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Generation of lymphokine-activated killer cells does not require DNA synthesis

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SUMMARY

We studied the role of DNA synthesis in the induction of lymphokine-activated killer (LAK) cells by recombinant interleukin-2 (IL-2) and the dependence of this phenomenon on DNA synthesis. Doses of gamma-irradiation (1000–5000 rads) that profoundly reduced DNA synthesis in human peripheral blood mononuclear leucocytes (PBL) also effectively suppressed the development of cytotoxic activity in the absence of IL-2. However, the same doses of irradiation affected the induction of LAK activity by IL-2 to a much lesser extent. Blocking the formation of deoxyribonucleotides by hydroxyurea, which resulted in a complete inhibition of DNA synthesis in PBL or purified T lymphocytes, had virtually no effect on the generation of LAK cells. These results indicate that the expression of LAK activity is not dependent on DNA synthesis.

Interleukin-2 (IL-2) induces some T cells, termed lymphokine-activated killer (LAK) cells, to exert a powerful cytotoxic activity against various tumour (Rosenberg, 1985) or normal (Sondel *et al.*, 1986) cells *in vitro*. Successful animal studies (Cheever *et al.*, 1982; Mulé *et al.*, 1984) were shortly followed by encouraging therapeutic attempts, during which cancer patients were treated with autologous LAK cells in conjunction with large quantities of IL-2 (Rosenberg *et al.*, 1985; Rosenberg, 1986). The results of these therapeutic manoeuvres drew considerable attention to the LAK cell population(s), as it became apparent that these relatively non-specific cytotoxic cells were capable of reducing the growth of tumours *in vivo*. Moreover, it is conceivable that these or similar IL-2-induced cells could be involved in the elimination of tolerated alloantigens (Malkovský & Medawar, 1984; Malkovský *et al.*, 1984, 1985; Loveland, Hunt & Malkovský, 1986) and pathogenic microorganisms (Colizzi, 1984) *in vivo*.

Since the report of Grimm *et al.* (1982), it has been generally accepted that proliferation of (and therefore DNA synthesis in) peripheral blood mononuclear leucocytes (PBL), which are used as a source of LAK cells, is strictly required for LAK expression. This view is challenged by the evidence presented here that blocking the synthesis of DNA by physical (gamma-irradiation) or chemical (hydroxyurea) means was not accompanied by the expected loss of LAK activity.

In the first set of experiments, PBL were isolated from human peripheral blood using Ficoll–Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to the instructions of the manufacturer, and were irradiated with varying doses from a ^{60}Co source. The irradiated and control, non-irradiated, PBL were then both placed into round-bottomed 96-well microplates and tissue culture bottles (Nunclon, Roskilde, Denmark) and incubated in the presence or absence of 500 units/ml of human recombinant IL-2 highly purified by reversed-phase high performance liquid chromatography (Malkovský *et al.*, 1985). (Endotoxin was not detected in this IL-2 preparation using the *Limulus* amoebocyte lysate test.) These and all other incubations were carried out in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37° in a humidified atmosphere of 95% air and 5% CO_2. After 144 hr of culture, PBL in microplates (250,000 cells in 0·2 ml/well) were pulsed with [Me-^{3}H]thymidine (1 μCi/well) for 6 hr, and the incorporation of tritiated thymidine was measured by liquid scintillation counting as described elsewhere (Malkovský *et al.*, 1982). Cytotoxicity was measured in a 5-hr ^{51}Cr-release assay (Malkovský *et al.*, 1983) using T24 target cells, which are relatively resistant to natural killer cells (Bubeník *et al.*, 1978). In brief, target cells were labelled with $Na_2[^{51}Cr]O_4$ and washed four times in medium. Five thousand

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Abbreviations: HU, hydroxyurea; IL-2, interleukin-2; LAK, lymphokine-activated killer; PBL, human peripheral blood mononuclear leucocytes.

