Cancer Immunol Immunother (1987) 24: 138–143

Cancer Immunology Immunotherapy

Increased therapeutic effect of vinca alkaloids targeted to tumour by a hybrid-hybrid monoclonal antibody

J. R. F. Corvalan, W. Smith, V. A. Gore, D. R. Brandon, and P. J. Ryde

Lilly Research Centre Ltd., Eli Lilly & Co., Erl Wood Manor, Windlesham, Surrey GU20 6PH, U. K.

Summary. Unmodified vinblastine (VLB) targeted through one of the antigen combining sites of the hybrid-hybrid 28.19.8 monoclonal is potentially more effective in suppressing the growth of established MAWI tumour xenografts implanted on nude mice than free VLB in the absence of the targeting agent, presumably due to an increased local drug concentration. Our efficacy results in this study suggest that drug, specifically removed from the circulation by hybrid-hybrid antibody previously located to the tumour mass, can be made available in a pharmacologically active from. Histological analysis of the treated tumours revealed dramatic changes in the tumour organisation with only a few surviving tumour cells with altered morphology.

Introduction

We describe here the in vivo inhibition of tumour growth due to the specific localisation of unmodified drug to the tumour site. Homing of the cytostatic drug vinblastine specifically to the tumour has been achieved by the prior administration of a hybrid-hybrid monoclonal antibody with specificity for both the tumour antigen carcinoembryonic antigen (CEA) and vinca alkaloids, hence allowing the antibody to be specifically located on CEA-expressing cells with the ability to concentrate circulating vinblastine around the tumour mass. This approach could overcome the lack of specificity associated with normal cancer chemotherapy. Although the covalent coupling of drug to tumour specific monoclonal antibody has been shown to overcome these localisation problems [1, 8, 10, 11, 12] such conjugates have been shown to be far less potent in vivo than the parental free drug (in our own laboratories as low as 0.1% [12] when comparing absolute amounts of drug localised in the tumours). Our efficacy results suggest that drug which presumably binds specifically to hybrid-hybrid antibody already located on the tumour, is being released in a free and pharmacologically active form.

Materials and methods

Mice and xenografts. Outbred nu/nu athymic mice were bred and maintained in isolators (Isotec Ltd, Bicester, UK). Xenograft growth was initiated by s.c. inoculation of 0.2 ml of a suspension of MAWI, a human adenocarcinoma [9], as previously described [12].

Hybrid-hybrid monoclonal antibody. The characterisation, affinity purification and in vitro and in vivo functional properties of the hybrid-hybrid 28.19.8 monoclonal antibody have been described elsewhere [5, 6]. For in vivo administration affinity purified 28.19.8 monoclonal was diluted to 3.0 mg/ml in phosphate-buffered saline (PBS), filtered sterile (0.2 μm) and maintained at 4 °C until used.

In vivo tumour inhibition studies. The in vivo effects of vinca alkaloids targeted to tumour tissues following the administration of 28.19.8 monoclonal were studied as follows. Groups of 10–13 nude mice with 8 to 15 days established MAWI tumour xenografts received 2 or 3 cycles of treatment. Each cycle consisted of the i.p. administration of 28.19.8 monoclonal followed by two i.p. doses of vinblastine sulphate (VLB) 7 and 11 days after the hybrid-hybrid injection. Tumour volumes were estimated at frequent intervals by calculation of the product of 3 perpendicular diameters. Control groups included animals receiving VLB alone, hybrid-hybrid alone and animals receiving only PBS. At the end the experiments, tumours from the different groups were excised and weighed. Tumour volumes were analysed using the Duncan's multiple range test for the analysis of variances, and expressed as mean values. Tumour weights were expressed as means ± SE and analysed using the student's *t*-test. Differences between groups were considered significant at $P<0.05$.

