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Immunomodulation: NK Cells Activated by Interferon-conjugated Monoclonal Antibody against Human Osteosarcoma*

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Abstract—*A mouse monoclonal antibody raised against the human osteogenic sarcoma cell line 791T has been covalently coupled to purified human lymphoblastoid interferon α (IFN α). Conjugation does not interfere with antibody function, as the product binds to 791T cells and mediates complement-dependent tumour cell lysis to a degree equal to that of free antibody. The IFN activity, assessed by augmentation of natural killer (NK)-cell-mediated lysis, is reduced, but the conjugate does augment the killing of 791T and other tumour targets by peripheral blood NK cells. In admixture experiments the conjugate, when bound to unlabelled osteogenic sarcoma cells, also augments the killing of radiolabelled bystander cells. Neither free antibody nor the conjugate mediate antibody-dependent cellular cytotoxicity (ADCC), and augmented tumour cell lysis is a function of NK cell activation. This product provides for an alternative approach to cancer therapy via the activation of infiltrating hose effector cells using specifically targeted lymphokines.*

INTRODUCTION

THE DEVELOPMENT of monoclonal antibodies [1] and their potential for producing antibody–drug and antibody–toxin conjugates of high purity and high specificity [2, 3] has renewed interest in the immunological control of tumour growth. Several such antibody conjugates have been described which exhibit *in vitro* [4–7] and *in vivo* [5–7] activity against tumour cells, but as yet these products have not been developed to the point of clinical application. An alternative approach to cancer therapy has been the use of preparations such as the interferons (IFNs), which may act both directly as antiproliferative agents and indirectly via the activation of pre-existing cellular immune mechanisms, including natural killer (NK) cells [8]. To date, clinical trials with systemic IFN, whilst clearly enhancing peripheral blood NK activity [9, 10], have shown little benefit [10, 11], and this may be due in part to the failure of the IFN to localize when given intravenously. We report here the activity of human lymphoblastoid IFN coupled to a monoclonal antibody raised against the human osteogenic sarcoma cell line 791T. The antibody, designated 791T/36, is a mouse IgG2b and has been described previously [12, 13]. In antiglobulin binding tests using ^{125}I-labelled protein A this antibody reacts strongly with 791T cells and another osteogenic sarcoma, and weakly with two others from a panel of ten such tumours. It has also been established that 791T/36 antibody localizes in human osteogenic sarcoma xenografts maintained in immunodeprived mice [14], and we have recently reported the use of the ^{131}I-labelled antibody for detecting an osteogenic sarcoma in a patient by gamma camera scintigraphy [15]. In addition, 791T/36 reacts with colorectal carcinomas and a trial on the radioimmunodetection of patients with primary and metastatic disease by external

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imaging of ^{131}I-labelled antibody has been reported [16]. Recently we have been able to show that the IFN-conjugated antibody also localizes specifically in sarcoma 791T xenographs in immune-deprived mice [17]. The IFN-conjugated monoclonal antibody, which retains both antibody-binding and complement-fixing activities and which augments NK-cell-mediated lysis of human tumour cells, thus represents a new approach to immunotherapy of cancer. Such a product allows the *in vivo* localization of lymphokines capable of acting on the infiltrating lymphoid effector cells at the tumour site whilst minimizing the side-effects often associated with drugs and toxins.

MATERIALS AND METHODS

Antibodies

The mouse IgG2b monoclonal antibody 791T/36, purified by affinity chromatography using Protein A-Sepharose, has been described in detail elsewhere [12, 13]. The antibody binds to several osteogenic sarcoma cell lines, including 791T, but does not cross-react with the erythroleukaemia K562 or bladder carcinoma T24.

The rabbit anti-human serum used for antibody-dependent cellular cytotoxicity (ADCC) was prepared by multiple injections of *in vitro* cultured Chang liver cells and was kindly donated by Dr Andrew Campbell, Department of Immunology, University Medical School, Nottingham, U.K.

The mouse anti-hepatocyte monoclonal antibody RL23/36 has been described elsewhere [18], and was kindly donated by Dr Christopher Holmes, C.R.C. Labs, Nottingham, U.K.

All antibody preparations were stored at -80°C until used.

Interferon

Purified human lymphoblastoid IFNα (3.24×10^6 reference units/ml; 2.3×10^8 reference units/mg protein) was kindly donated by Dr Karl Fantes, Wellcome Research Laboratories, Beckenham, Kent, U.K., and was stored at -80°C until used.

