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Research paper

A scFv antibody fragment as a therapeutic candidate to neutralize a broad diversity of human IFN-alpha subtypes

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Abstract

Despite their significant role in maintaining the normal physiology, cytokines may cause pathological conditions when they are overproduced. In this way, the increased expression of human interferon alpha (hIFN-α) is associated with acute viral infections, inflammatory disorders and several autoimmune illnesses, where the cytokine may be a factor in either initiating or maintaining the disease.

Currently, there are several mAbs marketed for a variety of indications and many more in clinical trials, in which IFN-α represents a potential target for antibody-based therapy.

A panel of 11 murine mAbs was prepared using recombinant hIFN-α_{2b} as immunogen, all of which bound to the native form of the cytokine with affinity constants ranging from 1.7×10^{7} M^{-1} to 1.4×10^{10} M^{-1}. An epitope mapping protocol demonstrated four spatially distinct areas of the protein recognized by the mAbs. Taking into account the characterization of the antibodies and their ability to inhibit the IFN-α biological activity, four mAbs were selected to produce scFv fragments. One of these fragments (CA5E6) was able to neutralize a wide spectrum of subtypes of the IFN-α family, including the recombinant cytokines hIFN-α_{2a} and hIFN-α_{2b} and a heterogeneous collection of IFN-α produced by activated leukocytes and Namalwa cells.

With the aim of improving the affinity of the selected fragment, a standard error-prone PCR method was carried out. By using this strategy, it was possible to generate a new fragment (EP18) with increased affinity and ability to neutralize a broad diversity of IFN-α subtypes. Consequently, the scFv EP18 represents a potential therapeutic agent for those immune and inflammatory diseases which are associated with an increased IFN-α expression.

Keywords: scFv; Interferon alpha-2b; Neutralizing antibody; Autoimmune diseases

1. Introduction

Type I interferons form a group of structurally related cytokines, being IFN-α, IFN-β and IFN-ω the most representative ones (Kontsek, 1994). There are approximately 30 IFN-α genes (including several pseudo-genes) encoding for more than 10 different proteins with 77–98% sequence identity. On the other hand, there is only one IFN-β and one IFN-ω (Meager, 1998). All type I

Abbreviations: hIFN-α, human interferon alpha; mAbs, monoclonal antibodies; scFv, single-chain antibodies; IFN-β, interferon beta; IFN-ω, interferon omega; HIV, human immunodeficiency virus; SLE, systemic lupus erythematosus; IDDM, insulin-dependent diabetes mellitus; PDCs, plasmacytoid predendritic cells; PBS, phosphate-buffered saline; BSA, bovine serum albumin; T, Tween; MDBK, Madin–Darbin bovine kidney; VSV, vesicular stomatitis virus; $pd(N)_6$, random hexadeoxy ribonucleotides; V_H, heavy-chain variable region; V_L, light-chain variable region; G, glucose; A, ampicillin; K, kanamycin; IPTG, isopropyl β-D-thiogalactopyranoside; K_A, affinity constant; EC50, half maximal effective concentration.

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IFNs bind to a single receptor consisting of at least two membrane-spanning proteins (Roberts et al., 1998; Mogensen et al., 1999).

Although IFN-α exhibits anti-viral and anti-proliferative activities, and is thereby indicated for the treatment of several viral infections and tumors (Gutterman, 1994), its endogenous liberation after an acute viral infection represents a severe damage to the host.

Studies carried out with HIV infected patients showed a strong correlation between IFN-α levels and the progression and severity of the illness (Kramer et al., 1992). In this respect, it was demonstrated that the immunosuppressive Tat protein triggers the IFN-α expression and accumulation in lymphatic tissues infected with the HIV (Zagury et al., 1998).

There are also several autoimmune diseases associated with an increased expression of IFN-α. For example, in patients suffering systemic lupus erythematosus (SLE), increased levels of IFN-α induced by the interaction of immunocomplexes with peripheral blood cells have been observed (Batteux et al., 1999). Additionally, several cytokines, including IFN-α, have been implicated in the progression of insulin-dependent diabetes mellitus (IDDM) in studies carried out with animal and human models. Moreover, the presence of immunoreactive IFN-α in β cells of IDDM patients has been documented (Huang et al., 1995). Recent studies have shown that plasmacytoid predendritic cells (PDCs), the natural IFN-α-producing cells, infiltrate the skin of psoriatic patients and become activated to produce IFN-α early during disease formation. In a xenograft model of human psoriasis, it has been demonstrated that inhibiting the ability of PDCs to produce IFN-α prevented the T cell-dependent development of psoriasis (Nestle et al., 2005).

