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Improvement of DNA Repair in Lymphocytes of Breast Cancer Patients Treated with *Viscum album* Extract (Iscador)

Eva Kovacs, Tibor Hajto and Katerina Hostanska

We investigated alteration in DNA repair during therapy with an immunomodulator. 14 patients with advanced breast cancer were treated parenterally with Iscador, an extract of *Viscum album* (mistletoe). As a parameter for measurement of DNA repair the incorporation of (^{3}H) thymidine into DNA of unstimulated lymphocytes after ultra violet light (UV) damage was taken. The DNA repair values in the patients were very low before treatment and on day 1: on average 16% of those in a healthy control population. Values started to increase on day 2 and on days 7–9 were on average 2.7 times higher than before treatment. 12/14 patients showed an improvement in repair. The values of spontaneous DNA synthesis were not altered during the treatment. We suggest that the increase of DNA repair could be due to a stimulation of repair enzymes by lymphokines or cytokines secreted by activated leukocytes or an alteration in the susceptibility to exogenic agents resulting in less damage.
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INTRODUCTION

MAMMALIAN CELLS are normally able to repair the damage to DNA caused by different chemical or physical agents. However, there are a number of rare human inherited syndromes which are associated with apparent defects in DNA repair and with a greatly increased frequency of cancer [1–3]. The existence of such disorders is direct evidence that unrepaired or misrepaired damage to the DNA can be carcinogenic, and that there is a direct association between mutagenic and carcinogenic agents.

Cancer patients or cancer-prone persons are reported to have an impaired DNA repair ability in their lymphocytes [4–5]. Agrell [6] assumed that a cancer cell comes into existence because of an interaction between defective repair enzymes and an altered immunological system. Firstly, damaged DNA codes proteins of types which are abnormal, and very rarely, as a result of this, a cell becomes an antigen. Via its production of altered proteins this antigenic cell challenges the immune defence of the body. Secondly, those damaged DNA sections which code for immunological system may mutate. Thus the immunological cells also lose their function against antigenic cells such as tumour cells in the course of time. There is evidence to show that cancer patients, especially in stages III or IV of the disease, have a depression in several functions of host defence [7–9]. Because of these findings increasing numbers of substances with immunomodulatory or immunostimulatory effects have been used in the treatment of cancer. We asked ourselves whether immunomodulatory treatment leads to an alteration in DNA repair in cancer patients. For our investigation we chose Iscador, an extract of *Viscum album* (mistletoe), which has an immunomodulatory effect [10, 11]. We examined breast cancer patients mainly in tumour stages III and IV. The patients were parenterally treated with Iscador and DNA repair in the unstimulated lymphocytes was measured before treatment and several times during the following week. As a parameter for DNA repair we used the incorporation of ^{3}H-thymidine into UV-damaged DNA.

Correspondence to E. Kovacs.
E. Kovacs is at the Department of Research, University Clinic, Kantonsspital, CH-4031 Basel; and T. Hajto and K. Hostanska are at the Laboratory of Immunology, Lukas Clinic, Arlesheim, Switzerland.
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PATIENTS AND METHODS

