Pergamon

European Journal of Cancer, Vol. 34, No. 10, pp. 1628–1633, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00176-2

Original Paper

Aggressive Administration of Recombinant Oncotoxin AR209 (Anti-ErbB-2) in Athymic Nude Mice Implanted with Orthotopic Human Non-small Cell Lung Tumours

N. Skrepnik[1,2,3], A.W. Zieske,[4] E. Robert,[2] J.C. Bravo,[4] R. Mera[3,4] and J.D. Hunt[1,3]

[1]Department of Biochemistry and Molecular Biology; [2]Department of Medicine; [3]Stanley S. Scott Cancer Center; and [4]Department of Pathology, Louisiana State University Medical Center, New Orleans, Lousiana, 70112, U.S.A.

Lung cancer remains a significant public health problem in the U.S.A. and will result in an estimated 160 400 deaths in 1997. This appalling number is due in large part to the lack of adequate treatment for tumours that are refractory to surgery with curable intent, or of an adequate salvage therapy for those patients who recur after surgical resection. Because non-small cell lung cancer is refractory to traditional chemotherapy, non-traditional therapies have been developed to treat patients with this disease. Recombinant oncotoxins have been designed to target cells that express certain proteins as part of their cellular membrane. One such oncotoxin, AR209 (formerly OLX-209 [e23(Fv)PE38KDEL]), has the specificity of an anti-ErbB-2 antibody contained within a single-chain antibody domain (e23v) coupled to a portion of the *Pseudomonas* exotoxin A (PE38KDEL). Previous studies demonstrate that this drug is capable of significantly reducing the size of orthotopic lung tumour xenografts. However, most of the treated mice developed tumours once therapy was removed. In this study, mice were treated aggressively using one of four drug treatment schedules. Mice were treated with either intravenous or subcutaneous injections of AR209 over a 2 week period. The data indicate that AR209 significantly reduced the size of tumours and upon microscopic analysis at necropsy, some mice were cured. However, despite the treatment schedule used, many mice contained residual tumour. Residual tumours expressed the ErbB-2 protein, indicating that more aggressive treatment with AR209 may have resulted in higher rates of cure. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: HER-2, *neu*, *Pseudomonas*, exotoxin A, immunotoxin, adenocarcinoma
Eur J Cancer, Vol. 34, No. 10, pp. 1628–1633, 1998

INTRODUCTION

DESPITE THE many advances made in the diagnosis and treatment of most human cancers, several types remain refractory to treatment. Cancers of the lung kill more Americans than any other tumour type, despite higher incidence rates for breast cancer and prostate cancer [1], reflecting the lack of an adequate therapy. Only 13% of people diagnosed with lung cancer will survive for 5 years [1, 2]. Non-small cell lung cancer (NSCLC, includes adenocarcinoma, squamous cell carcinoma, adenosquamous cell carcinoma, and large cell carcinoma) accounts for approximately 75% of all cancers of the lung [3]. Surgical resection of the tumour is the standard treatment for stage I and II disease, as most NSCLCs are refractory to radiotherapy and chemotherapy [4]. However, the majority of patients present at an advanced stage and are not candidates for a surgical procedure with curative intent [4]. For those patients with advanced stage disease or with recurrent metastatic lesions following surgical resection, palliative radiotherapy often becomes the only option.

Biological therapy has become an attractive alternative for the treatment of tumours that prove refractory to traditional therapies [5]. Immunotoxins and recombinant oncotoxins are an important component of biological therapy. These drugs rely on the specificity of an antibody to direct precisely a toxin that irreversibly inactivates protein synthesis in the tumour cell [6]. One such oncotoxin, AR209 (formerly OLX-209 [e23(Fv)PE38KDEL]), has the specificity of an anti-ErbB-2 antibody contained within a single-chain antibody domain (e23Fv) coupled to a portion of the *Pseudomonas* exotoxin A (PE38KDEL) [7]. We have previously demonstrated that the recombinant oncotoxin AR209 effectively targets human

Correspondence to J.D. Hunt.
Received 9 Dec. 1997; revised 28 Feb. 1998; accepted 5 Mar. 1998.

adenocarcinoma cells *in vitro* [8] and *in vivo* in a subcutaneous (s.c.) mouse model [8] and an orthotopic model [9]. The results of these studies indicate that three intravenous (i.v.) injections administered to mice over a 5 day period at 86 μg/kg or 43 μg/kg significantly reduced the size of s.c. tumours, but did not completely eliminate them [8]. Increasing the number of injections to six (three i.v. injections administered to mice over a 5 day period at 86 μg/kg followed by three injections separated by 10 days) resulted in smaller orthotopic tumours, but did not result in 'cures' in most mice [9]. Also, the tumours present in AR209-treated animals increased in size once therapy was removed. To evaluate the effect of a more aggressive use of the drug, mice implanted with intrathoracic orthotopic human tumours were treated with one of four drug administration schedules. The lungs of mice treated with AR209 were resected and stained for ErbB-2 oncoprotein using immunohistochemistry.