Antibody-based therapy has gained increased acceptance with several mAbs currently used as therapeutic agents or in late-stage clinical trials. Thus, IFN-α represents an attractive target for antibody development to circumvent those situations where the excessive production of the cytokine can lead to pathological conditions. However, the effectiveness of the use of whole monoclonal antibodies (mAbs) is limited by several factors. The possibility of genetic variations introduced during repeated cycles of cell growth makes mAbs difficult to handle and potentially unreliable. Most importantly, repeated administration of murine antibodies is associated with "serum sickness" in recipients, due to a strong human anti-mouse immune (HAMA) response (Waldmann, 1991). The severity of the HAMA response can be diminished by the use of antibody fragments lacking the Fc domain, particularly single-chain Fv antibodies (scFvs; Bird et al., 1988). In addition, cloning and sequencing retain and immortalize the unique and extensively characterized specificity of mAbs, which can be crucial for the rescue of unstable hybridoma cell lines.

In this work, we describe the development and characterization of a panel of 11 murine mAbs, prepared by using recombinant hIFN-α as immunogen, some of which were used to engineer functional single-chain variable fragments by phage display technology, obtaining a particular scFv able to neutralize a wide spectrum of IFN-α subtypes, produced by stimulated peripheral blood leukocytes and Namalwa cells. Consequently, we report a potential candidate as a single therapeutic agent to counteract the activity of several IFN-α subtypes in pathological conditions where the increased expression of the cytokine correlates with different autoimmune diseases.

2. Materials and methods

2.1. Cytokines

E. coli-derived hIFN-α_{2a} and hIFN-α_{2b} were supplied by Protech Pharma S. A., Argentina. Stimulated peripheral blood leukocytes-derived hIFN-α and Namalwa cells-derived hIFN-α were the WHO reference standards (95/574 and 95/568, respectively) obtained from the National Institute for Biological Standards and Control (NIBSC, UK).

2.2. Animals

Two month-old female BALB/c mice were obtained from Comisión Nacional de Energía Atómica, Argentina. They were housed in a temperature-controlled room at 23 °C with 12 h light/dark cycle and free access to food and water. All animal experiments were in accordance with the "Guide for the Care and Use of Laboratory Animals", and efforts were made to minimize the number of animals used and their suffering.

2.3. mAbs production and purification

Hybridomas were obtained from BALB/c splenocytes after immunization with pure *E. coli*-derived hIFN-α_{2b} using a standard fusion protocol (Galfrè and Milstein, 1981). Hybrids resulting from hypoxantine/aminoptherin/thymidine selection were screened by specific ELISA using 96-well microtiter plates coated with 50 ng rhIFN-α_{2b}. Hybridoma cell lines were gradually expanded and cloned by the limiting dilution

method. Selected clones were injected intraperitoneally in BALB/c mice (2×10^6 hybridomas in 0.5 ml phosphate-buffered saline, PBS) primed with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane; Sigma, USA). After 7–10 days, ascitic fluid was daily drained and the mAbs were purified using protein A-Sepharose 4 Fast Flow (GE Healthcare, Sweden) following manufacturer's guidelines. Antibody concentration was determined spectrophotometrically by measuring absorbance at 280 nm with an $\varepsilon^{1\%}_{1\ cm}$ value of 12.5. The heavy and light chains isotypes were identified with the mouse typer® isotyping kit (Bio-Rad, USA) according to the manufacturer's protocol.

