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Measurement of Interferon Sensitivity in Tumour Cells from Fine-needle Aspirations

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Previous studies have shown significant correlations between interferon (IFN) induced enhancement of the enzyme 2′,5′-oligoadenylate (2′,5′-A) synthetase *in vitro* and response to IFN therapy. A limitation of this and other predictive tests is the availability of malignant cells for culture. Malignant cells can be obtained from most palpable solid tumours by fine-needle aspiration. We investigated whether malignant cells from such aspirations can be used in a 2′,5′-A synthetase assay. In 23/27 (85%) of the cases sufficient amounts of viable cells were obtained, containing a high proportion (≥ 90%) of tumour cells. In 13/23 tumour samples (57%) IFN-α significantly enhanced the 2′,5′-A synthetase levels. The use of cells from the fine-needle aspirations for prediction of IFN sensitivity, makes the 2′,5′-A synthetase test applicable in a wide range of tumours at a variety of disease stages.
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INTRODUCTION

FOR SEVERAL years attempts have been made to develop tests for prediction of treatment-response in malignant diseases. With respect to chemotherapy the results have so far been disappointing, one major reason being that most tests require *in vitro* growth of the malignant cells [1]. In the case of interferons (IFNs) and other cytokines there are possibilities of developing *in vitro* assays that can measure physiological responses of non-proliferating tumour cells. We have previously used such an approach to test the IFN sensitivity of primary malignant cells by measuring the capacity of IFN to induce the enzyme 2′,5′-oligoadenylate (2′,5′-A) synthetase *in vitro*. This enzyme has been implicated in the antiviral and antiproliferative actions of IFN [2]. The test is rapid (48 h), requires few cells and is not dependent on *in vitro* growth [3]. A further study was carried out on IFN-α treated metastatic mid-gut carcinoid patients. A pretreatment 2′,5′-A synthetase assay on tumour cells derived from coarse needle biopsies of liver metastases correlated closely to the subsequent clinical effects of IFN therapy [4]. Similar findings have been reported for chronic myelogenous leukaemia (CML), chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma [5–7]. These findings implicate that a 2′,5′-A synthetase assay can be used as a predictive test for IFN sensitivity, at least in some malignancies. In resistant cases other treatment modalities could thus be tried and furthermore the side effects from IFN are not suffered unnecessarily.

Malignant cells from surgical specimens or coarse needle biopsies are not always readily available. We therefore decided to study whether malignant cells from fine needle aspirations can be used for the 2′,5′-A synthetase assay. This aspiration technique is lenient on the patient and is possible to perform from almost all palpable tumours [8].

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PATIENTS AND METHODS

Patients

Malignant cells were obtained from a primary tumour (mammary carcinoma), superficial lymph node metastases or cutaneous/subcutaneous metastases from 27 patients with various solid tumours (for patient data see Table 1) using a fine needle aspiration technique as previously described [8].

Culture conditions

The cells were immediately transferred to culture medium, RPMI-1640 (Flow) with L-glutamine (200 mmol/l solution, 1% by volume), benzylpenicillin (100 IU/ml), streptomycin sulphate (100 μg/ml) and 10% of fetal calf serum. In 2 cases the cell suspensions were treated with aspiration through a thin needle to disperse large clumps of cells. In most cases, 3×10^4 cells if not otherwise stated, were thereafter transferred to the wells of 96-well microtiter plates in the absence or presence of 500 units/ml of IFN-α_{2b} (Intro A; Schering) and cultured at 37°C in a humidified 5% CO_2 incubator for 24 h. The proportion of viable cells after culture was determined by staining with trypan blue. Based on initial experiments, less than 10% viable cells was used as the limit for non-evaluable tests.

Aspirations from 4 of the 27 patients were not tested since less than 60 000 viable cells were found in the aspirates. Repeated aspirations, that could have provided sufficient quantities were not performed.

Morphological evaluation

3×10^4 cells in 0.2 ml of medium were deposited on slides with a cytocentrifuge (35 *g* for 5 min). The preparations were immediately fixed with water-soluble fixative (Becton Dickinson) and stained with haematoxylin eosin. The samples were evaluated by cytopathological examination, by scoring of 200–400 cells to determine the ratio of neoplastic cells to the number of leucocytes and other non-malignant cells. It was found that in all but 4 cases tested (patients 3, 8, 10 and 16), the percentage of malignant cells was ≥ 90%. The non-malignant cells were in all cases leucocytes, mainly of lymphoid origin. Initial experiments showed that low number (< 50%) of contaminating lymphoid cells did not influence the test result: (1) Lymphoid cells were found to contain much smaller amounts of 2′,5′-A synthetase than primary tumour cells. This results in

