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The Effect of some Cytotoxic Agents on Lymphocyte Subpopulations *in Vitro*

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Abstract—*The effect of melphalan, adriamycin and daunorubicin on E- and EAC-binding cells* in vitro *was studied. Melphalan and adriamycin reduced the frequency of EAC-cells but not that of E-cells. Daunorubicin used in the same concentrations as adriamycin exhibited a dose- and time-dependent killing effect on the entire cell population.*

INTRODUCTION

CHEMOTHERAPY for malignant disease may impair several types of immune responses [1]. A number of often contradictory reports dealing with different aspects of such drug induced immunosuppression have been published [2–9]. The discrepancies in the results may be explained by factors other than the treatment itself such as the disease and its clinical stage and intercurrent infections, which *per se*, can influence the lymphocyte population. In order to avoid such errors we have studied the *in vitro* effect of cytostatic drugs on peripheral lymphocyte preparations from healthy donors using different concentrations of the drugs and different exposure times. Three drugs were chosen, namely; melphalan, adriamycin and daunorubicin. Melphalan, a phenylalanine derivative of nitrogen mustard was studied because of its effectiveness in the treatment of plasma cell malignancy [10]. Adriamycin and daunorubicin are both anthracycline antibiotics. The former is active against malignant lymphomas and the latter is most useful in the treatment of acute leukemia [11].

In this investigation we have examined the effects of these drugs on the frequencies of T- and non-T-cells as assessed by E-, "active" E- and EAC-rosette formation tests.

MATERIALS AND METHODS

Lymphocyte donors

Eight healthy volunteers of both sexes, aged 22–60 yr, served as blood donors.

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Separation of lymphocytes

Heparinized venous blood was centrifuged on a layer of Ficoll–Isopaque (FIP) [12]. Phagocytic cells were removed by iron powder and a magnet [13]. The lymphocytes were then centrifuged again on FIP to remove remaining erythrocytes and phagocytes. The resulting preparations consisted of 99–100% of small lymphocytes.

Cytostatic drugs

Melphalan was purchased from Wellcome (The Wellcome Foundation Ltd, The Wellcome Building, 183 Euston Road, London NW1 2BP, England), adriamycin was a gift from Montedison (Montedison AB, Dalagatan 34, Stockholm, Sweden) and daunorubicin was purchased from Leo Rhodia (AB Leo Rhodia, Norrbroplatsen 2, Helsingborg, Sweden). The drugs were diluted in Eagle's Minimal Essential Medium supplemented with Earle's salts (MEM) and kept at 20°C before use.

Experimental design

Purified preparations of lymphocytes were suspended in MEM supplemented with penicillin, streptomycin and 10% of heat inactivated human AB^+ serum. Cell suspensions, containing 10^6 lymphocytes/ml, were incubated with or without a cytostatic drug at therapeutic concentrations for 2 or 24 hr in a humidified 5% CO_2–air atmosphere at 37°C. After incubation the cells were washed twice by centrifugation and the number of viable cells counted using the trypan blue exclusion test. The frequency of viable cells always

exceeded 90% and there was no appreciable difference between control and experimental cultures. Daunorubicin exhibited a pronounced cytotoxic effect on the cells. The lower concentration (2 μg/ml) of the drug killed approximately 25 and 50% of the cells after 2 and 24 hr of incubation, respectively. The higher concentration (4 μg/ml) killed approximately 60 and almost 100% of the cells during the same incubation periods. Incubation of lymphocytes with melphalan or adriamycin did not reduce the number of viable cells to any detectable extent.

Rosette tests

The methods for determinations of E- and EAC-rosette forming cells have been described before [14, 15]. Determination of the frequency of "active" E-rosette forming cells was performed according to the method described by Horowitz *et al.* [16]. Briefly, 0.1 ml of phosphate buffered saline containing 5×10^5 lymphocytes was incubated with 0.1 ml of fetal calf serum (Grand Island Biological Company) in a plastic tube at 37°C for 60 min. The fetal calf serum had previously been heat inactivated (56°C for 30 min) and absorbed against both sheep and human AB erythrocytes (at 37 and 4°C). After incubation the lymphocytes were resuspended and 0.1 ml of a 0.1% solution of sheep erythrocytes (SRBC) in normal saline was added. The cell suspensions were centrifuged at 200 ***g*** and kept at room temperature for 5 min. After resuspending the lymphocytes the frequency of SRBC binding cells was determined by scoring two hundred cells. Cells binding three or more SRBCs were considered positive.

RESULTS

Lymphocyte preparations were examined for the frequency of E-, "active" E- and EAC-rosette forming cells after *in vitro* exposure for 2 or 24 hr to two different concentrations of the drugs. The results of four separate tests for each drug using different lymphocyte donors are presented.

Melphalan and adriamycin exerted essentially similar effects: the frequency of EAC-binding cells was reduced whereas the frequencies of E- and "active" E-binding cells were not markedly changed. The effect on the EAC-binding cells seemed to be dependent on the exposure time using the highest concentrations of the drugs (Tables 1 and 2). Daunorubicin reduced the frequency of all cell types studied and the prolonged treatment with the higher dose killed almost all the cells (Table 3).

