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Studies on Cellular Immunity in Patients with Renal Carcinoma: Radiation-Induced Inhibition of Leukocyte Migration*

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Abstract—*Thirty-two patients with hypernephroma (renal carcinoma), untreated or preoperatively exposed to local radiotherapy, were examined for tumor-directed cellular hypersensitivity by means of the indirect leukocyte migration test (LMT).*

(a) When soluble tumor extracts from preoperatively radiated hypernephromas were tested with autologous lymphocytes, 17 of 19 cancer patients gave a positive response; 10 of 11 were positive with allogeneic lymphocytes from hypernephroma patients. In no instance could migration inhibition be induced with allogeneic lymphocytes from 14 normal donors. Similarly, in 9 of 10 patients there was no significant inhibition with allogeneic lymphocytes from patients with histologically different types of malignant tumors other than hypernephroma.

(b) Tissue extracts from untreated hypernephromas failed to react in 12 of 13 patients when tested with autologous lymphocytes. LMTs, however, became positive in 6 of 7 patients from this group by in vitro*-radiation of tumor samples (^{60}Co *or electrons) before preparation of tissue extracts. This radiation-induced effect was dose-related and specific, since radiation of normal kidney tissue did not significantly influence the migratory activity of leukocytes.*

Our data indicating that an in vivo *as well as* in vitro*-radiation of the hypernephroma will be suitable for the induction and the demonstration of a tumor-directed cellular immune response, may be considered as an additional perspective in the integration of radiotherapy in the management of this neoplasia.*

INTRODUCTION

THE LEUKOCYTE migration test (LMT) has been used by many investigators as an *in vivo* test of cellular immunity to a variety of soluble antigens, as well as to extracts of tumors (Review [1; 2–12]). Conflicting results and technical difficulties, however, also were described in several reports [13, 14]. The presently available methods for the *in vitro* demonstration of the migration inhibitory factor (MIF) vary not only in cell separation procedures, the number of cells used for the assay, the culture conditions and the culture period, but also in the preparation of soluble tumour extracts. The need to achieve high concentrations of active tumor antigen to cause immunologically specific inhibition of migration and yet keep the concentration of toxic substances at low levels is a problem of many tissue preparations.

The indirect leukocyte migration assay as described by Søborg and Bendixen [15, 16] has been more successful in our experience because an unspecific inhibition will occur in a significantly lower percentage when compared with the direct technique. Preliminary studies indicated a specific migration inhibition in 45–50% with soluble tumor extracts from patients with malignant lymphomas or carcinomas of different types. In contrast, positive responses were found with tumor extracts from hypernephromas (renal carcinoma) in 85–90%. This effect has been explained by the nature of this tumor or, alternatively, by the preoperative local radiation therapy, which had been administered to all patients described in our previous report [12].

The present study was undertaken (a) to extend and confirm our initial findings and (b) to determine by means of the LMT the

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effect of an *in vitro*-radiation of hypernephroma tissue obtained from untreated patients.

MATERIALS AND METHODS

(1) *Patients and preparation of tissue extracts*

Thirty-two patients with histologically proven diagnosis of hypernephroma, clinically free of tumor dissemination and suggestive for a total tumor removal are included in the study. Tumor fragments were obtained during surgical resection procedure and tissue extracts prepared as described earlier in detail [12]. Briefly, tissue specimens were cut into small pieces and disintegrated with ultrasound in 0.14 M NaCl (pH 7). Homogenates were kept at 4°C for 18 hr, then centrifuged in the cold (1300 ***g***; 15 min), collected and protein concentrations determined by the Folin-method. Tissue extracts were prepared from the following groups of patients:

Group I: 19 patients who had received preoperative local radiation therapy (42 MeV photons; 2100 rad given in 3 treatments in 3 days).

Group II: 13 patients without any preoperative treatment. Tumor samples from 7 patients of this group were divided into 11 equal aliquots, each of about $1 \times 1 \times 1$ cm size. Specimens were frozen and, except one (unradiated control) radiated before preparation of tissue extracts with different doses either of ^{60}Co or electrons (e^-).

Group III: According to the same protocol as in group II, normal kidney tissue was prepared as control samples.

(2) *Radiation techniques*

Each sample of tumor was frozen in the middle of a water (aqua bidest.) filled Petri dish (90 mm diameter). The dish fitted exactly in a suitable central recess in a Perspex phantom (s.g. 1.18). As maximum dose adapter a Perspex sheet was placed on the phantom (for ^{60}Co 0.5 cm; for 35 MeV electrons 3.0 cm). The phantom was positioned so that the tissue specimen was always in the central beam, and radiated with 1000, 2000, 2500, 3000 or 4000 rad from each of both devices (^{60}Co unit SSD 50 cm field size 12×12 cm; Betatron SSD 100 cm treatment cone 12 cm diameter).