Table 1. Diagnosis, 2′,5′-A synthetase levels, percentage of tumour cells and % viable cells after 24 h of culture in samples from 23 patients

Patient	Diagnosis*	No. of cells per aspiration	% tumour cells	Control†	500 IFN-α (U/ml)‡	5000 IFN-α (μ/ml)‡	% viable cells
1	OC	390 000	> 95	22	6.8	7.0	70
2	OC	820 000	> 95	947	0.9	0.8	100
3	OC	4 300 000	75	11	2.9	6.5	70
4	MC	600 000	> 95	207	5.3	6.8	10
5	MC	120 000	> 95	94	1.8	nd	40
6	MM	180 000	> 95	5	49.8	nd	30
7	MM	5 420 000	> 95	129	9.0	9.0	50
8	MM	1 940 000	75	4	7.3	15.8	100
9	MM	510 000	90	50	3.7	nd	100
10	MM	720 000	80	20	5.4	nd	100
11	LAC	140 000	> 95	6	1.5	nd	30
12	LAC	1 200 000	> 95	8	7.0	nd	30
13	ACL	150 000	90	385	2.1	nd	30
14	ACL	500 000	> 95	281	3.4	4.6	10
15	AC	190 000	> 95	244	1.3	2.3	70
16	AC	940 000	50	455	0.6	0.8	100
17	AC	270 000	90	81	5.7	nd	100
18	TC	nd	> 95	5	4.9	nd	10
19	GC	700 000	90	372	1.7	nd	50
20	LCLC	400 000	> 95	8	1.1	nd	20
21	PC	120 000	> 95	34	1.6	nd	50
22	THC	120 000	> 95	34	9.7	nd	10
23	UC	700 000	90	79	8.3	7.5	100
Mean (S.E.)		930 000(290 000)		151 (46)	6.2 (2.1)	6.1 (1.4)	

* OC = ovarian carcinoma, MC = mammary carcinoma, MM = malignant melanoma, LAC = laryngeal carcinoma, ACL = adeno carcinoma of the lung, AC = adenocarcinoma of unknown origin, TC = tonsillar carcinoma, GC = gastric carcinoma, LCLC = large cell lung carcinoma, PC = prostate carcinoma, THC = thyroid cancer, UC = uretary carcinoma.

† nmol ATP incorporated per 5×10^3 cells per 16 h.

‡ Relative 2′,5′-A synthetase concentrations (nmol ATP incorporated per 5×10^3 cells incubated with IFN divided by nmol ATP incorporated per 5×10^3 cells incubated in medium alone).

nd = not done.

lymphoid cells contributing minimally to the levels of 2′,5′-A synthetase observed after culture in the absence and presence of IFN [3]. (2) It was repeatedly found that the results did not differ when purified tumour cell populations were compared to unpurified populations. (3) Mixing experiments performed with mouse cells resistant to human IFN and lymphoid cells from healthy donors showed that up to 50% of lymphoid cells (30 000 cells/well in total) were unable to give any measurable enhancement of 2′,5′-A synthetase with IFN.

Assay for 2′,5′-A synthetase

The cytoplasmic levels of 2′,5′-A synthetase were determined in NP-40 lysed cells following incubation with poly (rI) poly (rC) agarose beads [3]. Briefly, the cells were lysed by the addition of 50 μl of NP-40 to the wells. The plates were then centrifuged for 3 min at 3000 *g* and the supernatants collected and frozen at −70°C. For the enzyme assay, 10 μl of the extract was added to poly (rI) poly (rC) agarose beads and the mixture was incubated for 15 min at 30°C. The beads were then washed and the reaction mixture, containing 10 mmol/l Hepes buffer pH 7.5, 5 mmol/l $MgCl_2$, 7 mmol/l dithiothreitol, 10% glycerol, 2.5 mmol/l [α-^{32}P] ATP (3.7–11.1 GBq/mmole), 3 mg/ml creatinekinase, 10 mmol/l creatine phosphate and 40 μg/ml poly (rI) poly (rC), was added after which the samples were incubated for 16 h at 30°C. One unit of bacterial alkaline phosphatase in 140 mmol/l Tris-base was then added. After 1 h at 37°C 20 μl of water was added after which the beads were removed by centrifugation. The samples were then run through 0.3 ml alumina columns equilibrated in 1 mol/l glycine–HCl buffer pH 2 and collected in scintillation vials which were counted in the ^{3}H-channel of a scintillation counter (Packard). Two to three wells were used for each determination. A standard prepared from normal lymphocytes as well as a blank containing NP-40 only was always included in the assay.