Histology. All immunocytochemistry was performed on 4-μm sections of MAWI tumours which were prepared from formalin-fixed, paraffin-processed tissues. The CEA content of the tumour cells was demonstrated using the parental monoclonal antibody, 11.285.14, that had been biotinylated. After dewaxing and hydrating the sections were washed in 0.15 *M* PBS pH 7.4 and incubated for 1 h in 20% fetal calf serum (FCS). All incubation steps were carried out inside a humidifying chamber at room temperature. The FCS was then replaced by biotinylated 11.285.14 at 50 μg/ml diluted in 20% FCS and incubated for 1 h. After a 30-min wash in PBS, the sections were reacted with avidin-peroxidase (Vector Lab) in 20% FCS for 1 h. A 30-min wash in PBS was followed by development of the peroxidase reaction using 0.05% diaminobenzidine in PBS pH 7.4 and 0.045% hydrogen peroxidase. Fi-

Offprint requests to: J. R. F. Corvalan

nally the preparations were counterstained in Mayer's haematoxylin, dehydrated, cleared and mounted in DPX synthetic mountant. The necessary reagent/material controls were performed alongside the test regimen.

CEA content and tumour extracts. MAWI tumours were homogenised in 0.15 *M* saline in a glass homogeniser, diluted with saline to 100 mg tumour/ml, sonicated for 30 s, and incubated for 1 h at room temperature before centrifuging in an Eppendorf 5412. As the parental anti-CEA monoclonal 11.285.14 would be inhibited by any hybrid-hybrid present in the tumour homogenates, CEA content was determined by enzyme-linked immunosorbent assay using a modification of the methods previously described [13]. Microtiter plates were coated with 14.95.55, a specific anti-CEA monoclonal which recognises a different epitope on CEA from 11.285.14 [4]. The detecting antibody was ars-goat (ars = 4-arsonophenylazo) anti-CEA followed by $F(ab')_2$ rabbit anti-ars conjugated to alkaline phosphatase.

Results

Comparison of the anti-tumour effects of free VLB and VLB targeted by hybrid-hybrid monoclonal

Nude mice with established MAWI tumour xenografts were injected 11 days after tumour implantation with 3.0 mg of affinity purified hybrid-hybrid 28.19.8 monoclonal followed by two i.p. injections of free VLB at 100 or 50 μg/mouse. This cycle of treatment was repeated once with 1.0 mg of hybrid-hybrid at day 25 followed by two i.p. injections of 100 or 50 μg of VLB. This treatment was compared with nude animals in which the hybrid-hybrid treatment was replaced by a PBS injection, but which received the same amounts of VLB at the times previously indicated, and with animals in which the treatment with hybrid-hybrid and VLB was omitted and replaced by PBS. The results illustrated in Fig. 1 indicate that VLB targeted to the tumour by one of the antigen combining sites of the hybrid-hybrid monoclonal had an increased therapeutic effect when compared with equal doses of free VLB. Both levels of VLB targeted by the hybrid-hybrid showed a significant suppression of tumour growth when compared not only with untreated controls but also with equivalent doses of free VLB. In the absence of hybrid-hybrid, only the higher dose of free VLB (100 μg) showed a significant effect on tumour volumes when compared with the PBS-treated control animals. At day 41 the animals were sacrificed and the tumours dissected and weighed. Figure 2 shows the mean tumour weights in the different treatment groups. Again VLB targeted by hybrid-hybrid significantly suppressed the growth of the MAWI tumours when compared with free VLB or untreated controls. Only the higher dose of free VLB showed any effect in suppressing tumour growth.

Further studies were conducted in order to evaluate the fate of the tumour on those animals treated with hybrid-hybrid and VLB. In the experiment illustrated in Fig. 3, 3.0 mg of affinity purified hybrid-hybrid 28.19.8 monoclonal was administered i.p. 8 days after tumour implantation, followed by two i.p. injections of 50 or 10 μg of VLB. The cycle of treatment was repeated once with 1.0 mg of hybrid-hybrid at day 21 followed by 50 or 10 μg of VLB. Controls included animals treated only with PBS, animals in which the hybrid-hybrid treatment was omitted but which received 50 or 10 μg of free VLB, and animals that received only hybrid-hybrid at the times and doses described. All animals were killed at day 52, with the exception of the group receiving hybrid-hybrid and VLB at the level of 50 μg. The tumours were dissected, weighed and samples were fixed in 10% buffered-formalin for further histological examination. The remaining group continued to be studied until day 80 when tumour volumes became similar to those in control animals at day 52 (Fig. 3). The results obtained from this experiment indicated that treatment with hybrid-hybrid alone produced a significant in-