Effector cells

Peripheral blood mononuclear (PBM) cells were isolated from the blood of normal, healthy donors by centrifugation of whole blood diluted 1:1 with 0.15 M saline at 400 *g* for 20 min on Ficoll-Hypaque (Lymphocyte Separating Medium, Flow Laboratories). The interface was washed 3 times in Hanks' balanced salt solution (HBSS) containing 2% foetal calf serum (FCS). Cell numbers were adjusted to the appropriate concentrations for pre-incubation or cytotoxicity assay in Eagle's minimal essential medium (MEM) containing 5% FCS. PBM cells pre-treated with antibody, IFN or antibody–IFN conjugate were washed a further three times in HBSS–2% FCS and resuspended in MEM–5% FCS.

Target cells

The human osteogenic sarcoma cell line 791T was grown as a monolayer culture in MEM supplemented with 10% FCS. The human bladder carcinoma cell line T24, kindly donated by Dr Michael Moore, Paterson Laboratories, Manchester, U.K., was grown as a monolayer culture in MEM 10% FCS. The human erythroleukaemic cell line K562, kindly donated by Dr David Brown, University of Southampton, U.K., was grown in suspension in RPMI 1640 medium supplemented with 10% FCS.

Antibody–IFN conjugation

The covalent coupling of 791T/36 antibody and IFN using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pharmacia, Uppsala, Sweden) [19] is described elsewhere [17]. Briefly, SPDP-modified ^{125}I-labelled IFN (0.4 mg, assuming $E^{1\%}_{1\,cm} = 10$) was incubated with reduced SPDP-treated ^{131}I-labelled 791T/36 antibody (0.35 mg, assuming $E^{1\%}_{1\,cm} = 14.3$) at room temperature for 3.5 hr and then at 4°C for a further 18 hr. Unconjugated IFN was removed by gel filtration on a Sephacryl S-200 column previously calibrated using protein standards and unconjugated ^{125}I-labelled IFN and ^{131}I-labelled antibody. Fractions eluted from the column and containing double radiolabelled material were pooled, concentrated and dialysed overnight against phosphate-buffered saline (PBS). The purified conjugate was stored in aliquots at -80°C for subsequent testing. Three such batches were prepared in this manner and all showed similar properties [17], but all experiments described below were performed on samples from the same batch.

Complement-dependent cytotoxicity assay

The antibody-mediated cytotoxicity assay was performed as described elsewhere [20]. Briefly, 791T sarcoma cells freshly derived from *in vitro* culture were labelled for 2 hr at 37°C with 2 × KBq ^{51}Cr, washed 3 times and aliquotted into Cooke M24A plastic U-well microtitre plates at 10^4 cells/well. The monoclonal antibody 791T/36, the 791T/36–IFN conjugate or the irrelevant antibody, the mouse monoclonal anti-hepatocyte RL23/36, were added at appropriate concentrations. Reconstituted lyophilized rabbit complement (The Buxted Rabbit Co., Sussex, U.K.) was added to a final concentration of 1/100 and the plate incubated at room temperature for 2 hr, after

which it was centrifuged at 280 *g* for 15 min and 100 μl of the supernatant taken from each well for counting in a gamma counter. Cytotoxicity was calculated from the expression:

$$\% \text{ cytotoxicity} = 100 \times \frac{a - b}{100 - b},$$

where $a = \%$ ^{51}Cr release in the presence of complement and $b = \%$ ^{51}Cr release with the same antibody in the absence of complement.

NK-cell-mediated cytotoxicity assay

This has been described in detail elsewhere [21]. Briefly, ^{51}Cr-labelled target cells in MEM 5% FCS were aliquotted into Falcon 3034 flat bottom microtitre plates at 5×10^3 cells/well. Appropriate numbers of effector cells were added to give effector:target ratios ranging from 50:1 to 1.5:1, 3–4 ratios being tested for each target. Cytotoxicity, measured after 4 hr at 37°C by release of ^{51}Cr into the supernatant, was expressed as the slope of the linear regression line fitted to the plot of cytotoxicity vs effector cell number (for details see reference [21]). Augmentation of cytotoxicity was thus calculated as the ratio of:

$$\frac{\text{slope}_{(\text{treated})}}{\text{slope}_{(\text{untreated})}} \times 100.$$

Statistical significance of augmented cytotoxicity was assessed using Student's *t* test.

