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Immunological reactivity of the lung

IV. EFFECT OF CYCLOPHOSPHAMIDE ON ALVEOLAR MACROPHAGE CYTOTOXIC EFFECTOR FUNCTION

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SUMMARY

The effect of cyclophosphamide on the absolute numbers and function of alveolar macrophages following either *in vitro* or *in vivo* drug exposure was studied in the guinea-pig.

Two separate regimens of *in vivo* cyclophosphamide administration (100 mg/kg/one dose or 20 mg/kg for 5 days) both of which produce identical decreases in each population of peripheral blood leucocytes 5 days after the initial injection did not produce any change in alveolar macrophage numbers when compared to control values. Neither a brief exposure to CY *in vitro* nor a brief exposure *in vivo* (100 mg/kg/one dose) caused any change in cytotoxic effector function of alveolar macrophages using the PHA-induced and antibody-dependent cellular cytotoxicity assays against sheep erythrocyte targets. In contrast, the more prolonged *in vivo* exposure to CY (20 mg/kg for 5 days), produced a significant decrease in the killer cell function of these cells. Thus, this study demonstrates that different regimens of cyclophosphamide administration, although producing similar degrees of peripheral blood leucopenia can produce markedly different effects on the functional capabilities of alveolar macrophages without quantitatively decreasing the absolute numbers of these cells.

INTRODUCTION

The effects of cyclophosphamide (CY) administration on pulmonary mononuclear cells in man and animals have not been well defined. In clinical situations, however, doses of CY large enough to produce significant neutropenia are associated with increased pulmonary infections (Rodriguez, Burgess & Bodey, 1973; Atkinson, Kay & McElwain, 1974). The alveolar macrophage (AM) has been shown to be the primary effector of host defence against infection in the lung. It is unclear, at this point, whether CY administration also causes an alteration of AM numbers and function.

We have recently described the effects of various regimens of CY administration on numbers and functions of various peripheral blood leucocytes (PBL) and mononuclear cell subpopulations in the guinea-pig (Hunninghake & Fauci, 1976a). In addition, a method of obtaining and characterizing relatively large numbers of pulmonary mononuclear cells (alveolar macrophages and lung lymphocytes) (Hunninghake & Fauci, 1976b) as well as the cytotoxic effector function of these cells (Hunninghake & Fauci, 1976c) has recently been described.

The present study was undertaken to investigate the mechanisms of immunosuppression by CY on AM function. Two well defined assays of non-specific mononuclear effector cell function are the PHA-induced (PICC) and antibody-dependent (ADCC) cellular cytotoxicity assays against red blood cell targets (Perlmann & Holm, 1969). *In vivo* CY-induced immunosuppression was assessed by the effect of administration of a single large dose or repeated smaller doses of CY on AM numbers and by the

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effect on functional capabilities as measured by PICC and ADCC against sheep red blood cell (SRBC) targets. In addition, the effect of *in vitro* CY on the cytotoxic capabilities of AM was studied.

MATERIALS AND METHODS

Animals. Inbred strain thirteen 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 i.p. injections of saline or 20 mg/kg of CY (Cytoxan, Mead Johnson Laboratories, Evansville, Indiana) for 5 consecutive days, or were given a single i.p. injection of saline or 100 mg/kg of CY. The 20 mg/kg per day dose of CY is equivalent to approximately 150 mg/m^2 body surface area per day (Freireich *et al.*, 1966). AM were harvested for study 5 days following the single i.p. injection of saline or 100 mg/kg of CY or 24 hr following the last daily i.p. injection of saline or 20 mg/kg of CY. The effect of these regimens of drug administration on guinea-pig peripheral blood leucocytes has been previously described (Hunninghake & Fauci, 1976a).

Preparation of serum containing 'Active' CY. Guinea-pig serum containing active cyclophosphamide (metabolized *in vivo* to its active form) was prepared as previously described (Balow, Parillo & Fauci, 1976). Previous studies have shown that this serum with 'active' CY can impair the ability of peripheral blood mononuclear cells to proliferate in response to mitogens Balow *et al.*, 1976).

