cytotoxicity at E:T ratios from 1:20 to 10:1 and PHA concentrations of 1 10 and 100 μg/ml. The results of both assays 24 h after injection are shown in Fig. 3. PMN-mediated cytotoxicity from both saline and CY-treated animals were further assayed daily for 5 days following injection at each E:T ratio, antiserum dilution and PHA concentration noted in Fig. 3. In each assay at each E:T ratio there was no significant suppression of cytotoxicity at any time point (0, 1, 2, 3, 4 and 5 days) when compared to control values. Cell viabilities in each group were similar at 0, 24 and 48 h.

The effect of repeated administration of either saline (control) or CY (20 mg/kg, i.p., daily for 5 days) was also studied in each cytotoxicity assay as noted above. As in the previous experiment, there was no significant suppression of cytotoxicity in the CY-treated group at any E:T ratio, antiserum dilu-

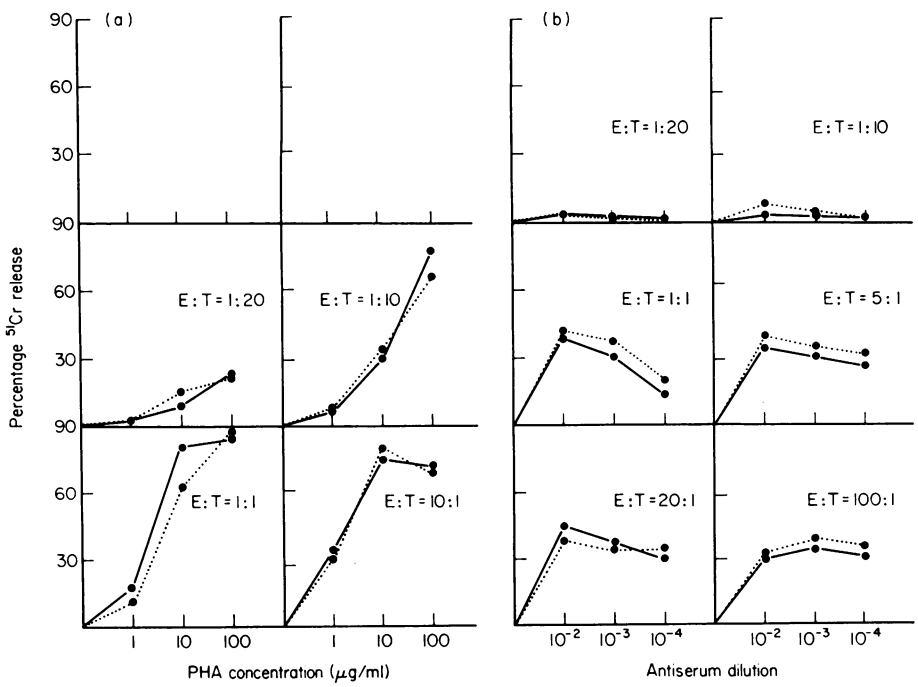

Figure 3. The effects of a single intraperitoneal injection of cyclophosphamide (100 mg/kg) on (a) polymorphonuclear leucocyte-mediated PHA-induced cellular cytotoxicity and (b) antibody-dependent cellular cytotoxicity at effector to target ratios from 1:20 to 100:1. Various concentrations of PHA and dilutions of antiserum are shown.

tion or PHA-concentration in either assay, when compared to the control group.

Hence, although both regimens of CY administration cause a severe neutropenia, neither regimen results in a significant suppression of the cytotoxic effector capabilities of the PMN surviving in the circulation.

