Biological activities of human recombinant interferon α/β targeted by anti-Epstein-Barr virus monoclonal antibodies

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The requirement of high doses of interferon (IFN) during therapy severely restrict its application. Thus a model using an Epstein-Barr virus (EBV) membrane antigen (MA) specific monoclonal antibody (MAb) was developed to assess the feasibility of coupling minimal amounts of IFN to a MAb and specifically delivering the IFN to the target cells. Coupled IFN was first shown to retain fully both its anti-viral and anti-proliferative properties when tested on human tumor cell lines QIMR-WIL (EBV-MA$^+$) and the U-266 (EBV-MA$^-$). A series of in vitro pulsing experiments demonstrated the specific targeting of both the anti-viral and anti-proliferative properties of IFN to the EBV-MA$^+$ QIMR-WIL cells and not EBV-MA$^-$ cell lines.

Recombinant interferon *Monoclonal antibody* *Targeting* *Growth inhibition* *Viral protection*

1. INTRODUCTION

The anti-viral and anti-tumour efficacy of both natural and recombinant interferons (nIFN, rIFN) currently available for clinical application has so far been disappointing [1–3]. This may in part be explained by the short half-life of IFN preparations, their lack of target cell specificity, rapid degradation and dilution effects in vivo. These all necessitate increased administration of IFN resulting in severe side effects [1,3,4]. An approach aimed at stabilising IFN or more specifically delivering the IFN to given target cells might help to overcome these problems.

Here we report our initial experiments demonstrating the feasibility of specifically targeting IFNs by coupling them to monoclonal antibodies (MAbs) specific for an Epstein-Barr virus (EBV) membrane antigen (MA) [5]. For this purpose the bifunctional reagent *N*-succinimidyl-3-(2-pyrridyl(dithio)propionate (SPDP) was used because of its very mild cross-linking ability which maintains protein activity and prevents the formation of intramolecular cross-links or homo-polymerization [6]. The targeting efficiency, anti-viral and anti-proliferative activities of the IFN-MAb conjugate were tested on an EBV infected human tumor cell line QIMR-WIL [7] which expresses the EBV-MA and control cells negative for EBV-MA.

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2. MATERIALS AND METHODS

MAb 53S80.17 (γ2b$\varkappa$) specific for EBV-MA p 340 was obtained and purified as in [5]. Human recombinant IFN α/β, used throughout these experiments, was kindly provided by Dr F. Meyer, Basel and affinity purified as in [8].

Human IFN was coupled to MAb 53S80.17 according to [7]. Briefly, to 1 ml of a stirred phosphate-buffered saline (PBS) solution, pH 7.2, containing 6 mg MAb or 100 ng IFN, 8 μl of a 20 mM solution of SPDP was added for 20 min at room temperature (RT). The IFN was then dialysed for 16 h against PBS. The MAb was dialysed against sodium acetate buffer, pH 4.5, followed by incubation with 25 mM dithiothreitol for 15 min and further dialysis against PBS. Derivatized IFN

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and thiolated MAb were then mixed and incubated for 2 h at RT and finally overnight at 4°C. The IFN-MAb conjugate was then passed through a 20 ml Sephadex G-200 (Pharmacia) column to remove any uncoupled IFN.

The activity of IFN was determined in a standard bioassay [9] by measuring the inhibition of Mengo virus challenge (800 PFU/well) on human Hep 2 cells (3×10^4 cells/microtiter well) using an international IFNα standard (B69/19, Natl. Inst. Biol. Stds., UK) as a reference value.

The anti-viral activity of IFN-MAb conjugates was tested on EBV-MA$^+$ QIMR-WIL and EBV-MA$^-$ U266 [10] (both 3×10^4/well in 100 μl). Target cells were incubated in RPMI plus 10% fetal calf serum (FCS) in microtiter plates (Falcon) with either IFN, MAb, IFN-MAb conjugates, MAb + IFN mixtures or medium alone. After overnight incubation Mengo virus (800 PFU/well for both target lines) was added to a test plate or the equivalent amount of medium to a second control plate. After a further 48 h incubation, cells were tested for viability using fluorescein diacetate (FDA). The anti-proliferative activity of IFN-MAb conjugates was assessed in a a similar way, but omitting viral challenge. Instead cells were pulsed for 2 h on day 3 of culture with 0.2 μCi [^{3}H]TdR/well, harvested (Titertek) and counted.