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Table 1. Effects of gamma-irradiation on DNA synthesis and cytotoxicity generated in 6-day cultures of PBL

Radiation dose (rads)	Without IL-2		With IL-2	
	[^{3}H]Thymidine incorporation (c.p.m. $\times 10^{-3}$)	% specific ^{51}Cr release*	[^{3}H]Thymidine incorporation (c.p.m. $\times 10^{-3}$)	% specific ^{51}Cr release*
0	6·59±0·54†	18·4±4·2†	25·65±1·61†	48·4±11·1†
1000	0·78±0·11	0·9±0·5	2·05±0·72	24·2± 9·0
2000	0·39±0·10	1·0±0·9	1·23±0·35	23·3± 9·1
3000	0·31±0·16	1·0±1·0	0·82±0·28	15·1± 2·8
4000	0·22±0·04	1·2±1·1	0·69±0·13	9·9± 1·5
5000	0·18±0·04	0·8±0·7	0·56±0·14	5·7± 1·9

* The corrected percentage specific lysis at a 15:1 effector to target cell ratio was calculated using regression analysis.

† The values represent means and standard errors calculated from four independent experiments using PBL from four different donors.

radiolabelled target cells were exposed to various numbers of effector cells (at least four different effector to target cell ratios per each sample of PBL) for 5 hr in a final volume of 0·2 ml, and the percentage specific lysis was calculated as described previously (Malkovský *et al.*, 1983). All the data were subjected to regression analysis, and the corrected percentage lysis was calculated from the curve at an effector to target cell ratio of 15:1. Table 1 shows that DNA synthesis was, as expected, highly radiosensitive, whereas the induction of cytolytic activity in PBL by IL-2 was remarkably resistant to gamma-irradiation and considerable LAK activity was induced even after initial exposure to 2000 or 3000 rads. There are two possible explanations of this phenomenon: (i) a substantial portion of the LAK-precursor population is relatively resistant to gamma-irradiation; (ii) the major LAK-precursor population is radiosensitive but there is a small radioresistant population with a high cytotoxic efficacy (i.e. multiple hits performed by a single cell). Interestingly, and in contrast to LAK activity, cytotoxicity induced by incubating cells in medium without IL-2 was very sensitive to gamma-irradiation (Table 1). Although at a 15:1 effector to target cell ratio the corrected lysis of natural killer-sensitive K562 cells by freshly isolated and uncultured PBL was easily measurable (and was above 30% specific lysis), their ability to lyse T24 target cells was negligible, around 3–4% specific lysis (data not shown), thus confirming the previously reported relative resistance of T24 to natural killer cells (Bubeník *et al.*, 1978). During these experiments, we noted that, in contrast to Grimm *et al.* (1982), who observed the peak of LAK activity around Day 6 of culture in the presence of relatively crude IL-2 preparations, the LAK activity reached its maximum after a 48–72 hr exposure to highly purified IL-2 and remained stable for at least 4 days (data not shown). Therefore, all subsequent measurements of LAK activity were performed after a 48-hr exposure to IL-2.

In the second set of experiments, we tried to induce the LAK activity in the presence of hydroxyurea (HU), which is known to inhibit DNA synthesis by interfering with ribonucleotide reductase—the enzyme that catalyses the formation of deoxyribonucleotides (Young & Hodas, 1964; Thelander & Reichard, 1979). PBL were incubated in the presence or absence of 500 units/ml IL-2 and/or 5 mM HU. After 48 hr of culture, the cytolytic activity of PBL against T24 was measured in parallel with DNA synthesis. Figure 1 shows that HU blocked DNA synthesis completely but had no effect on the development of LAK activity. Very similar results were obtained when the experiment was repeated using PBL from another two donors.