Antibody-dependent cellular cytotoxicity assay

The ADCC assay was modified from the NK cell assay described above. ^{51}Cr-labelled target cells were incubated at 4°C at 5×10^5 cells/ml in appropriate dilutions of antibody. After 30 min the cells were diluted 10-fold in MEM–5% FCS and aliquotted into flat bottom microtitre wells and appropriate numbers of PBM cells added to give final effector:target ratios of 6:1–0.8:1. Cytotoxicity was calculated from the ^{51}Cr released after 4 hr at 37°C by comparison of antibody-treated and untreated target cells at the same effector:target ratio, and again expressed as the slope of the linear regression line fitted to the plot of cytotoxicity vs effector cell number.

RESULTS

Antibody activity of 791T/36–IFN conjugate

Direct binding assays measuring ^{125}I-labelled IFN and ^{131}I-labelled antibody bound to 791T cells indicated that the conjugate bound to immobilized cells at levels greater than 80% of that shown by unconjugated, radiolabelled antibody or unconjugated antibody after radiolabelling, SPDP treatment and reduction. In addition, competitive binding assays using fluorescein-labelled antibody and assessed by flow cytofluorimetry on the Fluorescence Activated Cell Sorter (FACS IV) indicated conjugate binding to cells in suspension [17]. The 791T/36 antibody also mediates complement-dependent lysis of 791T cells [22], and the results in Table 1 indicate that the IFN conjugate exhibited no loss of this reactivity when cytotoxicity was assessed at comparable antibody concentrations. In all such experiments the conjugate exhibited a cytotoxic effect against these cells in the absence of added complement (an effect not observed with unconjugated IFN, and restricted to the 791T tumour cells). The reason for this is unclear, but it might be due to a direct effect of IFN bound to cells. The antibody concentrations at which this effect was observed (≥2 μg/ml) were far in excess of those used in subsequent tests, and this effect is therefore unlikely to have contributed to target cell lysis in later expleriments using 791T as radiolabelled targets.

The activity of 791T/36 antibody alone was also tested in two further systems. Figure 1(A) shows the results of preincubating PBM effector cells for 1 hr at 37°C with unconjugated antibody

Table 1. Complement-dependent lysis of 791T osteogenic sarcoma cells mediated by 791T/36 antibody and antibody–IFN conjugate

Monoclonal antibody	Antibody concentration (μg/ml)	% ^{51}Cr release (± S.D.) −complement	+complement	% cytotoxicity
791T/36	4	5.4 ± 0.3	25.6 ± 2.8	21.4
	2	5.8 ± 0.3	21.7 ± 0.7	16.8
791T/36-IFN	4	13.6 ± 0.5	36.1 ± 3.2	26.0
conjugate	2	8.7 ± 0.1	22.5 ± 2.9	15.1
RL23/36	4	5.7 ± 0.2	5.9 ± 0.1	0.2
	2	5.9 ± 0.1	5.6 ± 0.1	-0.3
Medium	-	6.0 ± 0.2	5.6 ± 0.1	-0.4

Cytotoxicity assessed in 2-hr ^{51}Cr-release test at room temperature (see text).

prior to testing for NK cell activity. It is clear that such pretreatment does not enhance target cell lysis in this system, and the killing of K562 and 791T cells is significantly reduced (by 24 and 70% respectively). There is no evidence, then, that the antibody alone enhances NK cell killing by activation of effector cells. Similarly, Fig. 1(B) shows that the 791T/36 antibody is not able to mediate ADCC against either K562 or 791T cells at concentrations of antibody ranging from 10 to 0.1 μg/ml, whereas both targets are equally susceptible to ADCC mediated by PBL and rabbit anti-Chang cell serum.

Augmentation of NK cell activity by 791T/36–IFN conjugate

Figure 2 shows the effects of preincubation of effector PBM cells with 791T/36–IFN conjugate against K562 and 791T cells in 4-hr NK cell assays. PBM cells incubated for 1 hr at 37°C with unconjugated IFN showed optimal augmentation of lysis of the erythroleukaemic cell line K562

