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ORIGINAL ARTICLE

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Treatment of human lung carcinoma xenografts with a combination of ^{131}I-labelled monoclonal antibody Po66 and doxorubicin

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Abstract Po66, a mouse monoclonal antibody, is directed against an intracytoplasmic antigen present in human lung squamous cell carcinoma cells. In previous work it was found that the co-administration of ^{125}I-radiolabelled Po66 and doxorubicin strongly enhanced the uptake of radioactivity by the tumour. The present-work was designed to evaluate, in a tumour-bearing mouse model of lung carcinoma, the ability of ^{131}I-labelled Po66 to retard tumour growth when injected alone, or in combination with doxorubicin (8 mg kg^{-1} at 1-week intervals). A single dose of 550 μCi ^{131}I-Po66 alone had no effect on tumour growth, whereas three fractionated doses of 250 μCi ^{131}I-Po66 decreased it over two doubling times from 14.5 ± 1.5 days for untreated control mice to 24.8 ± 2.7 days. Mice treated with doxorubicin alone had a double tumour doubling time of 22.6 ± 4.9 days, compared to 35.2 ± 2.9 days (1.55-fold increase) in mice treated with doxorubicin and a single dose of 550 μ Ci ^{131}I-Po66. Doxorubicin combined with three fractionated doses of 250 μCi ^{131}I-Po66 provoked a twofold decrease in tumour growth compared to mice treated with doxorubicin alone. The administration of fractionated doses of ^{131}I-Po66 simultaneously with doxorubicin resulted in a highly delayed mortality, which was not observed when ^{131}I-Po66 was administered after doxorubicin. Thus, in a non-small-cell lung tumour model, a ^{131}I-radiolabelled monoclonal antibody, directed against an intracellular antigen, significantly potentiated the effect of chemotherapy. Such a therapeutic approach could be used as an adjuvant therapy and improve the effect of chemotherapy on distant small metastases.

Key words Monoclonal antibodies · Lung cancer · Animal model · Radioimmunotherapy

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Introduction

Lung carcinoma is one of the most common and lethal forms of cancer. The poor prognosis is explained by the occurrence of tumour spreading and metastasis, even after complete surgical resection [7, 13], and the inefficacy of chemotherapy at the present time.

Monoclonal antibodies (mAb) have been developed in an approach to a more specific and effective therapy intended to target metastasis. Treatment with radiolabelled antibodies has been improved recently by the use of new radionuclides and by biochemical modifications of antibodies [8].

mAb directed against intracellular antigen have interesting properties in terms of tumour imaging and therapy. Epstein et al. [6] hypothesized that monoclonal antibodies directed against an abundant intracellular antigen showed preferential localization in malignant tumours because of the presence of abnormally permeable degenerating cells not found in normal tissue. The authors also showed that the antibody TNT, directed against histones, had a therapeutic effect by producing centrifugal killings of tumour cells around the deposit of the ^{131}I-radiolabelled antibody [2].

mAb Po66 is directed against a still unknown intracellular antigen present in human lung squamous cell carcinoma cells [4, 12]. The specific uptake by tumours of radiolabelled Po66, injected i.v., results first from the predominant expression of the antigen in the tumour, as compared to normal tissue [4]. Second, the intracytoplasmic localization of the antigen offers an additional mechanism of specific uptake because necrotic or degenerating cells constitute a pattern of tumoral tissues that can be detected even at an early stage of tumour development [3]. Thus, if the antigen was present in normal tissues, it would remain inaccessible to the antibody because cell membrane integrity is generally conserved, while the antibody could bind to the antigen present and accessible in degenerating tumour tissues. The proportion of degenerating cells in tumours is, however, unpredictable and a low incidence

of necrotic cells could result in weak or heterogeneous binding of the antibody.

Chemotherapy has long been known to induce tumour necrosis, and it has been shown in a mouse model that the co-administration of doxorubicin and Po66 increased the tumour uptake of the antibody and improved its intratumoral distribution [5]. The therapeutic efficacy of a radioiodinated mAb directed against a non-ubiquitous intracytoplasmic tumour antigen should, therefore, be increased by combined administration with chemotherapy. The present work was designed to verify this hypothesis, and the therapeutic effect of the administration of ^{131}I-radiolabelled Po66, alone or in co-administration with doxorubicin, was analysed in a tumour-bearing mouse model of lung carcinoma.