MATERIALS AND METHODS

Cell lines and culture conditions

The human lung adenocarcinoma cell line 201T was established from a poorly differentiated tumour using the method of Siegfried and Owens [10]. This cell line and its culture conditions have been described in detail elsewhere [8, 9, 11]. For orthotopic implantation, cells were isolated by trypsinisation (Life Technologies, Grand Island, New York, U.S.A.) and centrifugation at 500 ***g*** for 15 min, washed once in medium supplemented with 1% fetal bovine serum (FBS) (HyClone Laboratories, Logan, Utah, U.S.A.), and washed an additional two times with phosphate buffered saline (PBS) (Life Technologies). Cells were suspended in HBSS (Life Technologies). For injections, tumour cells were mixed with Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, Massachusetts, U.S.A.) [12, 13] at a ratio of 1:1, to a final concentration of 2×10^6, cells/0.1 ml.

Experimental animals

Specific pathogen-free, 4–6 week old female *nu/nu* (nude) mice obtained from Harlan Sprague Dawley Inc. (Indianapolis, Indiana, U.S.A.) were housed in sterilised filter-topped cages kept in laminar flow isolators (Forma Scientific, Marietta, Ohio, U.S.A.) and were fed autoclaved food and water *ad libitum*. The mice were acclimated for 1 week prior to use in study protocols. All procedures involving the animals were performed under sterile conditions in a laminar flow hood (Forma Scientific). All studies were approved by the LSUMC Institutional Animal Care and Use Committee.

Tumour cell implantation and drug therapy

Percutaneous orthotopic implantation of tumour cells was accomplished as described previously [9, 14], except that fluoroscopy was not used. Tumours were allowed to develop in all mice for 3 weeks prior to the initiation of therapy. This method has routinely resulted in the development of tumours in 100% of control mice [9]. Four drug administration schedules were used to test AR209. In schedule 1 (Figure 1), 14 mice were implanted with 201T cells orthotopically on day 0. An additional four mice were injected percutaneously with saline into the lung to mimic 201T cell implantation. After 3 weeks, the 14 mice containing tumours, were randomised into two groups. The experimental group received i.v. injections of 86 μg/kg AR209 (approximately 0. 1 ml/mouse) on days 25, 27, and 30. The placebo group received i.v. injections of 0.1 ml saline on the same days. All mice were sacrificed on day 32. In schedule 2 (Figure 1), 14 mice were implanted with 201T cells orthotopically on day 0. An additional four mice were injected percutaneously with saline into the lung to mimic 201T cell implantation. After 3 weeks, the 14 mice containing tumours were randomised into two groups. The experimental group received i.v. injections of 86 μg/kg AR209 on days 21, 23, 25, 27, 29, 31, 33, and 35. The placebo group received i.v. injections of 0.1 ml of saline on the same days. All mice were sacrificed on day 56. In schedule 3 (Figure 1), 21 mice were implanted with 201T cells orthotopically on day 0. An additional four mice were injected percutaneously with saline into the lung to mimic 201T cell implantation. On day 21, the 21 mice containing tumours were randomised into two groups. The experimental group ($n=11$) received s.c. osmotic pumps (ALZA Scientific Products, Palo Alto, California, U.S.A.) containing 35 μg AR209 (drug delivery 100 μl/kg/day). This resulted in the administration of 2.5 μg AR209/mouse/day. On day 35, the pumps were removed. The placebo group received osmotic pumps containing saline on the same day. All animals were sacrificed on day 56. In schedule 4 (Figure 1), 12 mice were implanted with 201T cells orthotopically on day 0. An additional four mice were injected percutaneously with saline into the lung to mimic 201T cell implantation. After 3 weeks, the 12 mice containing tumours were randomised into two groups. The experimental group ($n=7$) received s.c. injections of 86 μg/kg AR209 on days 21, 23, 25, 27, 29, 31, 33, and 35. The placebo group ($n=5$) received s.c. injections of 0.1 ml of saline on the same days. All mice were sacrificed on day 56. Tumour volumes were monitored using dial calipers. Tumour growth is reported as an average tumour volume, calculated as $\pi^{\star}(w^{\star}l^{\star}h)/2$, where w is the width, l is the length, and h is the height in mm. The mediastinum and lungs were weighed at autopsy, henceforth referred to as 'mediastinal blocks'.