Relative 2′,5′-A synthetase induction was calculated as nmol ATP per 5×10^3 cells with IFN/nmol ATP per 5×10^3 cells without IFN. The variability within duplicates/triplicates was with few exceptions less than 30% of the mean value.

Mainly based on previous experiments [4], induction of 2′,5′-A synthetase by a factor of 3 following treatment with IFN was chosen as a limit for IFN induced enhancement of 2′,5′-A synthetase.

Statistical analyses

Statistical significances was evaluated by linear regression.

RESULTS

Initial experiments were performed to find the optimal condition for culture. Human AB+ serum (10%) was compared with fetal calf serum (10 or 20%) and different microtiter plates

Table 2. 2′,5′-A synthetase levels in various amounts of tumour cells after incubation for 24 h in the absence or presence of 500 units/ml of IFN-α. Means (S.E.) from 9 patients

	Cells per well		
	1.5×10^4	3×10^4	6×10^4
No IFN*	119 (36)	274 (99)	504 (192)
IFN†	4.7 (1.2)	4.1 (1.0)	3.7 (0.8)

* nmol ATP incorporated in 2.5–10 × 10^3 cells during 16 h.
† Relative 2′,5′-A synthetase concentrations (nmol ATP incorporated in 2.5–10 × 10^3 cells incubated with IFN divided by nmol ATP incorporated in 2.5–10 × 10^3 cells incubated in medium alone).

(flat bottomed and U-shape bottomed) were tested. No major differences in baseline levels of induction of 2′,5′-A synthetase were found with different serum conditions nor type of microtiter plate (data not shown).

The minimal amount of cells (15, 30 or 60 × 10^3 cells/well) required for an assay was tested in 9 cases. The relative increase in 2′,5′-A synthetase following treatment with IFN-α was similar for the three cell concentrations employed (Table 2), which means that 15 × 10^3 tumour cells/well is sufficient for the test.

The levels of 2′,5′-A synthetase increased in a dose dependent manner following incubation with various concentrations of IFN-α. Maximal stimulation was observed at doses of 5000 units/ml (Fig. 1). There was also a statistically significant correlation between 2′,5′-A synthetase levels after incubation with 500 and 5000 U/ml IFN-α ($r = 0.83, P < 0.05$, Table 1).

Induction of 2′,5′-A synthetase was studied in 23 patients using 500 units/ml of IFN-α. Baseline concentrations of 2′,5′-A synthetase varied between tumours (from 4 to 947 nmol ATP per 5 × 10^3 cells, Table 1) and IFN-α induced enhancement of 2′,5′-A synthetase also showed a large variation from a factor of 0.6 to a factor of 49.8. In 13 out of 23 tumours, IFN-α induced a significant enhancement of 2′,5′-A synthetase (Table 1).

Cell viability after the 24 h incubation *in vitro* varied between 10 and 100% (mean 52%, Table 1). Linear regression analysis showed no significant correlation between cell viability and induction of 2′,5′-A synthetase or baseline concentrations of 2′,5′-A synthetase. No increase in 2′,5′-A synthetase was observed in the tumour samples with the highest baseline levels of 2′,5′-A synthetase, and there was an inverse correlation between baseline concentrations of 2′,5′-A synthetase and induction of 2′,5′-A synthetase using 5000 units/ml of IFN ($r = -0.70$, $P < 0.05$). Using a similar analysis for 500 units/ml of IFN showed no statistically significant correlation. In this limited material there was no obvious correlation between baseline or induced levels of 2′,5′-A synthetase and different tumour groups.

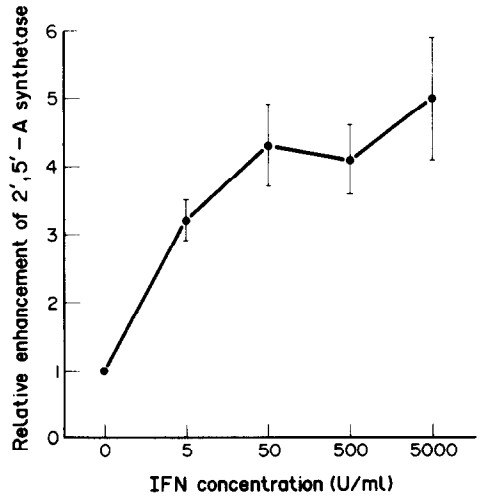

Fig. 1. Relative enhancement of 2′,5′-A synthetase following incubation with increasing doses of IFN-α for 24 h. Means (S.E.) from 4 patients.