Materials and methods

Production and radioiodination of monoclonal antibodies

mAb Po66, a mouse IgG1, was prepared as described before [4]. Briefly, Balb/c mice were immunized with enzymatically dissociated cells from a patient's lung squamous cell carcinoma. Mouse immune cells were fused with SP2/0 plasmocytoma and mAb Po66 was selected from the hybrids obtained. Po66 consistently reacted with squamous cell carcinomas and half of the adenocarcinomas tested, but not with small-cell lung carcinoma. mAb Po66 bound to a 47-kDa cytoplasmic glycoprotein [12]. It did not recognize normal tissues, except for distal renal tubules and gastric and bronchial serous glands. The Po66 batch used in the present work was purified from ascites obtained from Balb/c mice grafted i.p. with hybridoma. The ascites fluid was precipitated in 40% saturated ammonium sulphate, dialysed against 10 mM phosphate buffer, pH 8, and eluted from a DEAE ion-exchange column with a 10–150 mM, pH 8, phosphate buffer gradient. A mouse monoclonal immunoglobulin of the same isotype (IgG1), Py, without known specificity, was taken as the control and processed like Po66.

Samples of Po66 and Py were radioiodinated with iodine-131 by the iodogen method and separated from free iodine by elution through a Dowex anion-exchange column equilibrated with phosphate-buffered saline (PBS) containing 0.3% human serum albumin, as described. The specific activity of the radiolabelled antibodies varied between 4 mCi and 8 mCi ^{131}I mg protein^{-1}. The protein-bound radioactive fraction averaged 95%–98% as determined by trichloroascetic acid precipitation. The radiolabelled antibodies were stored at 4 °C and used within 3 h after labelling. They were diluted with saline to an appropriate volume before injection. The ^{131}I-radiolabelled Po66 (^{131}I-Po66) showed undiminished immunoreactivity in a radioimmunoassay.

Cell line

SK-MES-1, a human squamous cell carcinoma line (American Type Culture Collection HTB 58, 1990), was grown in RPMI-1640 medium (AES, Combourg, France) supplemented with 10% fetal calf serum (Anval, Betton, France), 2 mM glutamine and 80 μg/ml gentamycin, at 37 °C in a fully humidified atmosphere of 95% air: 5% CO_2. Cells at confluence were trypsinized, washed twice in PBS, and resuspended in RPMI-1640 medium prior to inoculation into mice.

Tumour model

Athymic mice (*nu*/*nu*) (8–9 weeks old; weight 28–30 g were purchased from Janvier (53590 St Berthevin, France). They were inoculated s.c. (0.1 ml) with 5×10^6 SK-MES-1 in the right flank. Tumours grew to 0.6–0.9 cm in diameter in 3 weeks. The tumour volume was estimated by the formula: (short dimension)^2 ×(long dimension) ×1/2. Therapeutic trials were started on well-established tumours, when their volume reached approximately 0.2–0.3 cm^3. Two days before and during the experiments, the animals had drinking water containing potassium iodide (0.2%) ad libitum.

Chemotherapy

Doxorubicin (Adriblastin, Farmitalia Carlo Erba, 92500 Rueil Malmaison, France) was chosen because it is active on the growth of non-small-cell lung carcinoma xenografts [1]. Doxorubicin was given i.v. in 0.9% saline at the dose of 8 mg kg^{-1} in two injections separated by 7 days (LD10).

Experimental design of therapeutic study

Two sets of experiments were performed. In each experiment, animals were divided into several groups: untreated controls (injected with 0.9% saline), and mice submitted to various treatments: doxorubicin alone, radiolabelled antibodies alone or doxorubicin in combination with radiolabelled antibodies. Each treatment group consisted of five to seven animals with tumours of comparable size on day 0 of each experiment. Radiolabelled antibodies were injected intravenously with a single dose (400 μCi or 550 μCi) or three fractionated (200 μCi or 250 μCi) doses at 1 week intervals. The amount of antibody injected varied but, according to the specific activity of ^{131}I, it was always under 100 μg.