2.4. Determination of antibodies association constants

A competitive ELISA described by Friguet et al. (1985) was used to determine the association constants of the complexes produced between the rhIFN-α_{2b} and the antibodies. The bivalent effect of the complete antibodies (Stevens, 1987) was considered. For the competition, different concentrations of soluble rhIFN-α_{2b} were pre-incubated overnight at room temperature with a constant amount of each mAb or scFv fragment. Then, an aliquot of each mixture was incubated in microtiter plates firstly coated with 50 ng per well of the antigen diluted in coating buffer (50 mM carbonate buffer, pH 9.6) and then blocked with PBS containing 1% (w/v) bovine serum albumin (PBS-BSA) to block non-specific binding sites. The plates were incubated for 2 h at 37 °C. After washing with PBS containing 0.05% (v/v) Tween 20 (PBS-T), bound mAbs scFv fragments were detected using peroxidase-labelled rabbit anti murine antibodies or peroxidase-labelled murine anti-E tag antibodies, respectively. The enzyme conjugates were properly diluted with PBS-T containing 0.1% (w/v) BSA (PBS-T-BSA) and incubated for 1 h at 37 °C. The plates were washed and incubated in the dark with 50 mM citric acid-phosphate buffer, pH 5.3, containing 3 mg *o*-phenylenediamine/ml and 0.12% (v/v) H_2O_2 (substrate solution). The absorbance was measured at 450 nm with a microtiter plate reader (Labsystems Multiskan MCC/340, Finland). The assay was reproduced in triplicate.

2.5. Mapping hIFN-α_{2b}-derived epitopes by competitive binding assay

To determine the number of distinct epitopes and the relationship among the epitopes identified by each mAb, competitive binding assays were carried out. Pure mAbs were conjugated to biotinyl-*N*-hydroxysuccinimide ester (Sigma, USA) according to the method of Bayer et al. (1979). A solid-phase ELISA was used, employing both biotinylated and non-biotinylated mAbs. Microtiter plates coated with 50 ng of rhIFN-α_{2b} per well were incubated for 1 h at 37 °C with PBS-BSA. After washing with PBS-T, plates were incubated with a constant amount of biotinylated mAb in the presence of increasing amounts of homologous or heterologous non-biotinylated mAbs (from 0.78 to 100 μg ml^{-1}) diluted in PBS-T-BSA. After incubating for 2 h at 37 °C and washing with PBS-T, the plates were incubated for 1 h at 37 °C with peroxidase-labelled streptavidin diluted 1/500 with PBS-T-BSA. Finally, plates were washed and incubated with the substrate solution as described above.

To quantify the competitive effect of each mAb, the binding of the biotinylated mAb to the immobilized antigen in the presence of the maximum amount of non-biotinylated (competitor) mAb was estimated by the following formula:

$$Binding\ (\%) = \frac{A-N}{A_0-N}100$$

where A, A_0 and N represent absorbance values corresponding to the binding of the biotinylated mAb in the presence of the maximum concentration of competitor mAb, the binding of the probe in absence of the competitor and the negative control, respectively.

2.6. Neutralization of rhIFN-α_{2b} biological activity by antibodies

A bioassay based on the MDBK cell line (ATCC CCL-22) challenged with vesicular stomatitis virus (VSV) Indiana strain (ATCC VR-158) was used. Briefly, MDBK cells were grown overnight in 96-well plates (2.5×10^4 cells per well). The following day, 1/5 serial dilutions of each purified mAb or scFv were pre-incubated for 2 h at 37 °C with equal volume of 4 U ml^{-1} of several subtypes of hIFN-α and added to each well containing the cells. Following an incubation period of 6 h, the supernatant was removed and an appropriate VSV suspension (which generates a 100% cytopathic effect after 24 h) was added to each well. After incubating overnight, viable cells were measured by the crystal violet stain method. The assay was reproduced in triplicate.

2.7. Construction of the scFv phage library

mRNA was isolated from 1×10^7 hybridoma cells using the QuickPrep mRNA Purification Kit (GE Healthcare) according to the manufacturer's recommended protocol.

All the steps involved in the construction of scFvs were carried out using the Recombinant Phage Antibody

System (RPAS™) kit (GE Healthcare) in accordance with the manufacturer's instructions. First-strand cDNA synthesis was performed separately for the light and heavy antibody chains using Moloney Murine Leukemia Virus reverse transcriptase and random hexadeoxy ribonucleotides [pd$(N)_6$] primers. Then V_H and V_L genes were PCR amplified from cDNA using partially degenerated primers which were complementary to the opposite ends of the variable region of each chain (primers were supplied by the kit). The PCR reaction program was run as follows: 30 cycles at 95 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min. The DNA encoding the heavy and light chains was gel purified and assembled into a single gene (scFv) by overlap extension PCR using DNA linker primers that encode the sequence $(Gly_4Ser)_3$. The V_H, V_L and the linker primers were mixed in equimolar ratio and after an initial denaturation for 5 min at 94 °C, the reaction proceeded for seven rounds at 94 °C for 1 min and 63 °C for 4 min. The scFv fragments were then amplified by a second PCR, using a mixture of V_H-specific primers containing a 5′-SfiI site and a mixture of V_L-specific primers containing a 3′-NotI site; the same conditions for amplification of V_H and V_L described above were used.