Pulsing experiments were performed aimed at demonstrating the specific targeting of both anti-viral and anti-proliferative activities of IFN by MAb. Target cells, EBV-MA$^+$, QIMR-WIL [7], EBV-MA$^-$ cell lines; Hep2 [11], Namalwa [12], U-266 [10] and ARH-77 [13] were incubated as above but for 15 min at 4°C with either medium, uncoupled IFN, IFN-MAb conjugate, IFN plus MAb mixture, then washed 3 times in a large excess of balanced salt solution and finally distributed at 5×10^4 cells in 100 μl into microtiter wells per test and per control plate for each cell line. Anti-viral and anti-proliferative activities were then assessed as above.

Table 1

Antibody coupled IFN maintains its anti-viral and anti-proliferative activities

Cells treated with	IFN dose (IU/ml)	% protection from lysis		[^{3}H]TdR incorporation			
		QIMR-WIL cells	U-266 cells	QIMR-WIL cells		U-266 cells	
				cpm $\times 10^{-3}$	% inhibition	cpm $\times 10^{-3}$	% inhibition
Medium	0	4	20	36.5	0	14	0
IFN alone	1	58	68	16.8	27	10.6	24
	10	95	100	13.4	34	6.7	52
	100	NT	NT	10.1	45	5.3	62
IFN-MAb conjugate	1	50	69	5.3	86	14.9	22
	10	100	100	2.8	92	10.2	32
	100	NT	NT	0.4	99	5.1	64
MAb alone	0	4	19		8		6

Target cells QIMR-WIL and U-266 were incubated for 2–3 days in the presence of different concentrations of IFN-MAb conjugate, IFN plus MAb mixture, IFN or MAb alone. The anti-viral and anti-proliferative activities were determined as described in section 2. The percent protection from viral challenge was calculated from the cell number found in the absence of virus (= 100% growth) for each IFN dose applied. This reference value was required for each IFN dose, because it was not possible to assay the anti-viral activity without simultaneously invoking IFN induced growth inhibition. Throughout this study all assays were performed in duplicate and all counts were conducted in a double blind manner. The percent inhibition of [^{3}H]TdR incorporation was determined from the controls representing maximal (i.e., 100%) incorporation from which the percent inhibition caused by the various IFN doses was calculated

3. RESULTS

Effective coupling of IFN to MAb was reproducibly found to be 85% as determined by IFN bioassay, protein measurements, and specific antibody determinations (cell binding radioimmunoassay). Radiolabelled IFN experiments demonstrated that Sephadex chromatography (see section 2) successfully separated uncoupled from coupled IFN. The resulting IFN-MAb ratio varied between 10^{-3} and 10^{-4}.

Table 1 demonstrates the maintenance of the anti-viral and anti-proliferative activities of the IFN-MAb conjugate on QIMR-WIL and U-266 cells. The conjugate conferred the same degree of viral protection compared to the uncoupled IFN: 1 IU/ml of uncoupled IFN or IFN-MAb conjugate protected 50–60% of the cells while 10 or more IU/ml IFN resulted in complete protection from viral lysis. Similarly the anti-proliferative effect of IFN-MAb conjugate compared to uncoupled IFN was maintained on QIMR-WIL and U266 cells (table 1). Further, an enhanced inhibition was observed with the relevant conjugate on QIMR-WIL cells in comparison to the uncoupled IFN indicating a specific targeting effect due to MAb.

Short-term pulsing experiments then demonstrated the specific targeting in vitro of IFN-MAb conjugate to the relevant EBV-MA$^+$ QIMR-WIL cells. Full protection from viral challenge was found only in QIMR-WIL cells when treated with IFN-MAb conjugate (table 2). Uncoupled IFN or MAb + IFN mixture did not confer a significant degree of protection in either QIMR-WIL or U266 cells (not shown). A dramatic growth inhibition (96% at 500 IU/ml IFN-MAb conjugate) was only demonstrated when the specific MAb targeted IFN to the EBV-MA$^+$ QIMR-WIL cells (table 3). In contrast no IFN-MAb conjugate mediated growth inhibition could be demonstrated on EBV-MA$^-$ control cells, i.e., Hep 2, Namalwa, U266 and ARH-77 (table 3). In addition, viability counts (FDA) on QIMR-WIL and U266 cells indicated that approx. 50% of the QIMR-WIL cells and not U266 were killed by the targeted conjugate while the remaining cells were arrested in growth for the duration of the culture (3–4 days). Furthermore, similar results were found when natural IFNα was conjugated to EBV-MA$^+$ MAbs using the same methods and assay systems (not shown).