Patients and treatment

14 patients, aged 38–68 years, with a histological diagnosis of invasive breast cancer were investigated (in 6 cases the tumour was diagnosed premenopausally, in 8 cases postmenopausally). 2 patients were in stage II, one in stage III and 11 in stage IV of the disease. All patients had been treated by surgery and 9 patients additionally by chemotherapy or radiotherapy 4 or more weeks prior to our investigation. All patients were treated with Iscador M (Hiscia, Switzerland, batch No 7F-7365). They received a single intravenous infusion in the morning of day 0 (beginning of treatment). The dose corresponded to 0.33 (S.D. 0.07) mg of fresh mistletoe plant (*Viscum album*) per kg body weight, given in 250 ml saline. The amount of mistletoe β-galactoside specific lectin (ML-I) in the applied Iscador, determined by an enzyme-linked lectin assay [12], was on average 1.6 ng/kg body weight. Preliminary experiments had shown this to be in the optimal range for immunomodulatory effects [13]. The extract was shown to be uncontaminated with endotoxin, using the limulus amoebocyte lysate (LAL) microtitre assay [14]. Starting 48 hours after the intravenous infusion the patients were given daily subcutaneous applications of Iscador in 1 ml distilled water. The average doses of Iscador and the amounts of ML-I were 0.09 mg/kg body weight and 0.24 ng/kg body weight (2nd day), 0.19 mg/kg and 0.49 ng/kg (3rd day), 0.2 mg/kg and 0.64 ng/kg (4th day), 0.21 mg/kg and 0.67 ng/kg (5th day), 0.26 mg/kg and 0.85 ng/kg (6th day), and 0.26 mg/kg and 1.0 ng/kg (7th day). Blood samples (20 ml) were taken into heparinised syringes between 8–9 am (always before parenteral applications) on day 0 then 6, 1, 2 and 7–9 days after. In 7 patients blood was also taken on day 3.

Blood from 92 healthy donors aged 21–68 years from the Basel Blood Transfusion Service served as reference [15].

Measurement of DNA repair activity

DNA repair was assessed at 0, 1, 2, 3 and 7–9 days. The DNA repair was measured in unstimulated lymphocytes after UV-C light exposure. As a parameter for DNA repair activity the incorporation of (^{3}H) thymidine into DNA was taken. This method has been described in detail [16]. Briefly, lymphocytes were separated on Ficoll–Ronpacon (d = 1.077), incubated overnight in Eagle's MEM (Gibco), then irradiated with UV-C light in doses of 2, 4, 8 and 16 J/m^2. UV irradiation was carried out with a germicidal lamp (Phillips) at 253.7 nm which produced 0.8 J/m^2/s from 30 W at a distance of 60 cm, and 0.2 J/m^2/s from 6 W at a distance of 38 cm (measured by Black Ray J 225 UV intensity meter).The cell cultures were then immediately incubated with 370 kBq/2 × 10^6 cells methyl-(^{3}H)thymidine (Amersham) for 2 h (repair time) in the presence of 2 mmol/l/2 × 10^6 cells hydroxyurea (HU, Sigma). This concentration was chosen to inhibit the spontaneous DNA synthesis without affecting the DNA repair. If more is used the baseline is lower, however repair could be affected [17].The DNA of the pelleted cells was precipitated with 5% TCA and filtered. The radioactivity was measured using a scintillation cocktail (Lumasolve:Lipopuma; Fakola) in a Packard liquid scintillation spectrometer and expressed as counts per minute (cpm) per 10^6 cells. To determine the degree of repair activity the cpm values of HU-inhibited unirradiated samples (baseline values) were subtracted from the cpm values of irradiated samples. We used eight dishes per person and per time point: four for the determination of spontaneous DNA synthesis with and without HU and four for the irradiation measurements (2, 4, 8 and 16 J/m^2). The individual regression lines for each patient at each time point were calculated. The DNA repair was classified as reduced or normal if the values on the individual regression line at 6 J/m^2 (middle dose in the logarithmically plotted dose range of 2–16 J/m^2) lay beyond or within the 99% confidence range of a control population (n = 92). Values on this average regression line were 1804 cpm/10^6 cells (2 J/m^2), 2212 cpm/10^6 cells (4 J/m^2), 2620 cpm/10^6 cells (8 J/m^2) and 3029 cpm/10^6 cells (16 J/m^2). The value of the middle dose (6 J/m^2) was 2410 cpm/10^6 cells (confidence interval 1900–2940) cpm/10^6 cells [15]. The methodological variation was 13–15% [16]. The maximum day to day variation, which was only found in a small proportion of the investigated persons was 30% (Ref. 15 and unpublished data).