Tumour growth of each mouse was expressed as the tumour volume at each assay time divided by the tumour volume on day 0. Therapeutic efficacy was determined from the slope of the growth curve and, because radiolabelled Po66 delayed tumour growth, from the time needed to obtain a median tumour volume of four times the volume at the start of the treatment on day 0 (two doubling times).

Statistical analysis

Statistical analysis was performed using Student's unpaired *t*-test.

Results

Toxicity

By monitoring the weight of the mice it was determined that a single dose of 550 μCi, or a cumulative dose of three weekly fractionated doses of 250 μCi, could be administered in combination with doxorubicin (8 mg kg^{-1} twice), ^{131}I-Po66 being injected 24 h after the last injection of doxorubicin. With these regimens, no mortality was observed during the 6 weeks of observation, but the maximum weight loss was about 18% and this was recovered within 2–4 weeks after treatment (Fig. 1). Only treatments with doxorubicin provoked a substantial weight diminution.

Therapy studies

Effect of a single dose (400 μCi or 550 μCi) of ^{131}I-Po66 with or without co-administration of doxorubicin

As shown in Fig. 2, the i.v. administration of 550 μCi ^{131}I-Po66 had no effect on tumour growth. However, the co-

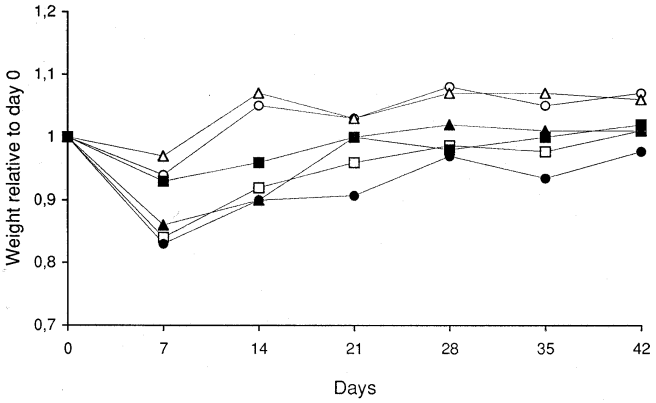

Fig. 1 Effect of the co-administration of doxorubicin and ^{131}I-Po66 on mouse weight. Doxorubicin was administered on days 0 and 7. ^{131}I-Po66 was administered either alone on day 0 for single doses and on days 0, 7 and 14 for fractionated doses, or combined with doxorubicin on day 8 for single doses and on days 8, 15 and 22 for fractionated doses. ○ Controll; □ doxorubicin alone, ■ 550 μCi ^{131}I-Po66; △ 250 μCi×3 ^{131}I-Po66; ▲ doxorubicin + 550 μCi ^{131}I-Po66, ● doxorubicin + 250 μCi×3 ^{131}I-Po66. $n = 7$ mice group. Error bars are not presented for reasons of clarity

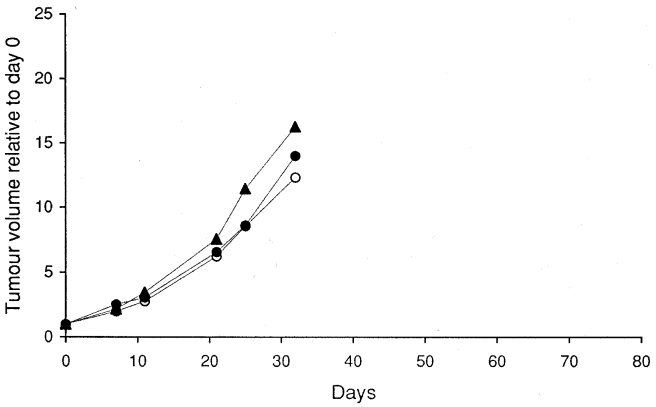

Fig. 2 Effect of a single dose of 550 μCi Po66 or of the unrelated antibody Py on SK-MES-1 tumour growth. ^{131}I-antibodies were i.v. injected on day 0. Results are given in mean tumour volume relative to day 0. ○ Control, $n = 5$; ● ^{131}I-Po66, $n = 7$; ▲ ^{131}I-Py, $n = 7$. Error bars are not presented for reasons of clarity

