Influence of tumour physico-chemical conditions on interleukin-2-stimulated lymphocyte proliferation

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Summary The proliferative response of murine lymphocytes to interleukin-2 (IL-2) was examined under physico-chemical conditions present in solid tumours, namely low oxygen and glucose concentrations and acidic pH. Lymphocytes were cultured for four days in 30 U ml−1 IL-2 to simulate IL-2 concentrations attainable with high-dose systemic IL-2 therapy. Lymphocyte proliferation was significantly (P<0.05) reduced by low oxygen concentrations (both anoxia [9% O2] and hypoxia [10%], low glucose (6 mg dl−1), or acidic pH (6.7 or 6.4). Moderate glucose concentration (32 mg dl−1), or neutral pH (7.0) did not impair proliferation. This study indicates that impairment of lymphocyte proliferation by tumour physico-chemical conditions may be a factor in the relatively poor success rate of IL-2/LAK cell immunotherapy.

Materials and methods

Mice

Male and female BALB/c mice from 2–6 months of age were used in these experiments. The mice were originally obtained from the Cancer Research Laboratory, University of California at Berkeley, and were maintained in our animal facility by brother-sister mating.

In vitro lymphocyte proliferation

Mice were sacrificed by cervical dislocation, and their spleens were dispersed by pushing through a 40 mesh wire screen. The cells were allowed to adhere for 45 min on 100 mm tissue culture dishes. Nonadherent cells were collected and erythrocytes were lysed with NH4Cl (0.14 M in Tris base), followed by two washes with Hanks' balanced salt solution (HBSS, Gibco Laboratories, Grand Island, NY). Lymphocytes were resuspended to 106 live cells ml−1 in appropriate media (see below) together with 30 U ml−1 of recombinant human IL-2 (Cetus Corporation, Richmond, CA) and 4 × 10−5 M mercaptoethanol (2-ME). The specific activity of this IL-2 preparation was 3 × 106 U ml−1. This concentration of IL-2 was chosen because 30 U ml−1 IL-2 is reported to be achievable in serum during systemic treatment (intravenous infusion over 6 h) with IL-2 at 106 U ml−2 (Mertelsmann & Welte, 1986). For proliferation assays in varying pH or glucose, 2 × 105 cells (200 μl) were dispensed per well in a 96-well round-bottom microtiter plate. For evaluation of lymphocyte proliferation in hypoxic conditions alone, cells were dispensed in 1 ml volumes into 6 x 50 mm borosilicate glass test tubes. All conditions were evaluated in triplicate or quadruplicate in each assay, and each experiment was performed on at least three separate occasions.

Production of tumour-like physico-chemical conditions

Low oxygen concentrations Lymphocytes were resuspended to 106 ml−1 in RPMI-1640 (Gibco) supplemented with 10% foetal calf serum (FCS), 100 U ml−1 penicillin, 100 μg ml−1 streptomycin, 10% NCTC-109 (Whittaker M.A. Bioproducts, Walkersville, MD), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1 g ml−1 NaHCO3, and 20 mM Hepes buffer. (Hereafter, this medium will be referred to as SRPM1). The procedure for production of hypoxia has been described previously (Loeffler et al., 1990). Briefly, 1 ml of cells was dispensed into 6 x 50 mm glass test tubes as described above; the tubes were placed in a 96 well
microtiter plate, inside a plexi-glass chamber connected via Tygon tubing to a tank containing 95% N\textsubscript{2}/5% CO\textsubscript{2} (anoxia) or 1% O\textsubscript{2}/94% N\textsubscript{2}/5% CO\textsubscript{2} (hypoxia). The chamber was placed within a 37°C incubator. Oxygen concentrations were measured via an MI-730 Micro-Oxygen electrode (Microelectrodes, Inc., Londonderry, NH) and determined to be 0.2% and 1.3% when cells were gassed with 0% O\textsubscript{2} and 1% O\textsubscript{2}, respectively. Aerobic controls were incubated at 37°C in room air/5% CO\textsubscript{2}. Lymphocytes were cultured in IL-2 for 72h, then \textsuperscript{3}H-thymidine (0.5μCi/tube) was added. (Tubes were briefly removed from the hypoxia chamber in order to add the radioisotope). Eighteen h later, cells were harvested with a cell harvester, and \textsuperscript{3}H-thymidine incorporation into cells was determined with a beta counter. Addition of \textsuperscript{3}H-thymidine and harvesting of lymphocytes was similar for the other proliferation assays described below.

**Low glucose** Glucose-free Dulbecco’s Minimal Essential Medium (DME, Sigma Chemical Company, St Louis, MO) was supplemented as described above for RPMI-1640, except that pyruvic acid and NCTC-109 were omitted, and the concentration of FCS was reduced to 2%. The glucose concentration in this medium (as measured with the Glucose HK-10 Test Kit from Sigma) was approximately 6mgdl\textsuperscript{-1}. Glucose (alpha-D(+)-glucose, Sigma) was added to yield a final concentration of either 32mgdl\textsuperscript{-1} or 125mgdl\textsuperscript{-1}. IL-2-stimulated lymphocyte proliferation in varying glucose concentrations was evaluated by resuspending lymphocytes to 10\textsuperscript{6} cells/ml in each of the three media, together with IL-2 and 2-ME as described above. 2x10\textsuperscript{5} cells (200μl/well) were dispensed into 96 well round-bottom microtiter plates. Plates were centrifuged daily and half of the medium was replaced with new medium in order to compensate for utilisation of glucose by the actively proliferating cells.