Measurement of immunological parameters

The immunological parameters were determined as previously described, in peripheral blood samples taken before treatment, and 6 hours, 1 and 2 days after treatment. Polymorphonuclear (PMN) leucocytes were isolated and their activity was assayed by luminal-dependent chemiluminescence during zymosan phagocytosis [12, 18]. The number of large granular lymphocytes (LGL) was determined by a leucocyte-concentrating method [19]. The natural killer (NK) activity of peripheral blood mononuclear (PBM) cells was measured by the inhibition of DNA synthesis in proliferating K_{562} cell line [12, 20]. Numbers of T-helper (CD_{4+}) and T-suppressor (CD_{8+}) cells were assessed with an indirect immunofluorescence method using a kit according to the instructions of the manufacturer (Johnson & Johnson AG, Switzerland).

Table 1. Effect of Iscador treatment on spontaneous DNA synthesis in unstimulated nonirradiated lymphocytes

(^{3}H) thymidine incorporation in cpm/10^6 cells

Patients	Without hydroxyurea					With hydroxyurea				
Day	0	1	2	3	7–9	0	1	2	3	7–9
1	2590	2270	1962	-	2272	1668	2011	1668	-	1053
2	1711	1898	1925	-	1670	1093	1123	485	-	708
3	1736	2807	2355	-	-	765	939	1627	-	-
4	1224	1489	1502	-	1811	1087	814	681	-	987
5	1904	2288	2211	-	2218	646	787	757	-	938
6	815	721	931	-	731	605	513	512	-	551
7	1026	1348	1098	-	1367	867	898	589	-	804
8	2563	2144	1890	1237	1340	1818	1104	1374	1954	1018
9	1070	1223	1282	982	1180	801	763	885	925	757
10	1802	1781	-	869	-	864	800	771	627	-
11	1487	1286	893	859	654	791	711	723	361	764
12	1576	1225	1000	-	-	1471	870	591	624	-
13	800	1234	810	902	986	674	1015	485	514	741
14	914	779	606	806	810	731	810	606	417	411

Statistical analysis

DNA repair activity. For nonirradiated cells; the Mann–Whitney U test was used for comparison of the values of spontaneous and HU-inhibited DNA synthesis. For irradiated cells, the Mann–Whitney U test was used for comparison of the values during the course of treatment. To determine the significance of alterations of DNA repair following the therapy we applied the paired Wilcoxon signed-ranks test. Immunological parameters were evaluated with the Mann–Whitney U test.

RESULTS

Baseline values in nonirradiated cells

Table 1 shows the individual values of spontaneous DNA synthesis with and without HU during the course of the investigation in unstimulated lymphocytes which have not been irradiated. There were no significant differences between values on day 0 and subsequent values except on the 3rd day ($P < 0.05$). The reason for this might be because only 7 out of 14 patients were investigated.

Alterations in the DNA repair in irradiated cells and immunological parameters during treatment

The individual values of DNA repair during treatment with Iscador are shown in Table 2, together with the clinical data of the 14 patients. 9 patients (64%) had a positive family history of cancer in first degree relatives, according to information given by the patient. This proportion is similar to that (76%) which we found in a former study [5]. Because of the small number of patients it was not possible to establish any relationship between DNA repair and stage of the disease or type of therapy.

Before treatment all of the patients had reduced repair compared with the healthy control population [15]. The average values of patients (n = 14) was only 16% of those of controls. In 3 patients, values were too low to be measured accurately against the background and are reported as < 30 cpm/10^6 cells. There was no significant change on day 1 after intravenous infusion, a slight but significant ($P < 0.05$) increase on day 2 and day 3 and a larger increase ($P < 0.05$) on days 7–9 (Wilcoxon signed-ranks test). However using the Mann–Whitney U test, values

Table 2. Clinical data and alteration of DNA repair in UV-irradiated lymphocytes of breast cancer patients before and during Iscador therapy.