administration of doxorubicin 7 days and 1 day before injection of ^{131}I-Po66 (Fig. 3) significantly delayed tumour growth over two doubling times (35.2 ± 2.9 days), compared to untreated control mice (14.5 ± 1.5 days), doxorubicin-treated mice (22.6 ± 4.9 days; $P < 0.05$), and ^{131}I-Py + doxorubicin-treated mice. This tumour growth delay induced by the combination of ^{131}I-Po66 and doxorubicin was proportional to the amount of radiolabelled antibody administered (mean of two doubling times of 28.5 ± 3.2 days for 400 μCi and 35.2 ± 2.9 days for 550 μCi).

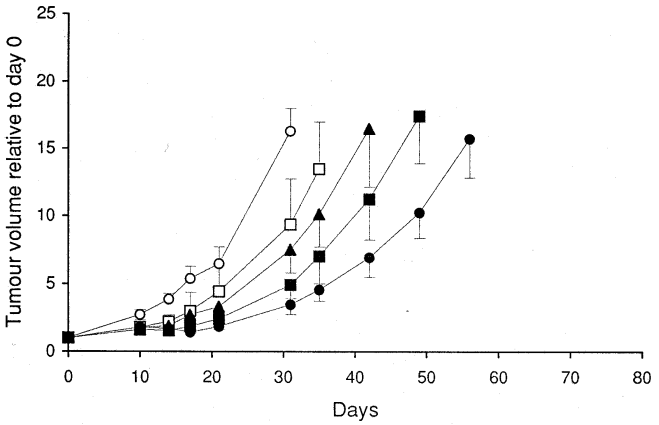

Fig. 3 Effect on SK-MES-1 tumour growth of a single dose of ^{131}I-Po66 or of the unrelated antibody Py, in combination with doxorubicin. Doxorubicin was administered i.v. on days 0 and 7. ^{131}I-antibodies were i.v. injected on day 8. Results are given in mean tumour volume relative to day 0. ○ Control, $n = 5$; □ doxorubicin alone, $n = 5$; ■ doxorubicin +400 μCi ^{131}I-Po66, $n = 7$; ● doxorubicin +550 μCi ^{131}I-Po66, $n = 7$; d ▲ doxorubicin +550 μCi of ^{131}I-Py, $n = 7$

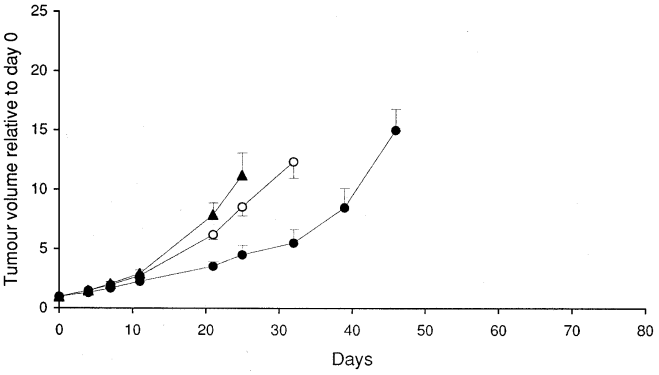

Fig. 4 Effect of fractionated doses (250 μCi×3) of ^{131}I-Po66 or of the unrelated antibody Py on SK-MES-1 tumour growth. ^{131}I-antibodies were i.v. administered on days 0, 7 and 14. Results ar given as mean tumour volume relative to day 0. ○ Controll, $n = 5$; ● ^{131}I-Po66, $n = 7$; ▲ ^{131}I-Py, $n = 7$

Effect of dose fractionation (200×3 μCi or 250×3 μCi) of ^{131}I-Po66 on tumour growth with or without co-administration of doxorubicin

In the same experiment, three fractionated doses of 250 μCi ^{131}I-Po66, administered to tumour-bearing, mice significantly delayed tumour growth (Fig. 4). Over one tumour doubling time there was no statistical difference between untreated controls (5.9 ± 1.4 days) and ^{131}I-fractionated Po66-treated mice (9 ± 2.32 days), whereas over two tumour doubling times the growth delay induced by fractionated doses of ^{131}I-Po66 was statistically different (14.5 ± 1.56 and 27.7 ± 3.9 days for untreated and ^{131}I-Po66 treated mice, respectively; $P < 0.02$). Lower doses of ^{131}I-Po66 (200×3 μCi) had no effect on tumour growth (data not shown). The ^{131}I-radiolabelled unrelated immunoglobulin Py also had no effect on tumour growth (Fig. 4).