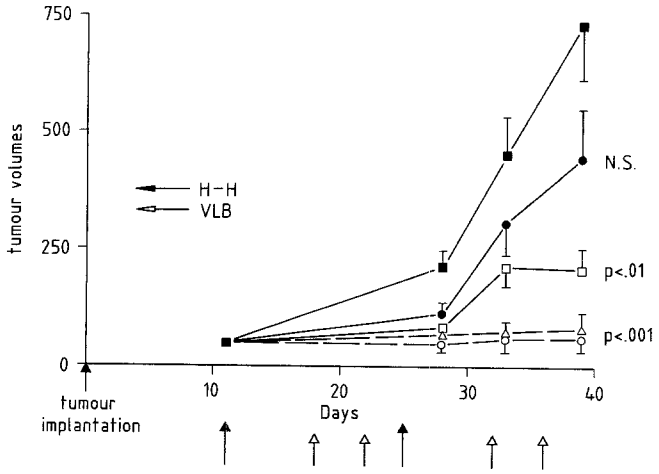

Fig. 1. Effects of pre-administration of hybrid-hybrid monoclonal on the anti-tumour effects of vinblastine (VLB). Results are means (± SE) of tumour volumes from groups of 12 nude mice bearing the MAWI human tumour xenograft. The effects of two cycles of treatment with hybrid-hybrid and VLB at the 100 (○) or 50 μg level (Δ) were significantly different from the VLB alone groups at the 100 (□) or 50 μg level (●) and from the phosphate-buffered saline (PBS) control group (■). Only at the higher dose of 100 μg was VLB alone significantly different from the PBS control group

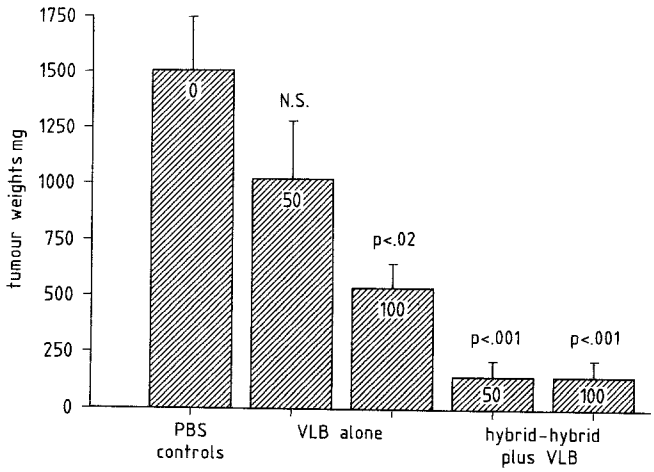

Fig. 2. Same experimental details as in Fig. 1. Results are expressed as mean tumour weight at day 41, when the experiment was terminated. Administration of hybrid-hybrid 28.19.8 followed by VLB at either 100 or 50 μg significantly inhibited tumour growth, as compared with VLB alone at either dose and the PBS control group. Again VLB had a significant effect only at the higher dose of 100 μg when compared with the PBS control group