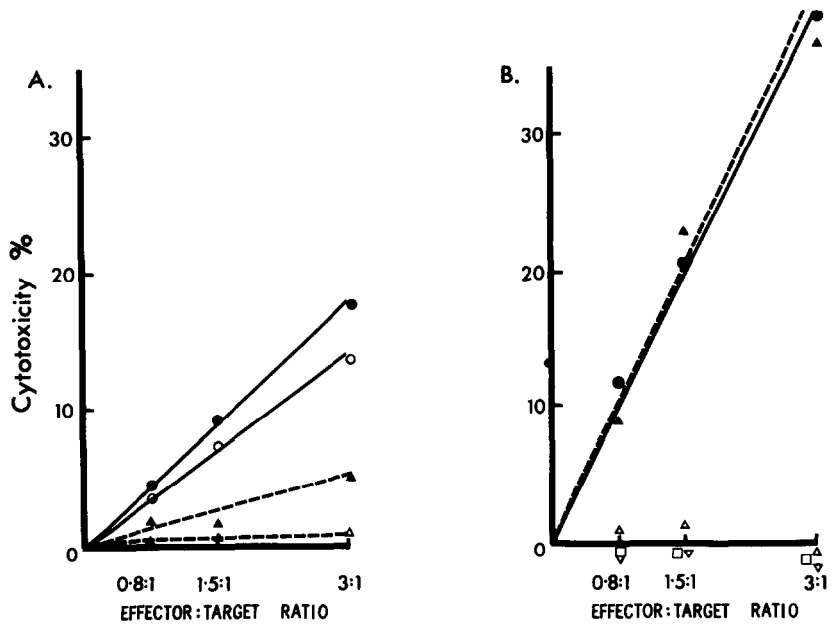

Fig. 1. (A) Effect of preincubating PBL with 791T/36 antibody prior to assay for NK cell activity. PBM cells pretreated with medium alone (solid symbols) or 791T/36 antibody (open symbols) and reacted against K562 (●,○) or 791T (▲,△). (B) ADCC against K562 (●) or 791T (▲) using rabbit anti-Chang cell serum, or 791T using 0.1 μg/ml (△), 1 μg/ml (□) or 10 μg/ml (▽) 791T/36 antibody. Cytotoxicity assessed by ^{51}Cr-release in 4-hr assays (see text).

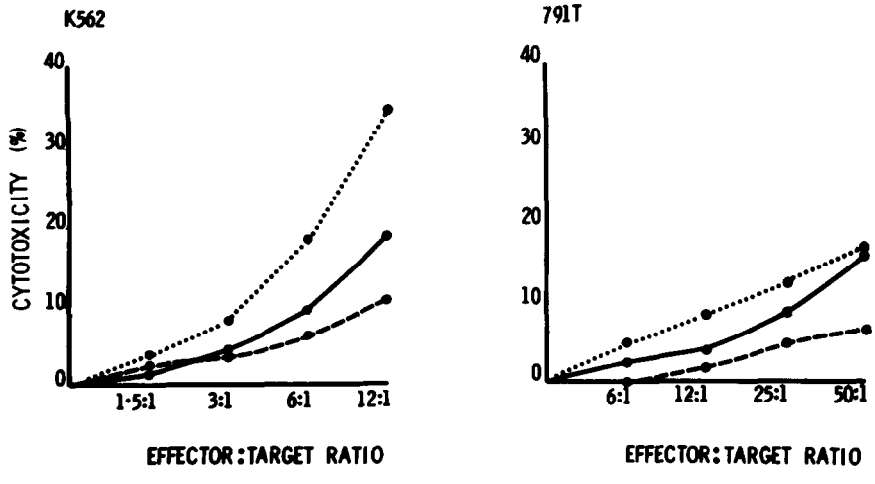

Fig. 2. Augmentation of NK-mediated lysis of K562 (left panel) and 791T (right panel) target cells following preincubation of effector PBM cells for 1 hr at 37°C with medium (– – –), IFN–antibody conjugate (——) at 200 μl/ml/5 × 10^6 cells or unconjugated IFN (· · · ·) at 100 units/ml/5 × 10^6 cells. PBM cells were washed three times and tested in a 4-hr ^{51}Cr-release assay (see text).

(approximately 2-fold) and of the osteogenic sarcoma cell line 791T (approximately 4-fold) at IFN concentrations of 100 units/ml/5×10^6 effector cells. Preincubation of PBM cells with conjugated IFN resulted in augmented killing of both targets, using 200 μl/ml/5×10^6 effector cells. The level of augmentation was, for each target, less than that obtained with unconjugated IFN (approximately 50 and 75% respectively) and was not increased by preincubation of the effector cells with larger volumes of the conjugated material (Table 2). This reduction in the ability to augment NK cell killing may reflect simple steric hindrance by the immunoglobulin molecules or may indicate alterations in the configuration of the IFN itself. (The conjugate exhibited high levels of background toxicity in assays of inhibition of viral replication [Fantes, personal communication] and consequently could not be assessed in terms of standard reference units of IFN.)