Immunohistopathology

Sections were prepared from formalin-fixed paraffin-embedded resected lungs. After staining with haematoxylin and eosin, each section was examined by a pathologist who was blind to the experimental conditions used to treat the mouse from which the lung section was obtained. Sections were also examined for expression of ErbB-2 protein using rabbit anti-human ErbB-2 polyclonal antibody (Biomeda #225M, San Mateo, California, U.S.A.). Immunohistochemistry was performed using the Ventana Medical Systems gen II automated immunohistochemistry system and reagents (Tucson, Arizona, U.S.A.). Diaminobenzidine (DAB) substrate kits supplied and customised by Ventana for horseradish peroxidase as an enzyme marker were used. Immunohistochemical analysis was performed by counting 200 cells (unless the tumour was too small) then taking the percentage of the cells that stained positively and applying the following scale (Table 1): +, 0–25% positive; ++, 26–50% positive; +++, 51–75% positive; and ++++, 76–100% positive. A breast adenocarcinoma containing 75–100% positively staining cells was used as a positive control for anti-ErbB-2 staining.

Statistical methods

The size of the tumours and the weight of the mediastinal blocks were considered the dependent variables. The fixed factors are the type of treatment (placebo, AR209 or PBS)

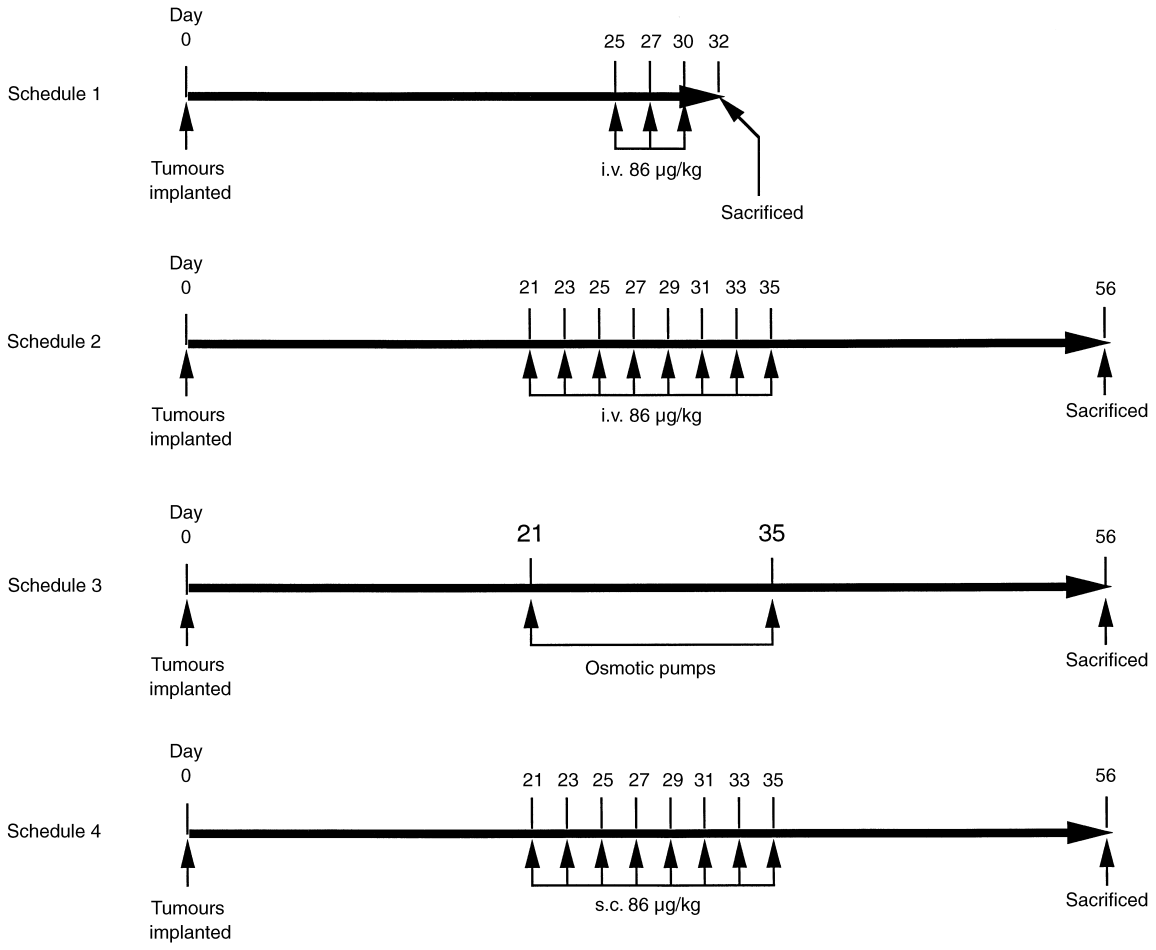

Figure 1. Drug administration schedules used in this study.

and the schedule. A two-way analysis of variance was used to determine the treatment effect after controlling for schedule and the schedule effect after controlling for treatment effect. The interaction between the factors was included in the model. *Post-hoc* analyses were performed using the Student–Newman–Keuls method.