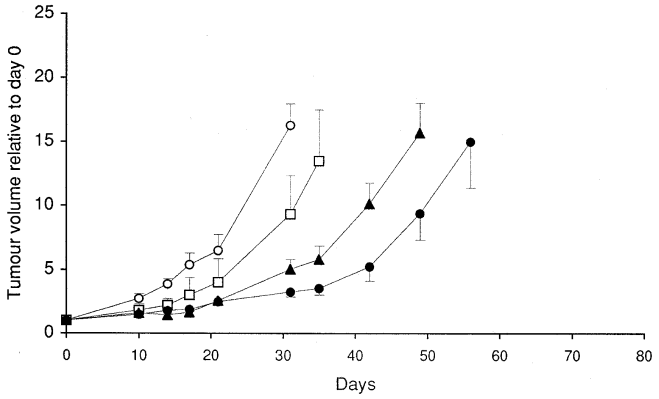

Fig. 5 Effect of the combination of doxorubicin and fractionated doses (250 μCi×3) of ^{131}I-Po66 or ^{131}I-Py on SK-MF-S-1 tumour growth. Doxorubicin was administered on days 0 and 7. ^{131}I-antibodies were i.v. injected 24 h after the last injection of doxorubicin on days 8, 15 and 22. Results are given as mean tumour size. ○ Control; □ doxorubicin alone, $n = 5$; ● doxorubicin +250×3 μCi ^{131}I-Po66, $n = 7$; ▲ doxorubicin +250×3 μCi ^{131}I-Py, $n = 7$

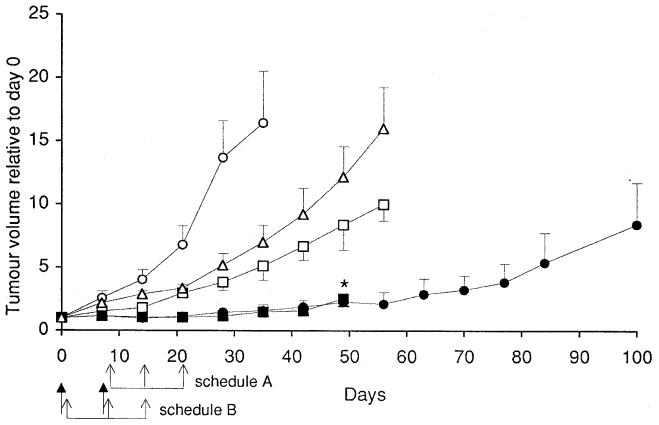

Fig. 6 Comparison of two schedules of administration of ^{131}I-Po66. Three weekly fractionated doses of 250 μCi ^{131}I-Po66 were started 24 h after the last administration of doxorubicin (schedule A, ●; $n = 7$), or 24 h after the first injection of doxorubicin (schedule B, ■; $n = 7$). ▲ Doxorubicin administration, △ ^{131}I-Po66. Tumour growth delay was compared to untreated controls (○) $n=5$, doxorubicin-treated mice (□) $n = 7$, and mice treated with 250 μCi×3 ^{131}I-Po66 alone (△) $n = 7$. ★ The growth curve was stopped at 7 weeks in schedule B because of mortality during the following 3 weeks

Doxorubicin combined with three fractionated doses of 250 μCi ^{131}I-Po66, administered the day after the last injection of doxorubicin (Fig. 5), decreased tumour growth 1.76-fold over two doubling times (39.8±4.4 days) when compared to doxorubicin-treated mice (22.6±4.9 days; $P < 0.05$). In this particular schedule, dose fractionation of ^{131}I-labelled unrelated immunoglobulin Py combined with doxorubicin resulted in a minor delay in tumour growth. In order to investigate the role of dose fractionation compared to that of a single dose, the effects of doxorubicin with a single dose of 550 μCi or three fractionated doses of 200 μCi ^{131}I-Po66 (a total of 600 μCi) were compared. Tumour growth did not differ (35.2±6 days for a single dose and 35.6±2.2 days for fractionated doses).