The amplified product was gel purified and sequentially digested with SfiI and NotI restriction enzymes (GE Healthcare). After purifying the digested product, 100 ng was used for ligation with 125 ng of the phagemid pCANTAB 5E, treated with the same enzymes. The sequences of all constructions were verified by dideoxy DNA sequencing.

The ligated vector was transformed into competent *E. coli* TG-1 cells (GE Healthcare) using the calcium chloride procedure (Sambrook et al., 1990).

2.8. Expression of phage-displayed scFv fragments

TG-1 transformants were grown in 2×YT broth containing 2% (w/v) glucose (2×YT-G) and then infected with 4.10^{10} pfu of M13KO7 helper phage. Phage rescue was performed in 2×YT broth containing 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin (2×YT-AK). Recombinant phage particles obtained in culture supernatant were concentrated by precipitation with 10% (v/v) poly(ethylene glycol) 8000 (Sigma), 14% (w/v) NaCl. The precipitate was resuspended in 2×YT broth and the suspension was used in biopanning.

2.9. Selection of antigen-positive recombinant phage antibodies by biopanning

Three rounds of panning were performed in 96-well microtiter plates coated with 10 μg ml^{-1} rhIFN-α_{2b}. Blocking was performed with PBS containing 2% nonfat dry milk followed by washing with PBS. Then, phages were diluted in blocking buffer, applied in aliquots of 200 μl to each well and incubated for 2 h at 37 °C. After extensive washing with PBS containing 0.1% Tween 20 and PBS, bound phages were used to reinfect *E. coli* TG-1 cells. After incubation for 1 h at 37 °C, the contents of each well were pooled and plated out on SOB agar (supplemented with 100 μg ml^{-1} ampicillin and 2% glucose). Individual colonies were grown overnight in 2×YT-AG. After phage rescue with M13KO7 helper phage, the culture was incubated for 2 h at 37 °C with shaking. Cells were spun down and resuspended in 2×YT-AK and incubated overnight at 37 °C. Recombinant phage particles produced in culture supernatant were screened to detect hIFN-α_{2b} positive clones by ELISA. Briefly, supernatants were incubated for 1 h at 37 °C in microtiter plates firstly coated with 100 ng per well of the antigen diluted in coating buffer and then blocked with PBS-BSA. After washing with PBS-T, bound scFv was detected using peroxidase-labelled murine anti-E tag antibodies properly diluted with PBS-T-BSA. Finally, the plates were washed and incubated with substrate solution as described above.

2.10. Infection of E. coli HB2151 cells

Recombinant phages from positive clones were used to infect non-suppressor *E. coli* HB2151 strain for the production of soluble scFv antibody. HB2151 cells were grown in 2×YT medium at 37 °C. Aliquots of 400 μl of log-phase cells were mixed with 2 μl of positive phage containing supernatant and incubated with gentle shaking for 30 min at 37 °C. Infected cultures from each clone were streaked on individual SOB agar plates (supplemented with 100 μg ml^{-1} ampicillin, 100 μg ml^{-1} nalidixic acid and 2% (w/v) glucose) and incubated overnight at 30 °C.

2.11. Preparation of soluble antibodies

Individual colonies from SOB agar plates were transferred to 5 ml 2×YT-AG medium and incubated overnight at 30 °C with shaking. The culture was diluted to 50 ml and incubated for 1 h at 30 °C. Then, cells were centrifuged, resuspended in 50 ml 2×YT-AI medium (2×YT medium containing 100 μg ml^{-1} ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside, IPTG) and grown for at least 4 h at 30 °C. Each culture was split into two equal samples and centrifuged. The supernatants were collected, filtered through 0.45 μm and stored at 4 °C.

To prepare periplasmic extract, one of the two cell culture pellets was resuspended in 0.5 ml ice-cold 1× TES (0.2 M Tris–HCl, pH 8.0; 0.5 mM EDTA; 0.5 M sucrose) followed by the addition of 0.75 ml ice-cold 1/5× TES and incubated on ice for 30 min. The contents were transferred to a 1.5 ml microcentrifuge tube and spun at full speed for 20 min at 4 °C. The supernatant was carefully transferred to a clean tube and stored at −20 °C. Whole cell extracts were prepared resuspending the second pellet in 0.5 ml PBS and boiling for 5 min. Cell debris was pelleted as described above and the supernatant was stored at −20 °C.