RESULTS

To determine if macroscopic tumours are present in the lungs of mice immediately after the administration of AR209, athymic nude mice were treated according to schedule 1 in Figure 1. This exactly mimics the schedule used in our previous study with a s.c. tumour model [8]. No adverse effects were noted for mice treated with this schedule or with similar schedules in previous studies [7–9]. The LD_{10} for AR209 was previously established to be 90 μg/kg/dose and the LD_{50} was established to be 160 μg/kg/dose [8]. As seen in Figure 2(a), the weight of the mediastinal blocks of control mice ($n = 4$) was 414.5 ± 19.3 mg (mean ± standard error), which was not statistically different from the mean weight of AR209-treated mice ($n = 7$, 440.9 ± 42.7 mg). However, the mediastinal blocks of the PBS-treated mice (560.0 ± 14.5 mg, $n = 7$) were significantly heavier than those from control mice and from AR209-treated mice ($P < 0.0001$, Student–Newman–Keuls test). Upon necropsy, the lungs of PBS-treated mice revealed that 7/7 (100%) had gross tumours that averaged 141.1 ± 35.4 mm^3 (Figure 2b). However, only 2/7 (29%) AR209-treated mice had tumours that could be detected upon gross inspection of lungs and they were significantly smaller (13.4 ± 9.1 mm^3) than those from PBS-treated mice (141.1 ± 35.4 mm^3, $P = 0.002$, Student–Newman–Keuls test). However, upon microscopic examination of resected lungs, 6/7 mice contained some tumour. The data demonstrate that the drug is effective in reducing the rate at which tumours form in the lungs of mice and that those tumours that do form are substantially smaller.

To determine if a more aggressive use of the drug would be effective, nude mice were treated according to schedule 2 (Figure 1). As seen in Figure 2(a), the mean weight of the mediastinal blocks from AR209-treated mice ($n = 7$) was 460.6 ± 23.8 mg, which was not statistically different from tumours observed in control mice. However, as with the mice treated according to schedule 1, the mean weight of the mediastinal blocks from PBS-treated mice (538.1 ± 7.9 mg, $n = 7$) was significantly larger compared with control mice and with AR209-treated mice ($P = 0.001$, Student–Newman–Keuls test). Again, upon necropsy 7/7 (100%) mice treated with PBS were shown to have gross tumours with a mean volume of 97.0 ± 18.3 mm^3 (Figure 2b). For AR209-treated mice, 5/7 had detectable tumours upon gross inspection that measured 21.0 ± 6.7 mm^3. Although the tumours were significantly smaller ($P < 0.0001$), upon microscopic examination 4/7 mice had tumours, indicating that the drug had not completely eliminated tumour formation.

Table 1. *Immunohistochemical (IHC) analysis for ErbB-2 expression in selected resected mouse lungs*

Treatment	Tumour weight (mg)*	IHC†
Schedule 1		
PBS	192.5	++++
PBS	124.5	+
AR209	69.5	++++
AR209	0	+
AR209	62.5	++++
AR209	48.5	++++
AR209	51.5	++
AR209	7.5	++++
AR209	0	No tumour
Schedule 2		
PBS	88.5	+++
AR209	65.5	+
AR209	102.5	+++
AR209	3.5	No tumour
AR209	151.5	+++
AR209	8.5	No tumour
AR209	15.5	+++
AR209	0	No tumour
Schedule 3		
Control	No tumour	No tumour
PBS	1279	++
PBS	316	+
AR209	489	+++
AR209	33	++
AR209	147	++
AR209	42	+++

*Tumour weight = weight of mediastinal block-weight of mediastinal block from control mice. †+, 1–25% positive; ++, 25–50% positive; +++, 51–75% positive; ++++, 76–100% positive.

To determine if a continuous infusion of AR209 s.c. over a 2 week period would increase efficacy, 25 nude mice were treated according to schedule 3 (Figure 1). As seen in Figure 2(c), the mean weight of the mediastinal blocks from control mice was 469.5 ± 3.3 mg ($n = 4$). The mean weight of the mediastinal blocks for both PBS-treated mice (936.6 ± 108.8 mg, $n = 10$) and for AR209-treated mice (692.4 ± 60.6 mg, $n = 11$) were significantly larger than those from control mice ($P = 0.014$, Student–Newman–Keuls test). The weight of mediastinal blocks from AR209-treated mice were not significantly different than the weight of those from PBS-treated mice. As expected, 10/10 (100%) mice treated with PBS were found to contain tumours upon necropsy. Surprisingly, 11/11 (100%) mice treated with AR209 also contained tumours. The data suggest that a continuous infusion of AR209 via a s.c. route did not control tumour growth as well as i.v. delivery of the drug. Therefore, to determine if AR209 could be delivered by s.c. injections, mice were treated using the procedure outlined in schedule 4 (Figure 1). As seen in Figure 2(d), the mean weight of mediastinal blocks resected from AR209-treated mice (689.4 ± 15.9 mg, $n = 7$) was significantly less than for PBS-treated mice (783.2 ± 28.6 mg, $n = 5$, $P < 0.0001$, Student–Newman–Keuls test). However, the mediastinal blocks from AR209-treated mice were significantly heavier than those from control mice (575.8 ± 12.3 mg, $n = 4$, $P < 0.0001$, Student–Newman–Keuls test).