Patients	Stage	Therapy after surgery	Family history*	(^{3}H)dThd incorporation in cpm/10^6 cells Before	Day 1	Day 2	Day 3	Days 7–9	Evaluated repair
1	IV	Chemo/radio	yes	<30‡	<30	<30	-	800	Increased
2	IV	Radio	no	300	330	450	-	760	Increased
3	III	Radio	yes	780	500	1880	-	-	Increased
4	IV	Radio	yes	480	580	740	-	1880	Increased
5	IV	No	no	1250	480	1030	-	2850	Increased
6	IV	Chemo/radio	no	780	800	670	-	170	Reduced
7	IV	Radio	yes	130	620	50	-	450	Increased
8	IV	No	no	<30	600	180	160	100	Unchanged
9	III	Radio	yes	200	110	80	280	580	Increased
10	II	No	no	42	310	360	480	-	Increased
11	IV	No	yes	220	360	360	640	800	Increased
12	IV	Chemo/radio	yes	<30	<30	180	240	-	Increased
13	IV	Radio	yes	170	340	380	240	280	Increased
14	II	No	yes	100	180	350	700	750	Increased

* Family history = first degree relatives with cancer.
† cpm/10^6 cells = after substraction of hydroxyurea values.
‡ < 30 = minimal measurable value after subtraction of hydroxyurea value.
§ Patient 3, (^{3}H) thymidine incorporation 5 weeks after start of therapy: 500 cpm/10^6 cells.
Patients 1, 2, 4, 6, 7, 9, 10 and 13 were postmenopausal; all others were premenopausal.

were significantly different only on days 7–9 ($P < 0.05$), the mean value being on average 2.7 times higher than on day 0.

Out of the 14 patients 12 had an increased repair on days 7–9 compared with day 0; 2 patients (nos 4 and 5) reached practically the normal range of the healthy controls [15] on day 7; they both had had an extra Iscador infusion a week before the investigation.

Blood samples could not be collected from 3/14 patients after 3rd day. The DNA repair could be measured after 5 weeks in only 1 patient; the repair values had returned to that before therapy.

Figure 1 shows the mean values for DNA repair for all patients at each time point during therapy and for the immunological parameters before and after intravenous treatment, taking the individual values of day 0 as 1 in both cases (values were calculated individually at each time point). The phagocytic activity of PMN leucocytes increased 2.2-fold ($P < 0.05$) 6 hours after infusion, returning to the baseline values at days 1 and 2. The numbers of large granular lymphocytes (LGL) and (NK) activity of PBM cells increased 2.5 and 1.9-fold, respectively at day 1. On day 2 LGL showed a slight decrease (not significant) compared with day 1, whereas NK activity returned to baseline. The ratio of T-helper/T-suppressor cells was significantly increased on day 1 ($P < 0.05$), with a slight decrease (not significant) on day 2 compared with day 1. These variations were due to significant increases in the numbers of T-helper cells ($P < 0.05$), combined with significant decreases of T-suppressor cells ($P < 0.05$). The results presented here for the immunological parameters were similar to those already published [10, 11, 18]. We measured the viability of the lymphocytes by trypan blue exclusion method in 4 patients before, and 1 and 3 days after intravenous application. There were no differences in the viability and values were in the same range as controls ($n = 6$)(range: 86–93%)

DISCUSSION

Many studies have indicated that unrepaired or misrepaired damage in DNA has a high carcinogenic potential. It is known that damaged DNA, which can act as an initiator, plays an important role in the two-stage carcinogenesis. Serum factors affect the cellular response and can lead to alterations in DNA repair [21]. It has been reported that lymphokines and cytokines are involved in the repair mechanism [22, 23]. Interferon reduces chromosomal aberrations [24] and stimulates repair processes [25, 26].