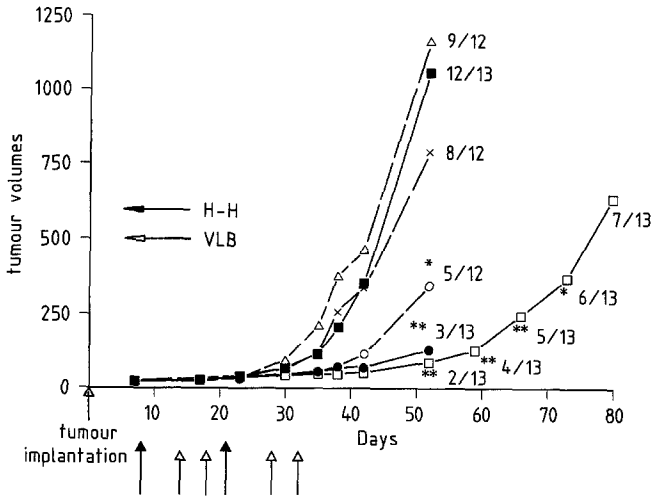

Fig. 3. Effects of hybrid-hybrid plus VLB on tumour growth, and study of tumour growth in the absence of further treatments. Groups of 12–13 nude mice bearing MAWI tumour xenografts were treated with two cycles of treatment with hybrid-hybrid 28.19.8 at the times indicated followed by either 50 (□) or 10 μg VLB (●) and compared with treatments with VLB alone at the 50 (Δ) or 10 μg level (×), and with animals receiving only hybrid-hybrid (○) and with PBS control animals (■). The experiment was terminated at day 52 with the exception of the group treated with hybrid-hybrid and 50 μg VLB which was studied until day 80 with no further treatment. *($P<0.05$) and **($P<0.01$) indicate significant tumour suppression as compared with the PBS control group values at day 52. Results are expressed as mean tumour volumes and, for clarity, standard error bars are omitted. The numbers refer to positive tumour growth (greater than 100 mm^3) per experimental group

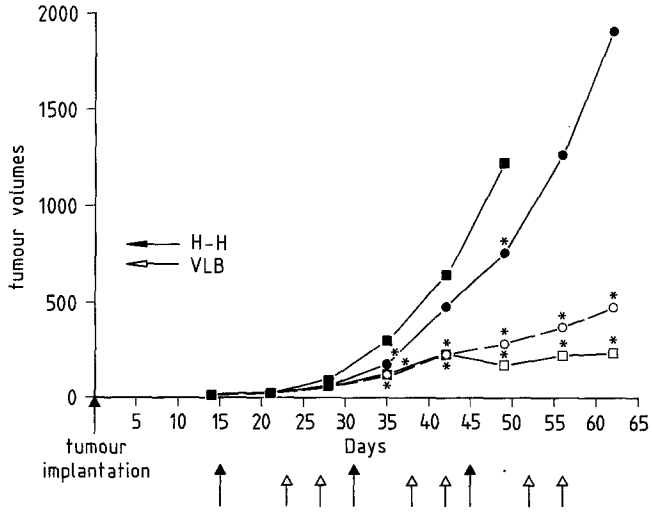

Fig. 4. Effects of a further cycle of treatment with hybrid-hybrid 28.19.8 plus 50 μg VLB. Groups of 10 mice bearing MAWI tumour xenografts were treated with two cycles of hybrid-hybrid and VLB (○), or three cycles of hybrid-hybrid and VLB (□) at the times indicated, and compared with groups receiving only VLB (●) or PBS (■). Results are expressed as mean tumour volumes, and for clarity, standard error bars are omitted. *Significant tumour inhibition ($P<0.05$) in comparison with untreated controls

hibition ($P<0.05$) of tumour growth with 5 out of 12 animals developing tumours growing larger than 100 mm^3. An increased therapeutic effect was highly significant ($P<0.01$) in animals treated with hybrid-hybrid and VLB at the 10 μg level with tumours growing in 3 out of the 13 animals at day 52. Therapy in the hybrid-hybrid plus 50 μg VLB group was equally significant with tumours growing in 2 out of 13 animals at day 52. Animals receiving only VLB at the 50 or 10 μg level, however, showed no significant inhibition of tumour growth when compared with the negative control group receiving only PBS as treatment.

The data from the experiment described (Fig. 3) indicated that tumours treated with hybrid-hybrid and VLB, when left without further treatment, eventually escaped the growth inhibition although with a considerable time delay. CEA was determined on all excised tumours with no significant differences within the different groups (data not shown).