In each case, despite the treatment schedule used, residual tumour remained in many of the treated mice. There are at least two explanations for this result. First, the tumour cells may heterogeneously express the ErbB-2 protein, and the AR209 is only able to inhibit protein synthesis in those cells that express ErbB-2. Alternatively, the drug may have an overall net static effect on the tumour because it may not be able to penetrate the tumour mass adequately. To differentiate between these possibilities, mediastinal blocks from mice were fixed in formalin and embedded in paraffin. Sections of the lungs were stained with a monoclonal antibody (MAb) specific for human ErbB-2. The data are presented in Table 1. Note that tumours in mice treated with PBS alone had a large range of expression of ErbB-2 protein (schedule 1, PBS). Also note that in mice treated with an aggressive schedule of i.v. injections (schedule 2), several of the mice had no evidence of microscopic tumours, indicating that they had been cured (all PBS-treated mice developed a tumour, indicating a 100% take rate—also seen in other studies [9]). In those mice that did contain tumours after treatment with AR209, most of the cells in the tumour mass (> 50%) expressed the ErbB-2 protein, suggesting that they would be susceptible to further treatment with AR209. Therefore, it seems unlikely that the presence of tumours is due to the heterogeneous expression of ErbB-2.

DISCUSSION

The ultimate goal of targeted therapy is to design drugs that specifically recognise protein markers expressed exclusively on the surface of tumour cells. Unfortunately, despite a great deal of effort to identify tumour-specific markers, little progress has been made in this area [15]. However, tumour cells often express normal protein on their cell surface in excess amounts, creating a large therapeutic window and making the use of drugs based on MAbs an attractive option. One such therapeutic molecule is the so-called recombinant oncotoxin. This type of drug consists of the antigen-binding region of a MAb linked to a toxin [6]. The recombinant oncotoxin used in this study, AR209, has the specificity of an anti-ErbB-2 antibody contained within a single-chain antibody domain coupled to a portion of the *Pseudomonas* exotoxin A. This compound has been extensively described elsewhere [7]. The modified *Pseudomonas* exotoxin A found in AR209 ADP-ribosylates eukaryotic elongation factor 2, thereby completely inhibiting protein synthesis in targeted cells. This ADP-ribosylation is catalysed by domain III of *Pseudomonas* exotoxin A, which usually contains the carboxyl-terminal sequence REDLK that directs endocytosed toxin into the endoplasmic reticulum. The modified toxin found in AR209 replaces this carboxyl-terminal sequence with KDEL, resulting in a toxin that is not only directed to, but is retained in the endoplasmic reticulum and is more toxic to cells [16]. We have previously found that this drug is highly toxic to NSCLC cell lines *in vitro* with extremely low IC_{50} concentrations ranging from 2 to 30 ng/ml; the IC_{50} for normal bronchial epithelial cells was > 1000 ng/ml [8]. Further, the drug was very effective at reducing the size of s.c. NSCLC xenografts [8], and orthotopic xenografts [9]. In our previous *in vivo* studies, treatment schedules were used to evaluate the potential efficacy of the drug, but were not aggressive as compared with actual phase I/II trials in humans [17–21]. Because very low concentrations of AR209 effectively killed cells *in vitro*, it was surprising that more mice were not cured