Table 2

Anti-viral effect of IFN targeted by anti-EBV MAb

Cells pulsed with	IFN dose (IU/ml)	Number of viable QIMR-WIL cells ($\times 10^{-3}$)	% protection from lysis	Number of viable U-266 cells ($\times 10^{-3}$)	% protection from lysis
Medium	0	60	6.6	80	20
IFN alone	5	100	20	60	24
	500	150	30	50	20
IFN-MAb conjugate	5	460	92	63	25
	500	490	98	65	26
MAb alone	0	80	16	30	12

Target cells were incubated in 0.2 ml RPMI/10% FCS and pulsed for 15 min at 4°C with varying dilutions of IFNs followed by copious washing. After viral challenge the viable cells remaining were counted using FDA. Results are expressed in terms of percent protection from viral lysis in comparison to the virus free control plate (i.e., no viral challenge = 100% protection) for each IFN dose. Cell numbers in the absence of virus were 900×10^3/ml for QIMR-WIL and 400×10^3/ml for U-266 cells. Note that Mengo virus was only capable of maximally lysing 80% of non-protected U-266 cells and this is seen as a 20% background protection level

Table 3

Anti-proliferative effects of IFN targeted by anti-EBV MAbs

Cells pulsed with	IFN dose (IU/ml)	Target cells/[^{3}H]TdR incorporation									
		QIMR-WIL		HEP 2		NAMALWA		U-266		ARH-77	
		cpm × 10^{-3}	% inh.	cpm × 10^{-3}	% inh.	cpm × 10^{-3}	% inh.	cpm × 10^{-3}	% inh.	cpm × 10^{-3}	% inh.
Medium	0	45–49	0	3–5	0	62–68	0	5–7	0	52–57	0
IFN alone	5	46–52	0	NT		NT		5–7	0	53–56	0
	50	48–51	0	NT		NT		NT		NT	
	500	41–48	6	3.7–3.8	8	61–69	0	5–6	17	54–56	0
IFN-MAb conjugate	5	46–49	0	NT		NT		5–6	17	47–56	6
	50	10–21	68	NT		NT		NT		NT	
	500	2–3	96	3.5–5.2	0	65–67	2	4–7	17	50–56	0
IFN + MAb mixture	5	40–52	2	NT		NT		NT		NT	
	50	44–51	0	NT		NT		NT		NT	
	500	45–50	0	NT		NT		NT		NT	
MAb alone	0	44–45	4	3.1–3.5	12	67–68	0	6–7	0	56–57	0

Target cells were pulsed as for table 2 but without viral challenge. Instead viable counts were performed on day 3 and the cells pulsed for 2 h with [^{3}H]TdR as described in section 2. Results are expressed in terms of percent inhibition (% inh.) of the maximal control values, i.e., in the absence of IFN

4. DISCUSSION

Our results clearly demonstrate the maintenance of IFN's biological activities on coupling to MAb also shown in [14] but more importantly illustrate a potential for specifically delivering these properties to a desired target cell population depending on the specificity of the carrier molecule, i.e., MAb. In this case IFN-MAb conjugate was specifically targeted to the EBV-MA$^+$ cell line QIMR-WIL under very short pulsing conditions (15 min) at low temperature (4°C), to avoid binding of IFN to its receptor and was able to provide full protection from viral challenge and inhibit cell growth. Viable counts indicated a direct cytotoxic effect of the IFN-MAb conjugate on QIMR-WIL cells in contrast to uncoupled IFN which even after very long incubation periods could only arrest but not kill these cells. Further the surprisingly low molar ratio of IFN to MAb (10^{-4}) did not limit the targeting of IFNs potent activities. These findings seem to indicate that once IFN is specifically delivered it affects other cells in the vicinity as well and/or it can be repeatedly used by several cells.

The above observations and our recent data obtained by the use of radiolabelled IFN-MAb conjugates [15] lend hope to the potential application of extremely low doses of IFN in vivo thereby eliminating severe side effects and further imparting target cell specificity and thus localisation of therapeutic effects.

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