(3) *The leukocyte migration assay*

The indirect leukocyte migration technique was used as described in our previous report [12]. In essence, lymphocytes were collected from the peripheral blood over a Ficoll–Isopaque gradient (Ficoll, Pharmacia; Ronpacon, Cilag), washed two times in TC 199 medium (Difco) and the cell concentration adjusted to 1.5×10^6/ml. For elaboration of MIF, $4.5–5.0 \times 10^6$ lymphocytes were suspended in 3 ml TC 199 medium and incubated with 0.05 ml tissue extract for 90 min at 37°C. Controls were set up without tumor extract. After incubation, cell-free supernatants were collected and 0.6 ml samples transferred into Mackaness-type culture chambers each containing 1.0 ml TC 199 medium and a capillary tube (1.5 mm diameter) packed with $5–6 \times 10^6$ leukocytes from normal blood donors. Chambers were incubated for 24 hr at 37°C and thereafter the areas of migration projected with a microscope, drawn onto paper and measured by planimetry [17].

Each test was performed in triplicate and the degree of leukocyte migration expressed as the so-called "Migration Index" (MI): Average migration area in chambers incubated with supernatant from lymphocytes cultured in the presence of tumor extract divided by the average area in chambers incubated with supernatant from lymphocytes cultured in the absence of tumor extract. The range of "normal MIs" was calculated from two control groups:

(a) Lymphocytes from healthy donors were tested with extracts of several malignant tumors (including 6 hypernephromas shown in Fig. 1B) at concentrations from 50 to 800 μg of protein/ml. Leukocyte migration was still unaffected up to 400–500 μg. With two exceptions (1.35; 1.42), MIs of these controls were between 0.72 and 1.2. At higher concentrations, inhibition regularly became apparent for all tumor extracts tested.

(b) Similarly, the cut-off limit of normal leukocyte migration found for a group of extracts from several benign tumors tested with normal lymphocytes was between 450 and 500 μg of protein/ml. MIs of these controls were between 0.73 and 1.3.

As the result from both control groups, values of MI <0.72 for tests set up with 400–500 μg of protein/ml were considered as "positive responses".

RESULTS

LMTs with hypernephroma-extracts from untreated and preoperatively radiated patients

Autologous tests. When tumor extracts from

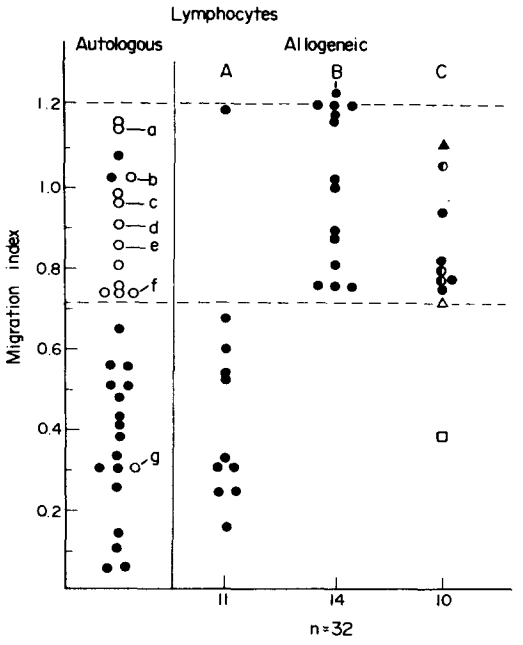

Fig. 1. Leukocyte migration with soluble extracts of hypernephroma tissue from untreated (○) and preoperatively radiated patients (●). Values of MI obtained with autologous lymphocytes (left column) and allogeneic lymphocytes from different donors: A. patients with hypernephroma; B. healthy probands; and C. patients with different types of malignant tumors (●: bronchogenic carcinoma, ◐: gastric carcinoma, △: colon carcinoma, ▲: Hodgkin's disease, □: reticulum cell sarcoma). Each value represents the mean migration index of triplicate cultures.

13 untreated hypernephroma patients were tested with analogous lymphocytes, only 1 (patient g) gave a positive response, while the others failed to react. However, 17 of 19 tumor extracts from preoperatively radiated patients tested with autologous lymphocytes were reactive (Fig. 1): Values for MI were between 0.05 and 0.65. Indices for the remaining two patients were within the "normal range" (MI of 1.02, respectively 1.07).