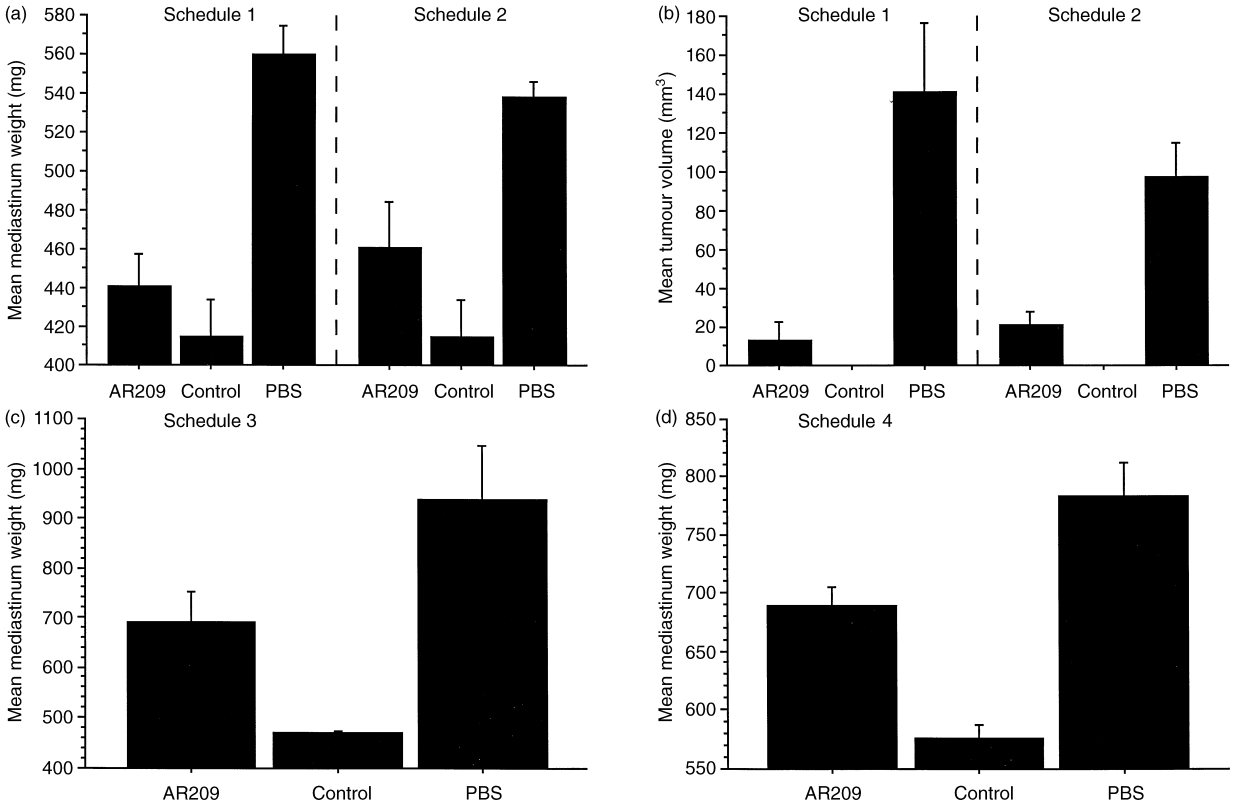

Figure 2. Comparisons of the mean weight of mediastinal blocks and the tumour volume in treated mice. A slight increase in the mean weight of mediastinal blocks was observed in lungs of mice treated according to schedule 1 and schedule 2 (a). This correlated with the presence of small tumours in the lungs of several mice that were amenable to measurement using dial calipers (b). Larger tumours were present in the lungs of animals treated with osmotic pumps (c) or with subcutaneous injections (d). Error bars=standard error.

in our initial *in vivo* orthotopic model [9]. Therefore, we tested new schedules for drug administration and evaluated the lungs of treated mice using immunohistochemistry.

For any drug to kill a tumour it must penetrate the tumour through its microvasculature. The obstacles for adequate drug delivery to tumours have been described [22, 23]. In addition to the problems associated with conventional drug delivery, another major barrier to the use of immunotoxins (whole MAbs or Fab fragments linked to toxin molecules) are their large size (~290 kDa) resulting in poor penetration of the drug into the tumour [6]. The development of small (~60 kDa) recombinant oncotoxins consisting of small single-chain (Fv) molecules linked to toxins predicted the end to tumour distribution problems [24]. However, our data indicate that the AR209 compound may not adequately penetrate the tumour. Tumours were present in the lungs of mice treated according to schedule 1; this is not surprising given that only three injections of drug were administered. The cells found within the tumours contained a high proportion of cells that remained positive for ErbB-2 staining. These data indicate that a more aggressive treatment schedule may have resulted in significant numbers of cures. Indeed, when mice were treated with the more aggressive schedule 2, 3/7 (43%) showed no evidence of tumour upon microscopic analysis of resected lungs. Of the four mice which contained tumours at necropsy, only one contained a tumour that had < 25% of cells staining positive for ErbB-2. Therefore, given that the NSCLC cells were allowed 3 weeks to recover in the absence of drug, but still had ErbB-2 present on their cellular membranes, even more aggressive therapy may have resulted in a higher rate of cure.