In an experiment designed to study the effect of a further cycle of treatment on tumour growth, two groups of 10 animals were given 3.0 mg of hybrid-hybrid 15 days after tumour implantation followed by 50 μg of VLB at the times indicated (Fig. 4). At day 31 they received a further dose of 1.0 mg of hybrid-hybrid followed by 50 μg of VLB at days 38 and 42. One group received a further cycle of treatment with 1.0 mg of hybrid-hybrid at day 45 followed by 50 μg VLB at days 52 and 56. Control groups included those receiving VLB but no hybrid-hybrid, and those receiving PBS instead of hybrid-hybrid and VLB. The results illustrated in Fig. 4 show that the animals receiving two cycles of treatment had significant inhibition of tumour growth when compared to either the VLB control group or the untreated control group. The group receiving a third cycle of treatment showed a more profound inhibition of tumour growth when compared to the animals receiving only two cycles.

Histological analysis of tumours

Tumour masses excised from animals of the experiment illustrated in Fig. 3 were studied histologically, and two morphological patterns were seen. The first was seen in the control groups receiving no treatment or those receiving only VLB. These xenograft masses were surrounded by a thin layer of fibroblastic tissue. Viable tumour cells were situated primarily in the periphery of the mass and in areas associated with vascularisation. The typical glandular organisation described for this mucoid adenocarcinoma xenograft [9] was present with well differentiated goblet cells, low columnar cells, polyhedral cells and signet ring cells (Fig. 5a). The majority of larger sized masses also contained central areas of necrosis (Fig. 5b). The second pattern was found on tumours from animals treated with hybrid-hybrid with or without VLB. Here the tumour cell organisation was disrupted. The relatively few surviving tumour cells were aggregated together forming small islands surrounded by a large concentration of mucilagenous material. This mass was encapsulated by a thin layer of fibrous cells and the whole resembled round balloon-like structures (Fig. 5c and d). When stained with haematoxylin or eosin, these tumour cells appeared hyperchromatic and highly basophilic lacking the morphological uniformity observed in the control groups. When CEA expression was immunochemically determined using the parental anti-CEA monoclonal 11.285.14 conjugated to biotin fol-