A new experiment was designed to explore the effect of interspersed injections of ^{131}I-Po66 and doxorubicin (Fig. 6). In this experiment, although the tumour growth of untreated mice was the same as in the first experiment (one and two doubling times of 6.6±2.5 and 14±3 days respectively), doxorubicin alone unexpectedly induced a greater tumour growth delay (30.6±3.4 days over two doubling times) than in the first experiment. Three ^{131}I-Po66 weekly injections were started either 24 h after the last injection of doxorubicin (schedule A), or 24 h after the first injection of doxorubicin (schedule B). The tumour growth curve was identical with the two protocols. Over two doubling times, the tumour growth decreased 2.4-fold when ^{131}I-Po66 was administered after the doxorubicin (schedule A; 77±13.9 days), compared to the results of doxorubicin alone. However, in the group treated with interspersed administration of doxorubicin and ^{131}I-Po66 (schedule B), a delayed toxicity was observed, and between 7 and 10 weeks after the start of treatment the seven mice lost weight rapidly and died without evidence of bone marrow or main organ toxicity (histologically evaluable only in two mice). With schedule A, no mortality was observed in the first experiment and only one mouse died at 7 weeks in the second experiment. Also, in the latter experiment, a schedule B treatment was performed with lower doses of doxorubicin (6 mg kg^{-1}). Only one out of six mice died. The effect on tumour growth was roughly equivalent to that in the group treated in schedule B with 8 m kg^{-1} doxorubicin in the 7 evaluable weeks (data not shown).

Discussion

This study was designed to investigate the therapeutic effect of the co-administration of chemotherapy and metabolic radiotherapy with the ^{131}I-radiolabelled monoclonal antibody Po66, directed against an intracellular tumour antigen present in non-small-cell lung carcinoma.

A single dose of 550 μCi ^{131}I-Po66 alone had no effect on the growth of SK-MES-1 tumours implanted in nude mice. This could be expected from biodistribution studies because Po66 binds to tumour necrotic areas and, because of the medium range of its β-emitter, ^{131}I is not able to destroy all viable cells of a well-established tumour (5–9 mm in diameter) from these necrotic areas [5]. It is, however, possible that ^{131}I could be more efficient on smaller tumours as it delivers 80% of its energy within 1 mm [9]. Fractionated doses of ^{131}I-Po66 alone were able to delay tumour growth. This therapeutic effect could be due to a centrifugal killing of viable tumour cells around the deposit of radiolabelled antibody in degenerating or necrotic tumour tissue, as described for antibodies directed against intracellular antigens [2]. However, this effect is probably minor with ^{131}I-Po66 because, with an almost identical amount of injected radioactivity (three fractionated doses of 200 μCi compared to a single dose of 550 μCi), dose fractionation was no more effective than

a single dose. So, dose fractionation with 250 μCi×3 ^{131}I-Po66 is probably more efficient because it allows delivery of a higher amount of radioactivity without enhanced toxicity [15].

Whatever kind of administration of ^{131}I-Po66 (single or fractionated doses) was used, the co-administration with doxorubicin significantly increased the therapeutic efficacy. Our hypothesis is that doxorubicin increases the amount of degenerating or necrotic tumour cells therefore enhancing antigen accessibility and distributing a larger amount of radiolabelled Po66 more evenly. By this mechanism, we were expecting to potentiate chemotherapy by metabolic radiotherapy, the latter destroying the remaining viable cells from numerous or larger areas of necrosis adjacent to these still viable, "drug-resistant" cells. The results obtained are in agreement with this hypothesis. This is particularly obvious because a single dose of ^{131}I-Po66 alone had no effect on tumour growth, whereas the combination with doxorubicin increased the tumour doubling time by 1.55 when compared to doxorubicin alone. Also, as dose fractionation was more efficient, its therapeutic effect with co-administered doxorubicin was also greater with a two-doubling-time increase of 1.75- and 2.4-fold in our two separate experiments. It is unlikely that total-body irradiation could play a role because the unrelated radiolabelled immunoglobulin Py had little or no effect on tumour growth.