The supernatant, periplasmic and whole-cell extract fractions were analyzed for the presence of soluble antibodies by ELISA as described above.

Besides, soluble recombinant scFvs were purified from *E. coli* periplasmic extract by affinity chromatography on the immobilized anti-E tag monoclonal antibody (RPAS purification module, GE Healthcare), according to the manufacturer's recommendations. Recombinant antibody concentrations were determined spectrophotometrically, measuring absorbance at 280 nm and ε values were calculated for individual sequences (Gill and von Hippel, 1989). ScFv purity was analyzed by SDS-PAGE followed by silver staining (Bio-Rad).

2.12. Random mutant scFv library

A mutant scFv library was generated by error-prone PCR. Conventional PCR conditions in the presence of Taq polymerase were applied by adding 0.150 mM $MnCl_2$ to induce a low rate mutagenesis (Cadwell and Joyce, 1992). Primers used to amplify the mutant library were epFw (5′ CCT TTC TAT GCG GCC CAG CC 3′) and epRv (5′ AGC GGA TCC GGA TAC GGC AC 3′), which hybridized with pCANTAB-scFv CA5E6 DNA. The PCR reaction program was run as follows: 30 cycles at 95 °C for 1 min, 58 °C for 2 min, 72 °C for 2 min. The amplified product was digested with SfiI and NotI enzymes and ligated with pCANTAB 5E phagemid. Then, the construction of the scFv phage library and the selection of antigen-positive recombinant phage antibodies were performed as already described with some modifications. After 3 rounds of panning using the standard protocol, a further round with stringent conditions was developed to allow the selection of improved clones by immobilizing 0.1 μg ml^{-1} IFN-α_{2b} in ELISA plates and washing with glycine 0.1 M pH 2 solution during the phage-scFv incubation. The high-stringency condition of pH 2 was selected, taking into account the work of Ceaglio et al. (2007), who demonstrated that IFN-α_{2b} could only be eluted by using this condition from an immunoaffinity matrix where mAb CA5E6 was immobilized.

Soluble expression and purification of mutated scFvs were carried out as it was described above.

3. Results and discussion

3.1. mAb characterization

Bearing in mind the capacity to bind to rhIFN-α_{2b} coated to plastic plates and the IgG immunoglobulin isotype, 11 mAbs were selected after the cloning process.

This method usually detects antibodies by recognizing insoluble and partially unfolded forms of rhIFN-α_{2b}. Therefore, association constants were determined to analyze whether mAbs could also bind to the soluble cytokine (Table 1). All antibodies exhibited significant binding capacity when antibodies and cytokine were in liquid-phase under experimental conditions. The antibody panel showed a wide range of affinity constants (K_A). mAbs CA1A3 and CB27H2 were those with the highest affinity constants.

3.2. rhIFN-α_{2b} epitope mapping

A solid-phase mutual competition assay (Kuroki, 1996) was developed to analyze the epitope specifity of mAbs in terms of their binding to analogue or overlapped epitopes (identity) or different epitopes (non-identity).

Firstly, rhIFN-α was used as biotin-labelled probe. Unfortunately, the majority of the mAbs could not interact with the biotinylated cytokine. Therefore, each mAb was conjugated with biotin and further analyzed for cytokine binding. After labelling, 9 of the 11 biotinylated mAbs preserved their ability to bind the cytokine (biotinilated

Table 1
mAb characterization

Antibody	Isotype	K_A (10^8 M^{-1})	Neutralizing activity (pM)
CA1A3	IgG_1/κ	140±10	45±13
CA2G7	IgG_{2a}/κ	1.5±0.1	126±30
CA5E6	IgG_1/κ	2.2±0.1	130±60
CA8B7	IgG_1/κ	0.47±0.04	–
CA9C3	IgG_1/κ	0.41±0.03	–
CB13A7	IgG_1/κ	1.5±0.1	–
CB15D7	IgG_1/κ	5.2±0.4	120±10
CB24D5	IgG_1/κ	0.17±0.01	–
CB27H2	IgG_1/κ	11±2	3±1
CB29D3	IgG_1/κ	1.3±0.1	873±200
CB33B3	IgG_1/κ	0.51±0.04	–

mAbs CA9C3 and CB13A7 could not bind the rhIFN-α_{2b}, data not shown).