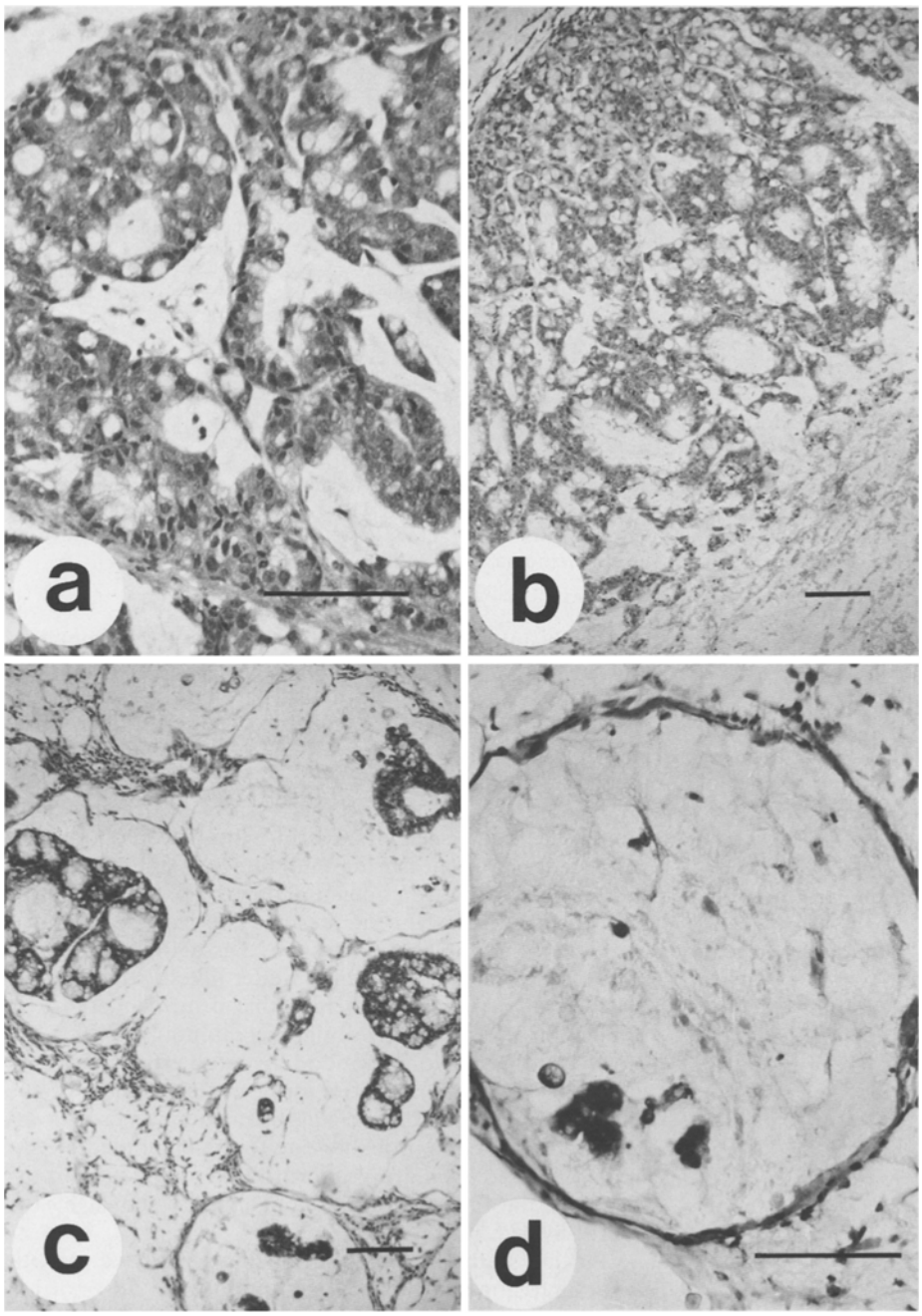

Fig. 5. Haematoxylin and eosin stained sections of the xenografts excised from animals of the experiment illustrated in Fig. 3, demonstrating the two dissimilar morphological patterns observed in the various treatment groups. **a** PBS control group. There is typical glandular organisation with differentiated goblet cells and low columnar cells. **b** PBS control group. Low power showing glandular organization at the periphery of the tumour mass, together with an area of central necrosis. **c** Hybrid-hybrid/VLB, 50 μg group. "Balloon-like" structures. The few surviving tumour cells are aggregated and seen as small islands surrounded by a large concentration of mucilagenous material. **d** Hybrid-hybrid alone group. Higher power showing a single "balloon-like" structure containing a small island of hyperchromatic tumour cells surrounded by mucilagenous material. Scale bar = 100 μm

lowed by avidin-peroxidase, the tumour cells of the hybrid-hybrid-treated groups showed a distinctively enhanced expression of CEA when compared with untreated controls (Fig. 6a and b).

Discussion

In a previous report [6] we have demonstrated specific drug localisation to MAWI xenografts implanted s.c. on nude mice, by using a hybrid-hybrid recognising both CEA and vinca alkaloids. At least 10 times more drug was specifically localised on tumour tissues when animals were pre-treated with the hybrid-hybrid 28.19.8 monoclonal.

The results obtained from the present series of experiments indicate that free VLB targeted through one of the antigen combining sites of the hybrid-hybrid 28.19.8 monoclonal is potentially more effective in suppressing the growth of the MAWI tumour xenografts when compared with free VLB in the absence of targeting agent (Figs. 1, 2, 3 and 4). There can be little doubt that this profound tumour growth inhibition was due to the effect of an increased local concentration of vinca alkaloids in the tumour mass combined with the intrinsic anti-tumour properties shown by the hybrid-hybrid monoclonal on its own (Fig. 3). Since the hybrid-hybrid, ($\gamma 1 - \gamma 2a$) [5], is not able to activate antibody-dependent cell cytotoxicity-mediated mechanisms for tumour inhibition, at least in vitro [5], the suppression of tumour growth observed with the hybrid-hybrid monoclonal was due, presumably, to a complement-mediated cytotoxicity. This latter mechanism can