Pretreatment of 791T target cells for 1 hr at 37°C with 200 μl of IFN-conjugate, followed by 3 washes, resulted in enhanced lysis by PBM cells (data not shown). Pretreatment of K562 target cells resulted in no augmentation, indicating the requirement for specific antibody binding (see also below).

The antibody–IFN conjugate, then, exhibited both antibody activity and the ability to augment NK-cell-mediated lysis. As an *in vitro* model, of *in situ* activation, the conjugate was further tested for its ability to augment NK cell lysis of 'third-party' target cells after binding to primary targets (Table 3). Unlabelled 791T tumour cells (10^6/ml), preincubated for 1 hr at 4°C with a mixture containing 1.5 μg 791T/36 antibody and 100 units IFN or 200 μl of antibody–IFN conjugate, were

Table 2. Augmentation of NK cell lysis of K562 and 791T cells following preincubation of effector cells with unconjugated IFN or IFN–antibody conjugate

PBM cells pre-incubated with:	Target: K562 Cytotoxicity (Slope ± S.D.)	Target: K562 Augmentation (%)	Target: 791T Cytotoxicity (Slope ± S.D.)	Target: 791T Augmentation (%)
medium	19.74 ± 1.31	—	2.15 ± 0.29	—
IFN(10^2 units/ml)	55.54 ± 1.04	281*	8.94 ± 1.10	415*
791T/36-IFN (200 μl/ml)	29.70 ± 0.55	150*	6.78 ± 0.16	315*

Peripheral blood mononuclear (PBM) cells from normal, healthy donors were preincubated for 1 hr at 37°C and washed three times before testing. Cytotoxicity assessed in 4-hr ^{51}Cr-release test and data subjected to linear regression analysis (see text).
*$P < 0.001$ (Student's t test).

Table 3. Augmentation of NK cell lysis in the presence of 791T osteogenic sarcoma cells preincubated with a mixture of 791T/36 antibody and IFN, or antibody–IFN conjugate

Experiment	Labelled target	Unlabelled 791T pre-incubated with:	Cytotoxicity (slope ± S.D.)	Augmentation (%)
1	K562	medium	6.68 ± 0.39	—
		791T/36, IFN mix	7.46 ± 0.57	112†
		791T/36–IFN conjugate	14.36 ± 2.04	215*
2	791T	medium	2.41 ± 0.31	—
		791T/36, IFN mix	2.29 ± 0.28	95†
		791T/36–IFN conjugate	3.96 ± 0.52	164*
Experiment	Labelled target	Unlabelled T24 pre-incubated with:	Cytotoxicity (slope ± S.D.)	Augmentation (%)
3	K562	medium	8.23 ± 0.20	—
		791T/36, IFN mix	7.81 ± 0.19	95†
		791T/36–IFN conjugate	8.68 ± 0.33	106†

Cytotoxicity assay performed in the presence of unlabelled tumour cells which had been preincubated with conjugate or mixture and then washed three times. Augmentation of killing determined by comparison with wells containing unlabelled tumour cells preincubated in medium alone.
*$P < 0.001$.
‡No significance.