Preparation of AM suspensions. Cell suspensions were obtained from teased lung tissues and purified mononuclear cells were obtained from these cell suspensions by methodologies described in detail in a previous paper (Hunninghake & Fauci, 1976b). The viabilities of all cell suspensions was >95% as determined by the trypan blue dye exclusion test. The macrophage suspension contained >85% macrophages as determined by morphology and neutral red dye uptake.

Cytotoxicity assays. ADCC and PICC was assayed by a previously described microcytotoxicity assay measuring the release of isotope from radioactive chromium ^{51}Cr-labelled SRBC targets (1×10^5/assay) (Hunninghake & Fauci, 1976a). Previous studies in our laboratory have confirmed that this target as well as other erythroid targets are killed predominantly by adherent cells, particularly at lower effector:target ratios. The degree of cytotoxicity was expressed as the per cent ^{51}Cr release in the presence of PHA or anti-SRBC antiserum minus the per cent ^{51}Cr release in the absence of PHA or anti-SRBC antiserum.

RESULTS

Effects of administration of a single dose or repeated daily doses of CY on numbers of AM

The effect of administration of a single dose or repeated daily doses of CY or saline on numbers of AM are shown in Fig. 1. There was no significant difference in the numbers of AM recovered from the lungs of saline, CY (100 mg/kg × 1 dose), or CY (20 mg/kg daily × 5 days) treated groups of animals ($n=6$ or more in each group).

Effect of in vitro *CY on AM-mediated PICC and ADCC*

The effects of *in vitro* CY on AM-mediated PICC and ADCC is shown in Fig. 2. In these experiments ($n=6$), AM from untreated animals were incubated at 37°C in 5% CO_2 in air at 100% humidity for

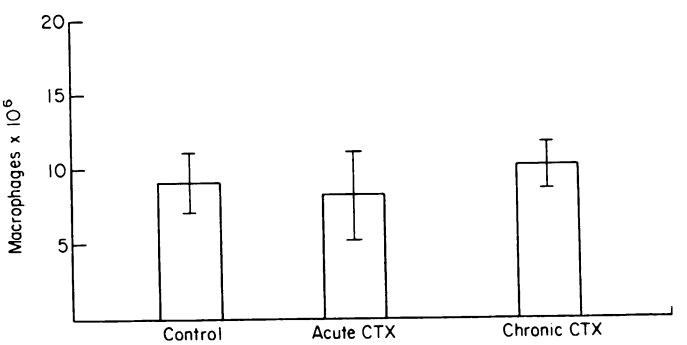

FIG. 1. The effects of a single i.p. injection of cyclophosphamide (acute CTX) (100 mg/kg) or five daily i.p. injections of cyclophosphamide (chronic CTX) (20 mg/kg/day) on the absolute numbers of alveolar macrophages measured 5 days following the initial injection. Controls received saline.

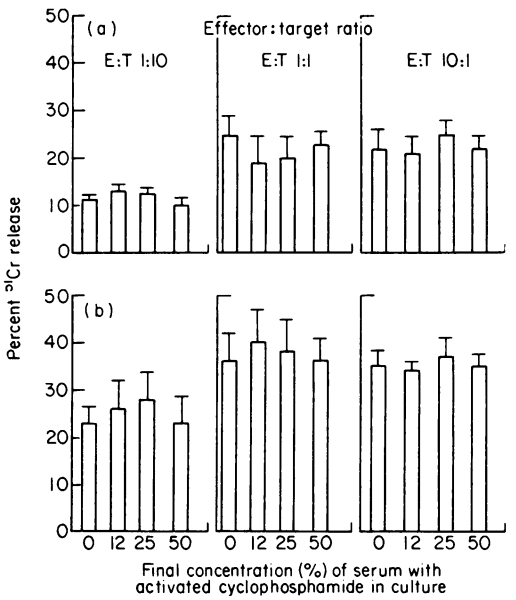

FIG. 2. The effects of *in vitro* cyclophosphamide (0, 12, 25, and 50% serum with activated cyclophosphamide in culture) on alveolar macrophage mediated—PHA-induced (a) and antibody-dependent (b) cellular cytotoxicity at effector to target ratios from 1:10–10:1. The concentration of PHA was 10 μg/ml and the dilution of antiserum was 10^{-3}.