DISCUSSION

In this investigation we have examined three different cytostatic drugs, commonly used in the treatment of human lymphoid malignancies, for their effects on the peripheral lymphocyte population *in vitro* as defined by cell viability and the expression of certain surface structures on the surviving cells. Two membrane markers have been studied: (i) The SRBC receptor which is expressed by T-cells [14] and (ii) the C'3 receptor which is expressed predominantly by B-cells [15]. Since the T-cell population is heterogenous with respect to its binding capacity of SRBC, tests were performed to learn whether those with a high binding capacity,

Table 1. Effect of melphalan treatment of human blood lymphocytes on the percentages of T- and non-T-cells

Dose of the drug and time of treatment		E-cells Exp. 1	2	3	4	'Active' E-cells Exp. 1	2	3	4	EAC-cells Exp. 1	2	3	4
Untreated cells		60	69	68	75	25	50	32	56	28	30	33	35
16 μg/ml	2 hr	50	60	64	65	34	52	34	50	16	14	20	14
	24 hr	52	59	60	60	30	60	33	49	15	10	12	14
32 μg/ml	2 hr	57	62	58	68	29	65	27	59	15	15	16	12
	24 hr	65	55	52	62	20	48	27	50	6	7	6	7

Four experiments using lymphocytes from different donors are presented. Two hundred lymphocytes were scored for each determination.

Table 2. Effect of adriamycin treatment of human blood lymphocytes on the percentages of T- and non-T-cells

Dose of the drug and time of treatment		E-cells Exp. 1	2	3	4	'Active' E-cells Exp. 1	2	3	4	EAC-cells Exp. 1	2	3	4
Untreated cells		67	60	68	58	25	39	32	34	38	28	35	34
2 μg/ml	2 hr	64	50	64	56	26	44	34	30	20	15	16	14
	24 hr	56	56	58	50	30	30	31	35	15	14	18	10
4 μg/ml	2 hr	63	58	55	54	36	49	37	25	10	10	11	17
	24 hr	52	50	58	48	32	38	38	30	6	4	4	7

Four experiments using lymphocytes from different donors are presented. Two hundred lymphocytes were scored for each determination.

Table 3. Effect of daunomycin treatment of human blood lymphocytes on the frequencies of T- and non-T-cells

Dose of the drug and time of treatment		E-cells Exp. 1	2	3	4	'Active' E-cells Exp. 1	2	3	4	EAC-cells Exp. 1	2	3	4
Untreated cells		67	70	62	75	40	36	30	41	38	35	38	30
2 μg/ml	2 hr	40	42	26	53	35	38	16	23	10	12	15	16
	24 hr	24	16	10	14	4	10	10	7	5	3	8	10
4 μg/ml	2 hr	29	33	20	24	18	20	11	10	11	8	10	9
	24 hr*	—	—	—	—	—	—	—	—	—	—	—	—

*85–93% of the cells were dead, as assessed by the trypan blue exclusion test. The rosettes were not counted.

Four experiments using lymphocytes from different donors are presented. Two hundred lymphocytes were scored for each determination.

"active" rosette forming cells, differ from the entire SRBC-binding cell population. The "active" rosette forming lymphocytes are of interest to examine since they have been found to be more reactive against PPD and allogeneic cells than T-cells with a low binding capacity for SRBC [17].

It was observed that the doses and the exposure times of the lymphocytes to melphalan or adriamycin did not kill them to any detectable extent as defined by their exclusion of trypan blue. However, both drugs reduced the frequency of EAC-binding cells. The E- and "active" E-binding cells were more resistant to melphalan and adriamycin. Daunorubicin used in the same concentrations as adriamycin exhibited a dose- and time-dependent killing effect on the entire cell population. This drug reduced the frequency of both E- and EAC-binding cells.

It is concluded that the expression of the C'3 receptor is highly sensitive to all three cytostatic drugs whereas the expression of the SRBC receptors is relatively resistant to melphalan and adriamycin. This receptor appeared to be sensitive to daunorubicin. The explanation for the differential sensitivity of the two receptors is unknown. One explanation could be that there is a general difference in the sensitivity of the T- and B-cell populations. For instance, previous work has suggested that B-cells are more radiosensitive than T-cells [18]. It is possible that the DNA-repair of T-cells is more efficient than that of B-cells [19]. It is also possible that the first step in adriamycin's and melphalan's action on lymphocytes is on membrane-associated DNA [20] which may lead to changes of the levels of cyclic nucleotides. Since adenylate cyclase synthesizing cyclic AMP resides in the external cell membrane [21] it is possible that changes in its activity may affect the cell's

capacity to synthesize and/or express receptors for SRBC or C'3. If this is true, the present results indicate that the latter receptor is more sensitive to decreased adenylate cyclase activity than is the SRBC receptor.

The only chemical difference between adriamycin and daunorubicin is a single hydroxyl group on carbon 14 and both drugs have similar pharmacologic characterisites and toxicities. Despite these similarities the *in vitro* effects of these drugs on lymphocytes were different. The reason for these different effects is unclear but pharmacologic factors such as the uptake of the drugs in the cells and their capacity to metabolize them may be important in explaining the pronounced killing effect of daunorubicin in comparison to adriamycin. Furthermore, this observation may be of significance in the context of the effectiveness of daunorubicin in the treatment of acute leukemias.

At present we do not know whether the sensitivity of lymphoid cells *in vitro*, as described in this article, correlates to their *in vivo* sensitivity to cytotoxic drugs. Possibly, however, this method may be useful in the screening of cytostatic drugs which are active against human lymphoid malignancies which frequently possess the membrane receptors studied.

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