DISCUSSION

The mechanisms whereby CY exerts its immunosuppressive effects are varied and complex (Gershwin *et al.*, 1974). It is clear that the administration of this drug results in a quantitative decrease in the absolute numbers of circulating leucocytes of all classes. The cytotoxic and antiproliferative effects of CY are most evident with populations of rapidly dividing cells. PMN with a relatively short half life of approximately 6–8 h (Athens, 1963) are highly susceptible to the leukopenic effects of this agent. In the clinical usage of CY, particularly at moderate doses used in inflammatory or non-neoplastic diseases, the resulting neutropenia with concomitant suppression of host defense and inflammatory responses is often the limiting factor in the use of this agent (Fauci and Wolff, 1973; Fosdick, Parsons and Hill, 1969). It is clinically relevant, although unclear at present whether the PMN remaining in the circulation at the time of drug-induced leukopenia are capable of functioning normally. It is clear, particularly from a host defence standpoint, that severe neutropenia (<1000 circulating PMN/mm^3) is associated with an increased susceptibility to infection (Rodriguez, Burgess and Bodey, 1973; Atkinson, Kay and McElwain, 1974; Levine, Graw and Young, 1972). It is uncertain, however, whether this suppression of host defences is related solely to the drug-induced decrease in absolute numbers of circulating PMNs or to a combination of a quantitative decrease in cell numbers and a qualitative decrease in functional capabilities of PMN remaining in the circulation.

There are relatively few studies in the literature directed at the functional capabilities of PMN in the face of neutropenia resulting from therapeutic regimens. Most of these studies have shown relatively little effect on a variety of PMN functions resulting from therapeutically induced neutropenia. In a study of a group of patients with neoplastic disease treated with various cytotoxic and immunosuppressive regimens, PMN function as measured by post-phagocytic oxygen consumption and nitroblue tetrazolium dye reduction was found to be normal (Deinard, Fortung, Theologidies, Anderson, Boen and Kennedy, 1974). In addition, chemotaxis and phagocytosis by PMN following *in vitro* irradiation, were normal (Holley, VanEpps, Harvey, Anderson and Williams, 1974).

The present study clearly demonstrates that during regimens of CY administration resulting in severe neutropenia, the capacity of the PMN surviving in the circulation to mediate two separate effector cell functions remains intact. The ADCC is mediated through a receptor for immunoglobin (Fc receptor) on the surface of the PMN (Gale and Zighelboim, 1975). This killer cell function is an energy requiring process (Perlmann and Holm, 1969) and is subject to bidirectional control by cyclic nucleotides (Gale and Zighelboim, 1974). The PHA-induced cellular cytotoxicity is mediated by mechanisms which at present remain unclear; however, deoxyribonucleic acid synthesis and mitosis are not involved (Perlmann and Holm, 1969). In any event, neither of these functions are suppressed as a result of drug administration. This lack of suppression of effector cell function is seen even at suboptimal effector to target cell ratios as well as throughout the entire range of both PHA and anti-target cell antiserum.

This resistance by PMN to CY-induced functional suppression is in contrast to the relative sensitivity of certain lymphocyte-mediated functions to the administration of high dose CY which results in a severe circulating lymphocytopenia. Previous studies have shown that administration of CY to guinea-pigs in doses sufficient to produce severe lymphocytopenia resulted in a significant reduction in mitogen and antigen-induced *in vitro* blastogenic responses and lymphokine (migration inhibitory factor) production of lymphocytes remaining in the circulation during the period of drug-induced lymphopenia (Balow *et al.*, 1975). In addition, CY administration in mice has been shown to selectively deplete certain lymphocyte populations (Turk and Poulter, 1972). The relative sensitivity of certain mononuclear cell functional capabilities to the administration of high dose CY was further demonstrated by the fact that both PHA-induced cellular cytotoxicity and ADCC of guinea-pig mononuclear cells was suppressed by CY administration in a dose-dependent fashion during a treatment regimen similar to that of the present study (Hunninghake and Fauci, 1975).

The evidence from the present study as well as from

previous studies indicates that the circulating PMN pool is quantitatively sensitive to a variety of cytotoxic and immunosuppressive regimens, but qualitatively quite resistant to suppression of the functional capabilities of surviving cells even with chemotherapeutic regimens which consistently result in severe neutropenia. These studies have clinical relevance in that they strongly suggest that the suppression of PMN-dependent phenomena by CY administration is directly and predictably related primarily to a quantitative decrease in the absolute numbers of circulating cells and not to functional impairment of the PMN surviving in the circulation during drug administration.

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