**Acidic pH** SRPMI was adjusted to pH 7.4, 7.0, 6.7, or 6.4 by addition of NaH\textsubscript{2}CO\textsubscript{3} or 1NNaOH, then sterile filtered. Lymphocytes were resuspended in IL-2 and 2-ME and dispensed in microtiter plates as described above. One-half of the medium in each well was replaced each day with fresh IL-2-containing medium (adjusted to proper pH immediately before use) in order to minimise pH variations. Values for pH were found to fluctuate by 0.1–0.2 pH units during the assay.

**Statistical analysis** Data from typical experiments under each set of physical conditions were chosen for statistical analysis. For assays in which pH or glucose were varied, the one-way analysis of variance (ANOVA) was used to determine whether treatment effects were present for pH or glucose levels. Where ANOVA indicated the presence of significant treatment differences, pairwise differences were evaluated by Tukey’s Multiple Range Procedure (Zar, 1984). For assays of lymphocyte proliferation in varying O\textsubscript{2} concentrations, Student’s t-test was used to compare pairwise differences.

**Results**

**Lymphocyte proliferation in low oxygen concentrations**

IL-2-stimulated lymphocyte proliferation under hypoxic (1% O\textsubscript{2}) and anoxic (0% O\textsubscript{2}) conditions was compared with proliferation in room air (20% O\textsubscript{2}). Proliferation was similar in both 1% O\textsubscript{2} and 0% O\textsubscript{2}, and for both conditions was significantly less than in room air (P<.0001 and P=.0049, respectively) Figures 1a–b).

**Lymphocyte proliferation in low glucose concentrations**

Lymphocyte proliferation in 6mgdl\textsuperscript{-1} and 32mgdl\textsuperscript{-1} glucose concentrations was compared with proliferation in normal serum glucose concentration (125mgdl\textsuperscript{-1}). Proliferation in 6mgdl\textsuperscript{-1} glucose was significantly less than at 125mgdl\textsuperscript{-1}

![Figure 1](attachment://figure1.png) **IL-2-stimulated lymphocyte proliferation in 1% O\textsubscript{2} (a) and 0% O\textsubscript{2} (b) compared to proliferation in room air (20% O\textsubscript{2}). Lymphocyte proliferation was significantly decreased in both conditions compared to room air (P<.0001 and P=.0049, respectively).**

**Discussion**

The effectiveness of IL-2 therapy presumably depends upon a variety of factors, including toxicity, concentration of IL-2 delivered to the tumour, extent of lymphocytic infiltration, response of TIL to IL-2, and sensitivity of tumour cells to cytotoxic effects of the activated TIL. The present study examined IL-2-stimulated lymphocyte proliferation under the range of physico-chemical conditions reported for experimental tumours in laboratory animals. The actual tumour microenvironment is clearly more complex than this in vitro model, with other factors (including suppressor cells and soluble immunosuppressing factors) influencing TIL responsiveness as well. Splenic lymphocytes rather than TIL were employed in these experiments because the IL-2 receptor is not well expressed on TIL, and in vitro proliferative response of TIL to IL-2 is poor (Miescher et al., 1986). It may be that TIL IL-2 receptor expression is down-regulated in part by tumour physico-chemical conditions, although this was not examined in the present study.

Oxygen concentrations in the range of radiobiological hypoxia (1% O\textsubscript{2}) as well as anoxia (0% O\textsubscript{2}) significantly reduced lymphocyte proliferation, as did extremely low glucose concentration (6mgdl\textsuperscript{-1}) and acidic pH (6.4 and 6.7). However, more moderate conditions (either 32mgdl\textsuperscript{-1} glucose or pH 7.0) did not decrease lymphocyte responsiveness to IL-2; in fact, lymphocyte proliferation at pH 7.0 was significantly increased relative to pH 7.4. These results suggest that the influence of tumour physico-chemical conditions on lymphocyte proliferation depends upon the severity of these conditions within the tumour, as well as the location of the TIL. Lymphocytes situated in the tumour periphery are not likely to be exposed to the extreme conditions which reduced proliferation in this study, while IL-2-responsiveness...
of more centrally located TIL may be down-regulated. However, tumour cells in hypoxic areas are more resistant to radiotherapy and some forms of chemotherapy than their well-oxygenated counterparts (Gray et al., 1953; Bush et al., 1978; Hill & Stanley, 1975; Tannock, 1982), and it is these cells which must be targeted if immunotherapy is to be a useful addition to standard therapy protocols. Inhibition of lymphocyte proliferation under tumour physico-chemical conditions is not specific for this cell type, as these same conditions interact to kill neoplastic cells in poorly perfused central areas of tumours (Rotin et al., 1986; Tannock & Kopelyan, 1986). Our results suggest that inhibition of IL-2-stimulated lymphocyte proliferation by tumour physico-chemical conditions may be a factor in the relatively poor success rate of IL-2/LAK cell immunotherapy. Short-term improvement of physical conditions within tumours during administration of IL-2, such as increasing tumour pO2 by Fluosol-DA plus carbogen (Fischer et al., 1986), should be examined in animal models to determine if TIL response to IL-2 may be improved. The potential benefit of increasing TIL responsiveness by alteration of the tumour microenvironment must be evaluated against the possibility of concomitant increase in tumour cell proliferation under more normal physico-chemical conditions.

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