DISCUSSION

Previous studies in mid-gut carcinoid, CML, CLL and non-Hodgkin lymphoma have implicated that induction of 2′,5′-A synthetase *in vitro* can be used as a predictive test for IFN therapy [4–7]. A problem in all predictive test systems is the availability of malignant cells for *in vitro* culture. Unlike most other predictive tests, a 2′,5′-A synthetase assay can be performed with small amounts of cells. Thus, theoretically a fine needle aspiration would suffice to give the cells required, and the present study shows that a 2′,5′-A synthetase test can be performed using tumour cells obtained through this technique.

With few exceptions the aspirates contained sufficient amounts of viable cells (≥ 60 000 cells) with a high percentage (usually ≥ 90%) of tumour cells. In a few cases, however, the aspirates contained > 10% contaminating leucocytes, almost exclusively lymphoid cells. Experiments have shown that less than 50% of contaminating lymphoid cells have no major influence on the test result; this being due to the relatively low levels of 2′,5′-A synthetase in these cells. Data from aspirates with up to 50% contaminating lymphoid cells have therefore been included.

We chose ≥ 3 times increment of 2′,5′-A synthetase as a level for sensitivity to IFN. This was partly based on previous experiments in mid-gut carcinoid in which we found a correlation betwen *in vitro* enhancement of 2′,5′-A synthetase and clinical effects [4]. Of course, it is not possible to generalise from one disease to others, and further *in vitro-in vivo* studies may show other levels of increment as significant. We find however, that a more or less arbitrary choice of a cut off level makes the data easier to interpret.

The 2′,5′-A synthetase assay is rapid (48 h), requires few cells and does not demand *in vitro* growth. In agreement with our previous studies, no major induction of 2′,5′-A synthetase was observed in approximately 40% of the samples. If this assay will prove to be a marker for resistance to IFN, it would identify tumour patients who are unlikely to benefit from IFN therapy. If available, other treatment modalities can be used and furthermore, the side effects from IFN therapy are not suffered unnecessarily.

The possibility of using cells from fine needle aspirations makes the test applicable in a wide range of tumours at a variety of disease stages.

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Interleukin-2 Bolus Therapy Induces Immediate and Selective Disappearance from Peripheral Blood of all Lymphocyte Subpopulations Displaying Natural Killer Activity: Role of Cell Adhesion to Endothelium

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As early as 10–15 min after the start of a 30 min interleukin-2 (IL-2) infusion, a rapid, virtually complete disappearance of all natural killer (NK) lymphocyte subpopulations (including both $CD3^-$ $CD56^+$ and $CD3^+$ $CD56^+$ cells with either alpha/beta or gamma/delta T-cell receptor) was observed from peripheral blood. In contrast, the number of T lymphocytes ($CD3^+$ $CD56^-$) was unmodified for at least 2 h after IL-2 injection. The IL-2-induced, rapid disappearance from peripheral blood of NK and NK-like lymphocytes may be related to their massive adherence to the activated endothelium. In this regard, IL-2 infusion caused a very rapid rise of tumour necrosis factor-alpha (TNF-α) plasma concentration, whereas other cytokines, such as interferon-gamma (IFN-γ), were induced only at later times. *In vitro* experiments indicated that IL-2, either alone or better combined with TNF-α, exerts a rapid and selective stimulatory effect on NK adhesion to endothelial cells. On the basis of these findings, we suggest that the activation of NK lymphocytes induced by IL-2, alone or combined with TNF-α, plays a key role in mediating the massive and selective adherence of NK and NK-like cells following IL-2 bolus infusion.
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INTRODUCTION

ADOPTIVE CANCER immunotherapy involves activation *in vitro* by recombinant interleukin-2 (rIL-2) of lymphocyte subpopulations (lymphokine-activated killer (LAK) cells) from a tumour-bearing patient and their subsequent administration to patients [1, 2]. LAK cells exhibit the *in vitro* capacity of lysing fresh tumour cells in a non-MHC restricted manner [2].

In the present study, we used the National Cancer Institute adoptive immunotherapy protocol [1, 2], which has been mainly applied in patients with metastatic malignant melanoma and renal cell carcinoma resistant to standard therapy [1, 2]. This protocol includes three phases: (i) high-dose IL-2 administration; (ii) autologous lymphocytes obtained by leukapheresis are collected and cultured with IL-2 to generate LAK cells; (iii) patients are reinfused with LAK cells together with high-dose IL-2.

In vitro infusion of IL-2 produces a variety of biological effects [3–5], ranging from induction of LAK cell activity markers (i.e. IL-2 receptors) [4] to alterations in the differential leukocyte count (i.e. eosinophilia) thought to be caused by the secretion of other cytokines [4]. Particularly, IL-2 infusion elicits dramatic fluctuations of the number of peripheral blood lymphocytes,