Since we know that monocytes similar to LAK cells can also display IL-2-induced cytotoxicity (Malkovský *et al.*, 1987), we depleted PBL of adherent cells by incubating in 50-ml tissue culture bottles (Nunclon) for 2 hr (10^6 cells/ml: 10 ml/bottle). The non-adherent population was then rosetted with sheep red blood cells (Weiner, Bianco & Nussenzweig, 1973), and E-rosetting cells were separated by centrifugation over a Ficoll–Paque gradient. The resulting cell population containing a

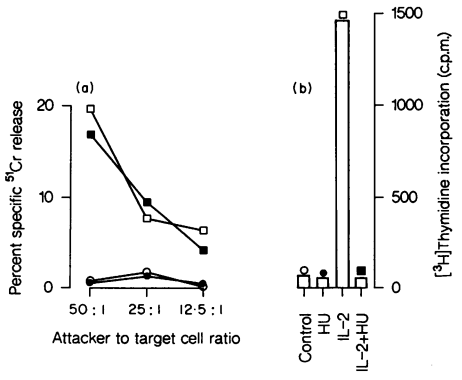

Figure 1. Blocking DNA synthesis does not prevent the development of LAK activity in PBL. PBL were incubated in 96-well tissue culture microplates (100,000 cells in 0·2 ml/well) with (squares) or without (circles) IL-2 (500 units/ml) in the presence (closed symbols) or absence (open symbols) of HU (5 mM). After a 48-hr incubation period, their cytotoxic activity against T24 cells (a) and DNA synthesis (b) was measured as described in the text.

Table 2. Effects of hydroxyurea on DNA synthesis and LAK activity of purified T lymphocytes

[^{3}H]Thymidine incorporation measured at:	T cells cultured in:*			
	Medium only	HU	IL-2	IL-2+HU
6–12 hr	81±21†	72±8	411± 10	76± 6
24–30 hr	122± 6	79±3	762±121	75± 6
48–54 hr	134± 7	75±5	1582±100	76±18
% specific ^{51}Cr release‡	0·5 (2·4)	0·7 (4·0)	39·8 (4·3)	38·8 (3·6)

* T lymphocytes were purified from PBL as described in the text, and were incubated in 96-well tissue culture microplates (100,000 cells in 0·2 ml/well). IL-2 (500 units/ml) and/or HU (5 mM) were present in some wells throughout the incubation period. The microcultures were pulsed with [Me-^{3}H]thymidine (1 μCi/well) at either 6, 24 or 48 hr. The cells were harvested 6 hr later and the incorporation of tritiated thymidine was determined by liquid scintillation counting.

† The values expressed are mean c.p.m. and standard errors.

‡ The cytotoxic activity of the T lymphocytes against T24 cells was measured after a 48-hr incubation period. The results of cytotoxic tests were subjected to regression analysis and the corrected percentage specific lysis at a 15:1 effector to target cell ratio is given. The numbers in parentheses indicate 95% confidence limits.

subset of LAK precursors was incubated in the presence or absence of 500 units/ml IL-2 and/or 5 mM HU. After 48 hr incubation, more than 99% viable cells expressed the T-cell receptor complex as judged by immunofluorescent staining with anti-Leu-4 monoclonal antibody (CD3). DNA synthesis in these cells was measured in the intervals 6–12, 24–30 and 48–54 hr after setting up the cultures, and their cytotoxic activity against T24 cells was assessed at 48 hr of culture. Table 2 illustrates the fact that the cells did not synthesize DNA in the presence of HU, but developed into potent LAK cells. On the basis of these results, we conclude that the generation of LAK activity is independent of DNA synthesis. Since all the permanent cell lines as well as certain mitogen-induced blasts we tested were sensitive to LAK cells, we believe that some of the so-called 'non-specific cytotoxicity' or 'autoreactivity' occurring in various limiting dilution assays for the estimation of cytotoxic cell precursor frequencies using IL-2 plus 2000 rads irradiated feeder layers as well as the variability of these results in different laboratories could be partly explained by our findings. Also, it appears that the potential therapeutic manoeuvres in cancer patients combining DNA synthesis-inhibiting cytotoxic drugs and IL-2/LAK cells are not mutually exclusive.

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