Allogenic tests. To demonstrate the specificity of positive responses obtained from the radiated group of patients, parallel studies were performed with hypernephroma extracts and allogenic lymphocytes from different donors:

A. With allogenic lymphocytes from 11 hypernephroma patients, a significant migration inhibition was found in all cases tested except one (i.e., one of the two radiated patients who had failed to react with autologous lymphocytes) (Fig. 1A).

B. In no instance could inhibition of leukocyte migration be induced with supernatants from cultures containing hypernephroma extracts and lymphocytes from 14 different normal donors (Fig. 1B).

C. Similarly, leukocyte migration was unaffected in tests with supernatants from cultures containing hypernephroma extracts and allogeneic lymphocytes from patients with histologically different types of malignant tumors (Fig. 1C). Of 10 different lymphocyte donors only one patient's lymphocytes (reticulum cell sarcoma) produced a positive response (MI of 0.35).

Changes in leukocyte migration produced by in vitro-*radiation of tumor samples from untreated patients*

Soluble tissue extracts from untreated hypernephromas (patients a–g; Fig. 1) as well as from normal kidneys were prepared after *in vitro*-exposure to different doses either of ^{60}Co- or e^--radiation and tested with autologous lymphocytes. MIs obtained with unradiated tissue samples are included for each individual and values for these controls are given as 0%. Indices for tests with extracts from radiated tissue samples are expressed as percentages of change in MIs as compared with the unradiated controls.

(a) Results presented in Fig. 2A show that with increasing radiation doses of ^{60}Co there was no significant effect upon leukocyte migration in tests set up with tissue extracts from normal kidneys. *In vitro*-radiation of tumor samples, however, caused a dose-dependent inhibition of leukocyte migration. Of 7 tumors tested 6 became positive after radiation with 1500–2500 rad. Lower as well as higher doses were less effective or without any effect. The lowest change in leukocyte migration was found for that patient who had already produced a positive response without radiotherapy (patient g; compare Fig. 1).

(b) The effects of e^--radiation are demonstrated in Fig. 2B. Results are similar to those described in the preceding experiment. No significant influence upon leukocyte migration was found in tests with extracts from normal kidney tissue, but the LMT became highly positive for 4 hypernephroma patients. Tumor specimens from patients d and b gave a poor response, and, again, values for patient g were only slightly different from the unradiated control. Similar to ^{60}Co-treatment, the inhibition of leukocyte migration induced by e^--radiation was dose-dependent. One thousand to two thousand five hundred radians were most effective; higher doses not only did not reduce, they even stimulated (patients a and d) the migratory activity.

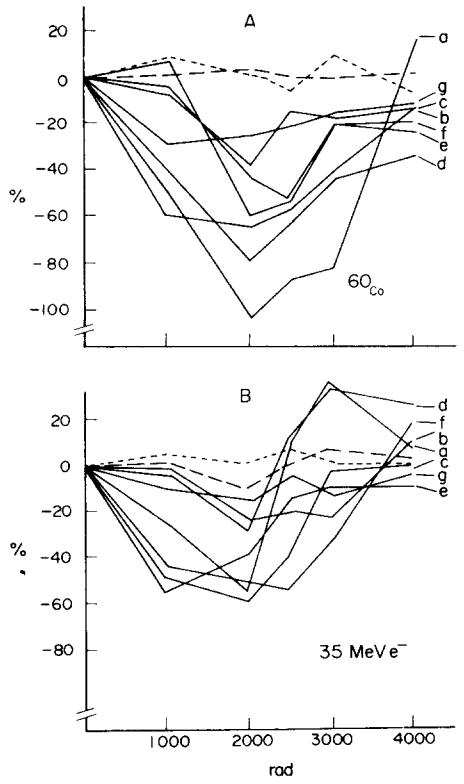

Fig. 2. The effects of in vitro*-radiation of tissue samples on leukocyte migration. Before preparation of soluble extracts, tumor specimens from untreated patients with hypernephroma (patients a–g; Fig. 1) and samples from normal kidneys (– – – –; — — — —) were frozen and exposed to different radiation doses either of* ^{60}Co *or* 35 MeV *electrons* (e^-). *Extracts were tested with autologous lymphocytes in triplicate cultures. Values of MIs obtained with one frozen unradiated control sample are included for each individual and given as* 0%. *Indices of tests with extracts from radiated tissues are expressed as percentages of change in mean MIs as compared with the unradiated control.*

DISCUSSION

Investigations concerning tumor-directed cellular hypersensitivity in patients with hypernephroma include the lymphocyte transformation test [18, 19], the colony inhibition technique [20], the target cell destruction assay [21–23], and the LMT [10–12, 24]. Our present results are comparable to the observations described by Kjaer [11]. Using the direct leukocyte migration technique, he found a positive response with soluble hypernephroma extracts for the majority of patients tested. In about 50% an inhibition of leukocyte migration was also obtained for foetal kidneys. Extracts from adult normal kidneys, however, did not react.