The aim of our study was to investigate whether immunomodulatory treatment leads to an alteration of DNA repair. DNA repair was very low before treatment, in most cases in a similar range as in xeroderma pigmentosum patients [27]. 7–9 days after the start of immunomodulatory treatment DNA repair was increased on average 2.7 times from this low level. This cannot be attributed to an increased viability of the cells due to Iscador treatment. The first increase on day 2 can be atributed to the intravenous treatment. The increase on days 7–9 might be due to the continuous subcutaneous therapy following intravenous application. Figure 1 shows that on day 1 the proportions of the subpopulations of lymphocytes (LGL, T-helper, T-suppressor) have been markedly changed compared with day 0. However DNA repair was unchanged. Therefore we suggest that the various types of lymphocytes have more or less the same repair capacities.

The method used for evaluation of DNA repair is the measurement of incorporation of (^{3}H)thymidine. It is important to be sure that any alteration in the incorporated values is a response to DNA damage and not to changes in DNA replicative synthesis, which could be a consequence of a mitogenic effect of the treatment. Our results provide no evidence for any mitogenic effect: the values of the spontaneous DNA synthesis without and with HU were in the same range before and after treatment.

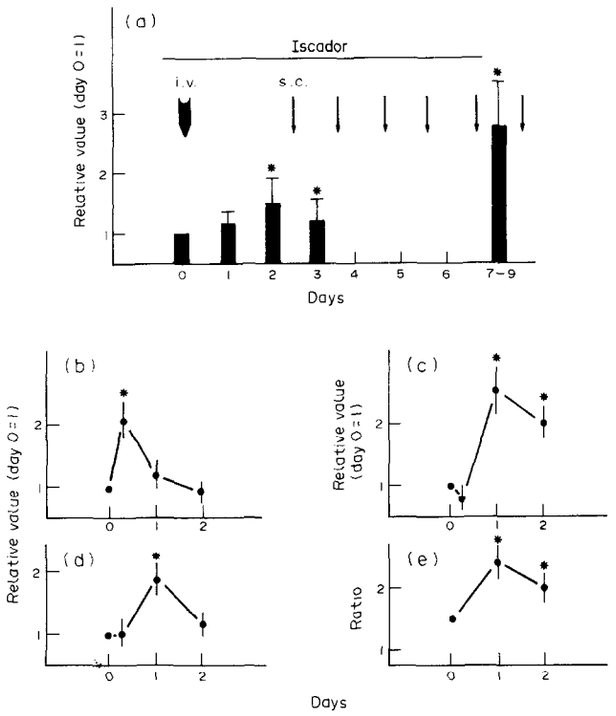

Fig. 1. Alterations of DNA repair and of immunological parameters in breast cancer patients treated with Iscador. Values at each time point were calculated individually relative to the corresponding values before treatment (day 0), and averaged (mean, S.E.), $n = 14$ except for day 3 ($n = 7$). (a) DNA repair in UV-irradiated lymphocytes, (b) phagocytic activity of polymorphonuclear leukocytes, (c) number of large granular lymphocytes, (d) natural killer activity of peripheral blood mononuclear cells and (e) ratio of T-helper and T-suppressor cells. i.v. = intravenous applications, s.c. = subcutaneous applications, $^*P < 0.05$ (Mann–Whitney U test).

However, as Iscador contains glycoprotein chains, we cannot exclude that it had a mitogenic effect on only a small cell population which could then express differentiated function(s). This might not be detectable in the large non-stimulated population. Preliminary results showed that a different batch of Iscador having no immunomodulatory effect after intravenous infusion did not lead to any improvement of DNA repair (unpublished data).

We suggest two possibilities for our findings. (1) Lymphokines or cytokines secreted by activated leukocytes stimulate the enzymes leading to improvement of repair. Recent results indicate that ML-I (mistletoe lectin) induces increased secretion of cytokines (interleukins 1 and 6, tumour necrosis factor) *in vitro* and *in vivo* (unpublished data). (2) There is an alteration in the susceptibility to exogenic agents, such as the UV irradiation used in this study, resulting in less damage; this would then lead to the improvement of repair. Gantt *et al.* [28] assumed that reduced repair in cancer patients is correlated with high damage. In a previous study [29] we found that DNA repair was negatively affected by chemotherapy or radiotherapy. We postulated that this treatment increased the susceptibility of the cells to exogenic agents resulting in high damage, thus leading to reduction of repair. Clinical experience has shown that immunomodulators such as Iscador or OK-432 have beneficial effects when combined with chemotherapy or radiotherapy [30, 31]. Present results indicate that the immunomodulators could counteract the negative effects of chemotherapy or radiotherapy on DNA repair. Therefore we plan an investigation to determine whether immunomodulators exert their beneficial effects via repair enzymes or via influence on the susceptibility of cells to damage. It would be of interest to find out whether this protective effect is direct and specific for active components in Iscador or if it is indirect e.g. by activation of lymphokines or cytokines.