To test this hypothesis, a more aggressive continuous infusion of AR209 was used in schedule 3. However, large tumours developed, despite the continuous presence of drug. Perhaps this is related to the poor transit of AR209 across capillary walls due to the large size of the conjugated toxin molecule yielding low serum levels of AR209, or the instability of AR209 when stored for prolonged periods at 37°C [25]. With s.c. administration, AR209 must be transported into the capillary, through the circulation and then again across the capillary wall to exit at the site of the tumour. It was previously demonstrated that immunotoxins can accumulate in s.c. xenografts [26]. Using radiolabelled immunotoxins, Sung and colleagues demonstrated that the spatial distribution for immunotoxins was punctate in appearance and showed large spatial heterogeneity [27]. They proposed that the heterogeneous distribution of their immunotoxins reflected retarded penetration due to binding to the tumour cells. Rippley and Stokes found that the affinity to which the drug binds to its receptor or the density of the receptor on the cellular surface affected permeation of extracellular drug [28]. Specifically, high receptor affinity or increased receptor density reduced permeation of the drug while increasing the peak concentration of intracellular drug. They termed this phenomenon 'internal trapping' and suggested that it could account for heterogeneous drug distribution often observed in experimental systems. Therefore, maximal drug distribution and optimal therapeutic effect may be mutually exclusive for

drugs that require internalisation to be effective [28], as does AR209. The human lung adenocarcinoma used in this study expresses 7.7-fold more ErbB-2 than normal bronchial epithelial cells [8]. This is far below the 330-fold increase in protein expression found in the lung adenocarcinoma cell line Calu-3. It seems unlikely, therefore, that AR209's effect is due to the presence of too many receptor molecules. The more likely explanation for the observed results is the high affinity to which AR209 complexes to the ErbB-2 protein.

The evaluation of tumours using immunohistochemical staining for ErbB-2 was an important component of the current study. Previously it was unknown if residual tumours that arose after therapy were composed of a subpopulation of cells that had lost the ErbB-2 protein marker and thus, would be refractory to further treatment with AR209. It is encouraging to note that the majority of tumours contained > 50% of cells expressing detectable levels of ErbB-2. Previous studies have demonstrated that when as few as 10% of tumour cells are killed, a significant 'bystander effect' results in the reduction of unaffected cells [29, 30]. Because so many cells in the tumour express ErbB-2, it is likely that the oncotoxin binds to ErbB-2-expressing cells on the periphery of the tumour and does not penetrate deeply into the tumour [27, 28]. This may explain the static effect of AR209 on large intrathoracic tumours.

It seems likely that the most effective use of AR209 will be in adjuvant therapy associated with surgical resection with curative intent. The drug has demonstrated the ability to prevent the development of small tumours and may be effective in eliminating undetected micrometastases. However, our data suggest that large tumours will be poorly targeted by AR209. Given that the overall 5 year survival rate for NSCLC remains at only 13% [1], aggressive adjuvant therapy with novel therapeutic agents may increase overall survival.