Most studies of tumor-directed cell mediated immunity in man by the LMT have employed the supernatants of homogenized fresh tumor tissue as "antigen". The need to achieve high concentrations of active tumor antigen to cause an immunologically specific response and yet keep the concentration of non-specifically, toxic substances at low levels presents one of the main problems of such tissue preparations. For soluble extracts from hypernephromas only low differences between concentrations with non-specific and specific activity have been demonstrated by Kjaer [24]. Using the direct migration test, the optimal protein content found was about 400 μg/ml culture fluid. In our present experiments, carried out with the indirect leukocyte migration assay, best results were obtained with comparable concentrations of hypernephroma extracts (400–500 μg protein/ml culture fluid). With one exception, positive responses, however, could be only induced with tissue extracts from radiated hypernephromas. *In vivo*-radiation (i.e., preoperative local radiotherapy) as well as *in vitro*-radiation of hypernephroma tissue did demonstrably facilitate the establishment of a migration inhibition. The type of radiation used seems to be less important for the induction of a positive LMT, since preoperative radiotherapy was performed with photons, while *in vitro*-radiation either with ^{60}Co or e^-. The degree of migration inhibition, however, clearly depended upon the given radiation dose, at least for treatment with ^{60}Co and e^- (for preoperative radiotherapy only one tumor dose was administered to all our patients). The specificity of the effect was shown (a) by the occurrence of a normal leukocyte migration in tests set up with hypernephroma extracts from radiated patients and lymphocytes from normal donors or patients with other types of malignant tumors; and (b) by the demonstration that *in vitro*-radiation of normal kidney tissue did not significantly influence the leukocyte migration. A radiation-induced non-specific toxicity of the tissue extracts and responses to histocompatibility antigens present in the extracts, therefore, can be ruled out.

Though our study described here does not attempt to define biochemical effects, there are several explanations of the radiation-induced change in leukocyte migration. The specific inhibition of leukocyte migration as a result of radiation treatment of tumor tissue indicates an increase in the tumor extracts specific reactivity. Possibly, radiation procedure will be suitable for the preparation of tumor extracts with a higher concentration of tumor-associated antigens. Alternatively, the demonstrated migration inhibition is probably due to reactions against fetal kidney antigens,

since it seems possible that the hypernephroma is more readily damaged by radiation than normal kidney tissue with release of fetal antigens to which sensitized lymphocytes may respond better. Such a cross-reactivity between the hypernephroma and the corresponding fetal tissue has been reported by Kjaer [11]. He demonstrated sensitization to an extract of fetal kidney in lymphocytes from patients with hypernephroma. Finally, it could be postulated that radiation will change the heterogenous, unknown composition of the tumor extract, in that these might have a lower content of substances masking tumor-associated antigens which may prevent sensitized lymphocytes from recognizing and reacting with these antigens. All these interpretations are not excluded by the unexpected observation of a complete abolishment of the migration inhibition with increasing radiation doses of ^{60}Co or e^-. Theoretically, this phenomenon could be due to a stimulating factor which may be released and overcome the inhibitory activity. Such a migration stimulating factor has been recently isolated and characterized by different authors [25, 26]. However, we would rather explain this effect by a release of toxic substances due to necrosis induced by high-dose radiation. Though the mechanism(s) of radiation-induced specific inhibition and stimulation of leukocyte migration remains to be clarified and further studies are necessary to investigate the factors supposed to be active, our experiments may be of practical interest for the *in vitro* detection of tumor-directed cellular immunity by means of the LMT.

The exact evidence of *in vitro* findings to the effector mechanisms operating in tumor immunity in man is not yet clearly defined, but there is some evidence that MIF (and other mediators) may be involved at the reaction site of delayed hypersensitivity *in vivo* [27–30]. If it is assumed, therefore, that the *in vitro* "MIF-model" will have its *in vivo* equivalent, then the inhibitory effect of radiation on leukocyte migration may also be of clinical interest.

There are conflicting reports in the literature about the value of preoperative and postoperative radiotherapy as an adjunct to radical nephrectomy in the treatment of hypernephroma (Review 31). Our results may be considered as an additional perspective in the integration of radiotherapy in the management of this neoplasia.

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