2 hr in RPMI-1640 with 50% guinea-pig serum. The percentages of serum containing 'active' CY in these cultures were 0, 12, 25 and 50%. These cells were then extensively washed and used in the cytotoxicity assays as previously described. In these experiments 'active' CY had no effect on the subsequent ability of these cells to kill target cells, when compared to untreated cells. Previous experiments, using this serum containing active CY in a similar fashion, demonstrated that it was capable of severely limiting the proliferation of peripheral blood mononuclear cells in response to mitogens (Balow *et al.*, 1976). The numbers and viabilities of cells from each treatment group were unaltered by this *in vitro* exposure to 'active' CY at the end of the cytotoxicity assays. AM were preincubated with CY for only 2 hr prior to the cytotoxicity assays since this is the approximate length of *in vivo* exposure to activated CY following a single dose of drug. Also previous experiments showed that continuous exposure of effector cells during the cytotoxicity assays to the serum containing active CY severely compromised their viability (Balow *et al.*, 1976).

Effect of a single dose of 100 *mg/kg of CY on AM-mediated PICC and ADCC*

The effect of a single large dose of saline or CY (100 mg/kg) on AM-mediated PICC and ADCC is shown in Fig. 3. With this regimen of CY administration there was no effect on AM effector function when compared to saline treated controls. Previous studies have shown that this regimen of CY administration causes identical decreases in each leucocyte class as well as mononuclear cell subpopulations in the peripheral blood, as does the administration of 20 mg/kg of CY for 5 days (Hunninghake & Fauci, 1976a).

Effect of repeated daily doses of 20 *mg/kg of CY on AM-mediated PICC and ADCC*

The effect of repeated daily doses of saline or 20 mg/kg of CY on AM-mediated PICC and ADCC measured 24 hr after the last injection is shown in Fig. 4. In contrast to the studies noted above, there was a highly significant ($P<0{\cdot}001$) suppression of cytotoxicity in each assay and at each E:T ratio. As seen in Fig. 4, the CY-induced suppressions of cytotoxicity could not be overcome at higher E:T

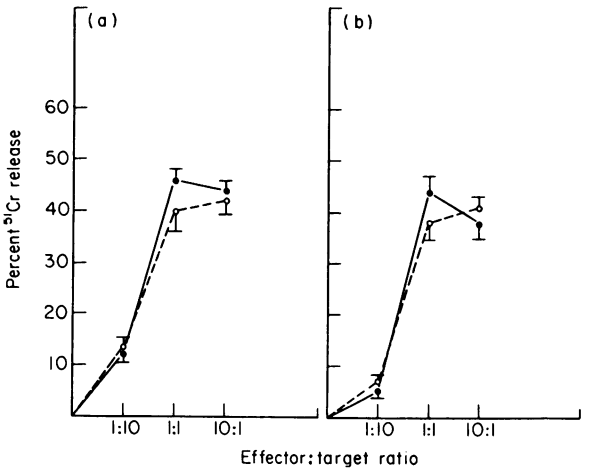

FIG. 3. The effects of a single i.p. injection of cyclophosphamide (100 mg/kg) on the alveolar macrophage—antibody-dependent (a) and PHA-induced (b) cellular cytotoxicity measured 5 days following injection. Effector to target cell ratios were 1:10–10:1. The concentration of PHA was 10 μg/ml and the dilution of antiserum was 10^{-3}. (●——●) Saline control; (○- - -○) cyclophosphamide (100 mg/kg/one dose).