1. *Cancer Facts & Figures—1997*. Atlanta, Georgia, American Cancer Society, 1997.
2. Ginsberg RJ, Kris MG, Armstrong JG. Cancer of the lung. In DeVita Jr VT, Hellman S, Rosenberg SA, eds. *Cancer: Principles and Practice of Oncology*. Philadelphia, J. P. Lippincott, 1993, 673–730.
3. Ihde DC, Minna JD. Non-small cell lung cancer. Part I: Biology, diagnosis, and staging. In Haskell CM, ed. *Current Problems in Cancer*. St. Louis, Mosby-Year Book, 1991, 63–104.
4. Ihde DC, Minna JD. Non-small cell lung cancer. Part II: Treatment. In Haskell CM, ed. *Current Problems in Cancer*. St. Louis, Mosby-Year Book, 1991, 107–154.
5. De Vita Jr VT, Hellman S, Rosenberg, SA (eds). *Biologic Therapy of Cancer*. Philadelphia, J. B. Lippincott, 1995.
6. Pai LH, Pastan I. Section 20.2. Immunotoxins and recombinant toxins. In DeVita Jr VT, Hellman S, Rosenberg SA, eds. *Biologic Therapy of Cancer*. Philadelphia, J. B. Lippincott, 1995, 521–533.
7. Batra JK, Kasprzyk PG, Bird RE, Pastan I, King CR. Recombinant anti-*erb*B-2 immunotoxins containing *Pseudomonas* exotoxin. *Proc Natl Acad Sci (USA)* 1992, **89**, 5867–5871.
8. Kasprzyk PG, Sullivan TL, Hunt JD, *et al.* Activity of Anti-*erb*B-2 recombinant toxin OLX-209 on lung cancer cell lines in the absence of *erb*B-2 gene amplification. *Clin Cancer Res* 1996, **2**, 75–80.
9. Skrepnik N, Araya JC, Qian Z, *et al.* Effects of anti-*erb*B-2 (HER-2/*neu*) recombinant oncotoxin AR209 on human non-small cell lung carcinoma grown orthotopically in athymic nude mice. *Clin Cancer Res* 1996, **2**, 1851–1857.
10. Siegfried JM, Owens SE. Response of primary human lung carcinomas to autocrine growth factors produced by a lung carcinoma cell line. *Cancer Res* 1988, **48**, 4976–4981.
11. DeMichele MAA, Davis ALG, Hunt JD, Landreneau RJ, Siegfried JM. Expression of messenger RNA for three bombesin receptor subtypes in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1994, **11**, 66–74.
12. Pretlow TG, Delmoro CM, Dilley GG, Spadafora CG, Pretlow TP. Transplantation of human prostatic carcinoma into nude mice in Matrigel. *Cancer Res* 1991, **51**, 3814–3817.
13. Fridman R, Kibbey MC, Royce LS, *et al.* Enhanced tumor growth of both primary and established human and murine tumor cells in athymic mice after coinjection with matrigel. *J Natl Cancer Inst* 1991, **83**, 769–774.
14. Hamide JP, Qian Z, Xu H, *et al.* Percutaneous implantation of non-small cell lung carcinoma: technique and observations. *Academ Radiol* 1997, **4**, 629–633.
15. Schreiber H, Ward PL, Rowley DA, Stauss HL. Unique tumor-specific antigens. *Ann Rev Immunol* 1988, **6**, 465–483.
16. Munro S, Pellham HRB. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 1987, **4**, 899–907.
17. Lynch Jr TJ. Immunotoxin therapy of small cell lung cancer. N901-blocked ricin for relapsed small cell lung cancer. *Chest* 1993, **103**, 4365–4395.
18. Grossbard ML, Lambert JM, Goldmacher US, *et al.* Anti-B4-blocked ricin: a phase I trial of 7-day continuous infusion in patients with B-cell neoplasms. *J Clin Oncol* 1993, **11**, 726–737.
19. Grossbard ML, Gribben JG, Freedman AS, *et al.* Adjuvant immunotoxin therapy with anti-B4-blocked ricin after autologous bone marrow transplantation for patients with B-Cell non-Hodgkin's lymphoma, *Blood* 1993, **81**, 2263–2271.
20. Amlot PL, Stone MJ, Cunningham D, *et al.* A phase I study of an anti-CD22-deglycoxylated ricin A chain immunotoxin in the treatment of B-cell lymphomas resistant to conventional therapy. *Blood* 1993, **82**, 2624–2633.
21. Grossbard ML, Freedman AS, Ritz J, *et al.* Serotherapy of B-cell neoplasms with anti-B4-blocked ricin: a phase I trial of daily bolus infusion. *Blood* 1992, **79**, 576–585.
22. Jain RK. Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J Natl Cancer Inst* 1989, **8**, 8570–8576.
23. Jain RK, Baxter LT. Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: significance of elevated interstitial pressure. *Cancer Res* 1988, **48**, 7022–7032.
24. Chaudhary VK, Queen C, Junghans RP, Waldmann TA, Fitzgerald DJ, Pastan I. A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature (London)* 1989, **339**, 394–397.
25. Reiter Y, Brinkmann U, Jung SH, *et al.* Improved binding and antitumor activity of a recombinant anti-erbB2 immunotoxin by disulfide stabilization of the Fv fragment. *J Biol Chem* 1994, **269**, 18327–18331.
26. Blumenthal RD, Stein R, Sharkey RM, *et al.* Antibody penetration of tumor GS-7 xenografts in nude mice: a model for mucinous adenocarcinoma of the colon. *Cancer Res* 1996, **56**, 3508–3515.
27. Sung C, Dedrick RL, Hall WA, Johnson PA, Youle RJ. The spatial distribution of immunotoxins in solid tumors: assessment by quantitative autoradiography. *Cancer Res* 1993, **53**, 2092–2099.
28. Rippley RK, Stokes CL. Effects of cellular pharmacology on drug distribution in tissues. *Biophys J* 1995, **69**, 825–839.
29. Freeman SM, Abbound CN, Whartenby KA, *et al.* The "Bystander Effect": Tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res* 1993, **53**, 5274–5283.
30. Hoganson DK, Batra RK, Olsen JC, Boucher RC. Comparison of the effects of three different toxin genes and their levels of expression on cell growth and bystander effect in lung adenocarcinoma. *Cancer Res* 1996, **56**, 1315–1323.

Acknowledgements—The authors thank Drs Charles Warner and Robert Rando of Aronex Pharmaceuticals Inc. for the gift of the AR209 compound and Claudina Alemán for critical review of the manuscript. The authors also acknowledge the support of Dr Ulrich Weidle of Boehringer Mannheim Therapeutics. This work was supported in part by a grant from the Stanley S. Scott Cancer Center. JDH is a Council for Tobacco Research Scholar.