washed three times and added to the wells of a microtest plate during a 4-hr ^{51}Cr-release test using normal PBM cells as effectors and radiolabelled target cells (experiments 1 and 2). The final ratio of unlabelled to labelled target cells was 1:1. In experiment 3 the unlabelled cells (T24 bladder carcinoma) did not bind the 791T/36 antibody [12, 13]. In these experiments NK cell killing of radiolabelled tumour cells (K562 or 791T) was augmented in the presence of unlabelled 791T cells preincubated with conjugate but not in the presence of unlabelled 791T cells preincubated with a mixture of unconjugated antibody and unconjugated IFN (Table 3). These results strongly suggest that IFN, bound via antibody to 791T cells, is capable of augmenting the lysis of other bystander targets to which the conjugate is not bound. That this response is immunologically specific is indicated by the failure to produce augmentation in the presence of target cells (T24 bladder carcinoma) to which the antibody does not bind, after preincubation with either conjugate or mixture. For these experiments preincubation of unlabelled tumour cells was carried out with optimal (100 units/ml) and supra-optimal (500 units/ml) doses of unconjugated IFN, determined in the previous experiments, in admixture with unconjugated antibody, and in no case was there sufficient carry-over, after washing, to provide augmented third-party killing. Preincubation of the tumour cells with 400 μl of conjugate failed to produce a greater augmentation than was observed with 200 μl, the volume required to produce optimal augmentation of preincubated effectors and equivalent to a saturating amount of antibody for the number of tumour cells used [23]. Similarly, increasing the ratio of unlabelled to labelled cells from 1:1 to 4:1 did not consistently result in greater augmentation. In this system, then, maximal augmentation of third-party killing apparently occurs with approximately 50% of target cells carrying bound conjugate after preincubation under conditions of antibody saturation. The failure to further increase the augmented lysis might, however, be explained by increased, but undetected, lysis of the unlabelled tumour cells carrying bound conjugates, acting as competitors and present in excess. The antagonistic tumour protective effect of IFN reported elsewhere [24, 25] is unlikely to have contributed since the IFN levels and time of exposure were significantly less than those shown to be necessary for this protection.

DISCUSSION

Immunotherapy using monokines and lymphokines such as IFN has thus far shown minimal benefit in human cancer [10, 11], although clear evidence has been obtained to indicate at least temporary enhancement of peripheral blood NK cell activity [9, 10]. One reason for this failure may be the inability of the reagent and/or the activated host cells to localize at the tumour site. The anti-tumour antibody–IFN conjugate described in the present work represents an attempt to overcome these problems of localization.

The 791T/36–IFN conjugate, prepared by SPDP covalent coupling, is stable at -80°C for several months, exhibits apparently unaltered antibody binding activity to target cells and retains the ability to mediate complement-dependent tumour cell lysis (Table 1). Although IFN-induced NK cell augmentation is reduced relative to that of free IFN, the conjugate does augment such lysis (Fig. 2, Table 2) and has a potential for localization [17] which the free IFN may lack. The conjugate tests *in vitro* also strongly suggest that the coupled IFN is capable of augmenting the lysis of bystander cells to which the antibody is not bound. There is little likelihood that the results of the later experiments are due to direct activation of effector cells by the antibody (Fig. 1A), nor is it likely that the antibody has mediated ADCC (Fig. 1B). The bystander killing is apparently a function of the conjugated IFN, and this is not the result of reduced competitive activity by IFN- or conjugate-treated tumour cells (data not shown). It seems, then, that the conjugated IFN has the potential for directly augmenting the lysis of bystander cells via NK cell activation.

In animal systems NK cells are demonstrable in the host cell infiltrates of virus- and chemical-induced tumours [26–29]. They are also present in the infiltrates of spontaneous tumours and are capable of further activation with IFN [Ferry and Flannery, unpublished findings]. Such activated effector cells exhibit enhanced cytotoxicity and resistance to the suppressive effects of infiltrating macrophages [Flannery *et al.*, unpublished findings]. Reports to date on human tumour infiltrates have been few and largely negative, and suggest that NK cells may be restricted to effusions [30] and ascitic fluids [31], although the localized injection of immunostimulants such as BCG results in the accumulation of NK cells within solid metastatic lesions [32]. The presence of other cytotoxic effector cells in solid human tumours is, however, well documented [33–36], and recent work suggests that these cells may be further activated by various agents [37, 38]. It would be of interest to determine, in this context, whether the IFN-conjugate was also capable of activating effectors such as cytotoxic T cells, and this is currently under investigation.

Radiolabelled monoclonal antibody 791T/36 has been used clinically to gamma-camera image ostoegenic sarcoma [15] and colonic carcinoma [16] in patients, and the IFN-conjugated antibody has been shown to localize preferentially in xenografts of 791T in immune-deprived mice [17]. Optimal augmentation of NK activity and bystander target lysis occurs with 200 μl of the IFN-conjugate. This material contains approximately 80 ng antibody protein [17], an amount well within the range deposited in xenografts following intraperitoneal infusion of conjugate [17]. It is likely, then, that this and similar conjugates may provide the basis for targeting immunomodulating agents to tumours as a novel approach to immunotherapy. The antibody-IFN conjugate may thus provide a model system for specific *in vivo* localization of various agents such as monokines and lymphokines to the environment of the tumour, leading to the activation, augmentation or recruitment *in situ* of an equally diverse range of host effector cells.

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