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Interleukin-2 Therapy for Refractory and Relapsing Lymphomas

Jean-Marc Tourani, Vincent Levy, Josette Briere, Rafaël Levy, Chris Franks and Jean-Marie Andrieu

Recombinant interleukin-2 (rIL-2) has been reported to be active in metastatic renal cell carcinoma and malignant melanoma. The purpose of this trial was to determine the efficacy and toxicity of rIL-2 administered in continuous infusion in patients with Hodgkin's disease (HD) and non-Hodgkin lymphoma (NHL). 21 patients with HD (4 patients), diffuse large-cell NHL (7) or low-grade NHL (10) in failure or relapse after multiple-conventional treatments were included in this trial. rIL-2 therapy consisted of an induction period of two cycles separated by 3 weeks of rest, and, in the absence of progressive disease or undue toxicity, a maintenance period of 4 monthly cycles. Each induction cycle comprised the continuous infusion of rIL-2: 18 × 10^6 IU/m^2 per day on days 1–5 and days 12–16. Each maintenance cycle comprised the continuous infusion of rIL-2: 18 × 10^6 IU/m^2 per day on days 1–5. Among the 21 treated patients, 5 (all of those with low-grade NHL) responded to the induction phase (1 complete response, 4 partial responses) and 2 patients had a mixed response. Conversely, no response was observed in patients with HD or large-cell NHL. The median duration of response was 4 months. rIL-2 administered as a continuous infusion was well tolerated and most patients received the full dosage, and management did not require intensive care. During the induction period, 2 patients experienced grade III cardiovascular or renal toxicity. During the maintenance period, rIL-2 had to be interrupted in 1 patient because of a myocardial infarction. This trial confirms the inefficacy of rIL-2 for the treatment of large-cell NHL and HD. Conversely, in low-grade NHL, rIL-2 activity needs to be explored by further studies. rIL-2 may have a place in the early phase of the disease, when the immune system is not compromised, as an adjuvant treatment in residual disease in order to improve the duration of response.
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INTRODUCTION

RECOMBINANT INTERLEUKIN-2 (rIL-2) has been first used in clinical trials in 1984 [1]. Since then, rIL-2, whether in bolus injections [2, 3] or in continuous infusions [4, 5] whether or not associated with lymphokine-activated killer cell (LAK) infusions, has been used for the treatment of solid tumours and particularly in metastatic renal cell carcinoma and malignant melanoma. With or without LAK infusions, rIL-2 can achieve a response rate of about 25% in metastatic renal cell carcinoma and 20% in malignant melanoma. Malignant lymphomas have sometimes been treated with rIL-2. However, histology is often not documented and the response rate cannot, at the present time, be determined. The purpose of this study is to determine the efficacy and toxicity of rIL-2 (18 × 10^6 IU/m^2 per day continuous infusion) in 21 patients suffering from refractory or relapsing lymphoma.

PATIENTS AND METHODS

Eligibility criteria and initial evaluation

21 patients were included in the study; 4 with Hodgkin's disease (HD) and 7 with large cell non-Hodgkin lymphoma (NHL) who failed to respond to second, third or fourth line therapy (refractory disease) or who experienced a second or third relapse. 10 patients with low-grade NHL were also treated. These patients were also relapsing or in failure after a second, third or fourth line therapy. All were free of treatment for at