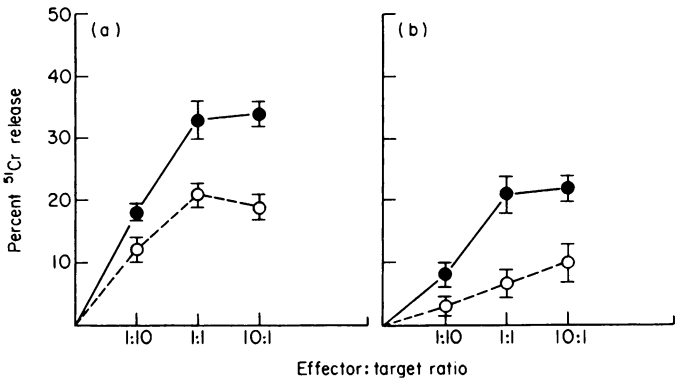

FIG. 4. The effects of five daily i.p. injections of cyclphosphamide (20 mg/kg/day) on the alveolar macrophage-antibody dependent (a) and PHA-induced (b) cellular cytotoxicity measured 24 hr following the last injection. Effector to target cell ratios were 1:10–10:1. The concentration of PHA was 10 μg/ml and the dilution of antiserum was 10^{-3}. (●——●) Saline control; (○- - -○) cyclophosphamide (20 mg/kg/day).

ratios. In addition, other experiments showed that alteration of antibody dilutions and PHA concentrations did not compensate for the defect in killing. The possibility that disproportionately greater rates of cell death *in vitro* subsequent to the initial preparation of the cultures could explain the differences in AM-mediated cytotoxicity between the saline and CY treated groups was unlikely since the numbers and viabilities of AM from each group cultured for 18 hr were the same.

DISCUSSION

CY has come to be recognized as a potent and clinically useful agent in the treatment of various pulmonary immunologically-mediated diseases (Fauci & Wolff, 1973) as well as other inflammatory and malignant diseases (Gershwin, Goetzl & Steinberg, 1974). CY, in large doses, is also associated with altered host defences often leading to pulmonary infections (Rodriguez *et al.*, 1973; Atkinson *et al.*, 1974). Little is known, however, about the effects, if any, of CY on the AM, a primary effector of both

host defences and immunological processes in the lung. A previous study in the rat suggested that CY may alter the ability of AM to kill ingested micro-organisms (Sharbaugh & Grogan, 1969). The effects of CY administration on other AM effector functions have not been well-defined. In clinical usage, CY can be given as bursts of single high dose administration or as chronic low dose administration. The present study of the effect of these two basic regimens of CY administration shows that neither regimen decreased the absolute numbers of AM, at least for the duration of this study. This is in contrast to the effects of the same regimens of CY administration on the peripheral blood where CY caused a decrease in numbers of each leucocyte class, including monocytes (Hunninghake & Fauci, 1976a). This suggests that any alteration of AM function seen *in vivo* is probably a result of actual alteration in cell functions rather than a decrease in cell numbers. These findings are consistent with previous studies in dogs which showed that neither CY treatment nor whole body irradiation caused a decrease in the numbers of AM recovered by bronchopulmonary lavage (Reynolds, Kazmierowski & Dale, 1976).

This study also demonstrates that these regimens of CY administration may have distinctly different effects on AM function. A brief exposure of AM to CY *in vitro* or *in vivo* simulating high dose 'burst' therapy, had no effect on AM cytotoxic effector function. Previous studies also showed that a single dose of 100 mg/kg of CY *in vivo* had no effect on peripheral blood mononuclear cell function (Hunninghake & Fauci, 1976a). In contrast, daily administration (20 mg/kg for 5 days) of CY (stimulating chronic low dose therapy) caused a marked impairment in AM killer cell function. This regimen of CY administration also resulted in altered peripheral blood mononuclear cell function (Hunninghake & Fauci, 1976a). The reason for the dichotomy in the effect on the functional capabilities of the AM from these two groups of animals with similar degrees of peripheral blood leukopenia is unclear at present. It is known, however, that the plasma half-life of active CY is short (5–6 hr) and that only the cells from the animals given daily doses of CY were exposed for more than a short period of time to the effects of CY. It is likely that this repeated exposure to low dose CY was the predominant factor responsible for the alteration of functional capabilities of these cells suggesting that suppression of distinct functional capabilities of certain cell types by CY administration can be distinguished from the more obvious effects of cell depletion and death. Thus, this study demonstrates that various regimens of CY administration may have similar effects on AM numbers but clearly different effects on certain well characterized functional capabilities of these cells. Recognition of these differential effects may provide the rationale for greater specificity in the design of therapeutic regimens for inflammatory or immunologically mediated diseases involving the lung.

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