Although biodistribution studies [5] suggest that the administration of doxorubicin doubles the amount of ^{131}I-Po66 in tumours, it is impossible to assume that this phenomenon could take into account all the therapeutic benefit of the combination of chemotherapy and immunoradiotherapy. It is very likely that the changes in distribution of ^{131}I-Po66 within the tumour induced by doxorubicin pretreatment play an important role in the increased therapeutic efficacy, and it is not certain that sequential scintigraphy [2] or semi-microdosimetry [14] would have been able to evaluate accurately the role of the increased ^{131}I-Po66 tumour uptake or the role of the distribution of ^{131}I-Po66 within tumours after doxorubicin.

The combination of chemotherapy and metabolic radiotherapy increased toxicity. With a high rate of delayed toxicity, the interspersed administration of doxorubicin and ^{131}I-Po66 (schedule B) was more toxic than when ^{131}I-Po66 was administered after doxorubicin (schedule A). Although this was not expected from toxicity studies, which did not evaluate the effect of concomitant administration of doxorubicin and three fractionated doses of 250 μCi ^{131}I-Po66, this toxicity was conceivable because doxorubicin increases radiation sensitivity. This toxicity was delayed (7–9 weeks after the start of treatment) and no evidence of major haematological or main-organ injury was detected in the mice killed because they lost weight. The doxorubicin is probably the more important factor because this drug induced the highest toxicity (evaluated only on the weight loss) compared to ^{131}I-Po66 alone. Also, when doses were lowered to 6 mg kg^{-1}, the delayed toxicity was reduced but no experiment was performed with the concomitant administration of doxorubicin at 8 m kg^{-1} and lower doses of ^{131}I-Po66. As the therapeutic efficacy was identical when fractionated doses of radiolabelled antibody were administered during or after the injections of doxorubicin, further experiments should be performed with the latter schedule.

The antigen recognized by Po66 is present at low levels in some human tissues, such as bronchial serous glands, the middle layer of the oesophagus and distal tubules of kidney [4]. One concern is that chemotherapeutic drugs could render accessible the target antigen in normal tissues. However, these tissues are not expected to be damaged by chemotherapy, which would therefore be unlikely to enhance toxicity in combination with radioimmunotherapy.

Although we have demonstrated that the therapeutic efficacy of doxorubicin can be enhanced by the co-administration of a radiolabelled monoclonal antibody directed against an intracellular antigen, toxicity remains a limiting factor. However, there are several possible ways of easily lowering the toxicity of the drug and the radioisotope, and of increasing the efficacy of this combination. First, although more efficient drugs should be tested, the use of new formulations of chemotherapy could be promising. For example, doxorubicin encapsulated in stabilized liposomes could very efficiently circumvent the toxicity due to the chemotherapy [16]. Second, the use of more appropriate isotopes like ^{186}Re with a high percentage β remission and a higher medium range than ^{131}I could destroy more cells distant from necrotic areas. Third, the use of antibodies directed against abundant intracellular antigens like histones [6] could concentrate more antibody within the tumour to better therapeutic effect. Finally, with long-term retention in tumours, Po66 could be a good candidate for a two-phase radioimmunotherapy, which is expected to decrease the residence time of the radionuclide in normal tissues [10, 11].

We have demonstrated, in a non-small-cell lung tumour model, that metabolic radiotherapy with a ^{131}I-radiolabelled monoclonal antibody directed against an intracellular antigen could significantly potentiate the effect of chemotherapy. Such a therapeutic approach could represent an adjuvant therapy, as it should destroy distant small metastases responsible for recurrences. This concept would also be particularly interesting in chemo- and radiosensitive tumours like small-cell lung carcinomas. It should be possible to improve the efficacy of this combination and reduce its adverse effects.

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