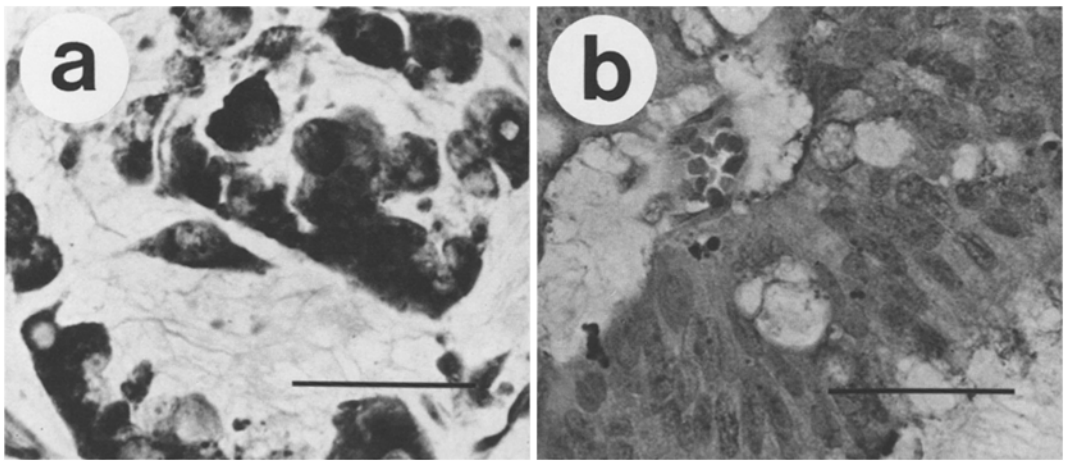

Fig. 6. Immunoperoxidase-treated sections of the xenografts demonstrating the differences in carcinoembryonic antigen (CEA) expression between treatment groups. **a** Hybrid-hybrid/VLB 10 μg group. Enhanced CEA expression. There is strong cytoplasmic staining within the tumour cells. **b** PBS control group. The distribution and intensity of immunoperoxidase staining is markedly different from that seen in **a** and its appearance resembles that of a primary colonic adenocarcinoma. Scale bar = 50 μm

have significant effects in vivo [2, 3]. In a recent report, Couderc et al. [7] demonstrated that a hybrid-hybrid monoclonal IgG_1-IgG_{2a}, monovalently recognising the hapten TNP, was able to activate the classical complement pathway lysing red cells conjugated with TNP, indicating that neither bivalent binding to antigen nor the presence of two complement fixing heavy chains in the Ig molecule are necessary requirements for complement activation. The hybrid-hybrid 28.19.8 monoclonal represents a similar example and its complement fixing properties are presently under study.

As shown in Fig. 3, tumours from animals treated with hybrid-hybrid and VLB gradually started growing when left without further treatment, escaping the inhibitory influence of the treatment although requiring until day 80 to reach tumour volumes equivalent to those of control groups at day 52. This tumour growth is not dependent on tumour cells being negatively selected for CEA expression since those tumours express similar amounts of CEA as untreated controls (data not shown). A further cycle of treatment with hybrid-hybrid and VLB was able to effectively inhibit this delayed tumour growth as shown in Fig. 4.

In the present set of experiments growth inhibition was achieved on an established, actively growing tumour cell population, representing a more realistic approach for a tumour therapy model. In contrast, when using the parental anti-CEA monoclonal 11.285.14 conjugated with vindesine [11, and unpublished results] or in other reports using drugs or toxins chemically conjugated to monoclonal antibodies [10], treatment had to be started almost simultaneously with the tumour implantation in order to obtain significant tumour growth inhibition, constituting an effect on tumour-take or an immunoprophylactic model.

When the initiation of treatment with hybrid-hybrid and VLB was delayed until 15 days after tumour implantation (Fig. 4) at a time when tumour growth was in the exponential phase, it became increasingly difficult to inhibit such growth. Nevertheless, a significant suppression was obtained shortly after the initiation of treatment when compared with untreated controls or with animals treated only with free drug (Fig. 4).

The histological analysis of tumours excised from animals in the experiment illustrated in Fig. 3, revealed that when treated with hybrid-hybrid or hybrid-hybrid plus free VLB, the tumour organisation was totally disrupted, showing balloon-like structures consisting of encapsulated mucilagenous material in which were small islands of surviving tumour cells. The fact that significantly fewer viable tumour cells are seen on hybrid-hybrid-treated tumours suggested that by measuring total tumour volumes and indeed weight, we were understimating the tumour suppression achieved. Experiments designed to test the tumourigenicity of these treated tumours by passaging to fresh untreated nude mice are currently under investigation.

We can reasonably conclude that drug delivered to a tumour target through an antigen combining site of a hybrid-hybrid monoclonal antibody is more efficacious than an equivalent amount of free drug in the absence of the bispecific monoclonal. The significant tumour growth inhibition observed is probably due to the combined effects of an increased local drug concentration on the tumour tissues with the intrinsic anti-tumour activity shown by the hybrid-hybrid.

Further studies are presently being conducted to optimise the treatment schedules, to optimise the doses of hybrid-hybrid and vinca alkaloids and to study therapeutic indices and toxicity.

Acknowledgements. We would like to thank Dr. R. G. Simmonds for helpful discussions and critical review of the manuscript, and Mrs. P. Whiteling for her excellent typing.

References

1. Baldwin RW, Byers VS (eds) (1985) In: Monoclonal antibodies for cancer detection and therapy. Academic Press
2. Bernstein ID, Tam MR, Nowinski RC (1980) Mouse leukemia:therapy with monoclonal antibodies against a thymus differentiation antigen. Science 207: 68
3. Capone PM, Papsidero LD, Croghan GA, Ming Chu T (1983) Experimental tumoricidal effects of monoclonal antibody against solid breast tumours. Proc Natl Acad Sci USA 80: 7328

4. Corvalan JRF, Axton CA, Brandon DR, Smith W, Woodhouse CS (1984) Classification of anti-CEA monoclonal antibodies. Protides of the Biol Fluids 31: 921
5. Corvalan JRF, Smith W (1987) Construction and characterisation of a hybrid-hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. Cancer Immunol Immunother 24: 127–132
6. Corvalan JRF, Smith W, Gore VA, Brandon DR (1987) Specific in vitro and in vivo drug localisation to tumour cells using a hybrid-hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. Cancer Immunol Immunother 24: 133–137
7. Couderc J, Kazatchkine MD, Ventura M, Thien Duc H, Maillet F, Thobie N, Liacopoulos P (1985) Activation of the human classical complement pathway by a mouse monoclonal hybrid IgG1–2a monovalent anti-TNP antibody bound to TNP-conjugated cells. J Immunol 134: 486
8. Kanellos J, Pietersz GA, McKenzie IFC (1985) Studies of methotrexate-monoclonal antibody conjugates for immunotherapy. J Natl Cancer Inst 75: 319
9. Lewis JCM, Smith PA, Keep PA, Boxer GM (1983) A comparison of the content and immunohistochemical patterns of CEA-like activity in human colorectal tumours and nude mouse xenografts. Exp Pathol 24: 227
10. Moller G (ed) (1982) Antibody carriers of drugs and toxins in tumour therapy. Immunol Rev 62
11. Rowland GF, Axton CA, Baldwin RW, Brown JP, Corvalan JRF, Embleton MJ, Gore VA, Hellstrom I, Hellstrom KE, Jacobs E, Marsden CH, Pimm MV, Simmonds RG, Smith W (1985) Antitumour properties of vindesine-monoclonal antibody conjugates. Cancer Immunol Immunother 19: 1
12. Rowland GF, Simmonds RG, Gore VA, Marsden CH, Smith W (1986) Drug localisation and growth inhibition studies of vindesine-monoclonal anti-CEA conjugates in a human tumour xenograft. Cancer Immunol Immunother 21: 183
13. Simmonds RG, Smith W, Corvalan JRF (1984) Affinity purification of anti-CEA antibody. Protides of the Biol Fluids 31: 917

Received August 27, 1986/Accepted October 14, 1986