As expected, homologous mAbs (the same biotinylated and non-biotinylated antibody) completely competed for binding to the cytokine. Contrarily, heterologous mAbs produced different competition patterns (Fig. 1): complete competition (0–20% biotinylated mAb interaction), partial competition (21–80%) and non-competition (more than 80% interaction). mAbs were thus classified into four groups according to their binding profile. Consequently, competition experiments among antibodies were useful to outline four non-overlapping rhIFN-α_{2b} epitopes.

3.3. Inhibition of rhIFN-α_{2b} biological activity

The ability of mAbs to neutralize the anti-viral biological activity of IFN-α and the correlation with their binding epitopes were investigated. The anti-viral activity of rhIFN-α_{2b} was evaluated in the presence of mAbs and compared with the anti-viral activity in absence of antibodies. The neutralizing antibody titer (EC50) was defined as the concentration of antibody which neutralizes 50% of the anti-viral effect induced by 4 U ml^{-1} of hIFN (Table 1). Antibodies from epitope groups A and B neutralized the rhIFN-α_{2b} biological activity in a dose-dependent manner (Fig. 2), showing mAb CB27H2 the highest inhibition capacity. Antibodies CA1A3, CA2G7, CB15D7, CA5E6 and CB29D3 showed lower neutralizing ability, being mAb CB29D3 the antibody with the lowest one. These results should be

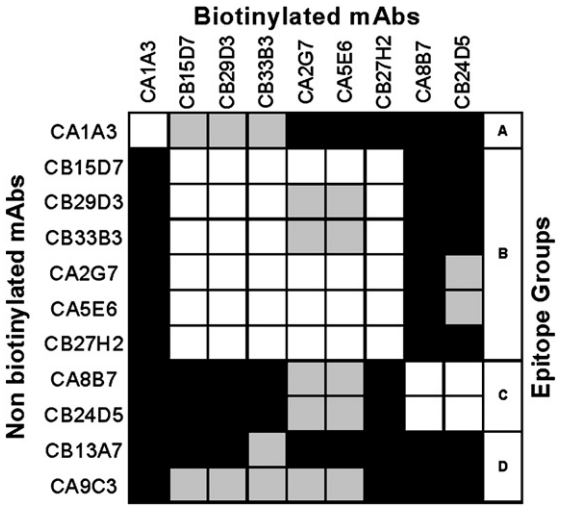

Fig. 1. Reciprocal competitive binding assays using biotinylated mAbs. Three competition patterns were defined: □ complete (0–20% of probe binding), ▒ partial (21–80%) and ■ non-competition (more than 80% of probe binding).

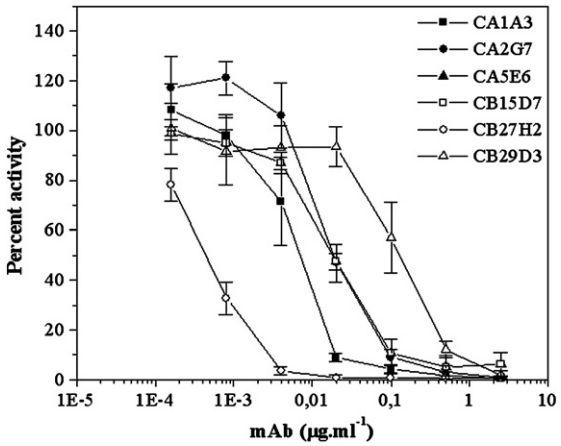

Fig. 2. rhIFN-α_{2b} was assayed for anti-viral activity in the presence of increasing concentrations of mAbs. Data points represent mean values ±SD (n=3).

considered with caution since neutralizing activity depends on both mAb affinity and mAb ability to block a receptor binding area of the cytokine. Besides, for those antibodies which recognize a common epitope, it is supposed that their increasing affinity is related to their increasing neutralization. In this case, considering group B, mAb CB27H2 with the highest affinity for rhIFN-α_{2b} showed the greatest neutralizing activity, whereas mAb CB33B3 with the lowest K_A was not able to inhibit the IFN biological function. However, mAbs CA2G7 and CB29D3, with similar cytokine affinity, neutralized the *in vitro* biological activity with different capabilities (89- and 1653-fold excess antibody was required to inhibit 50% of the rhIFN-α_{2b} anti-viral activity, respectively). Therefore, these antibodies might bind to distinct domains inside epitope group B, which would compromise IFN-α biological action in a different way.

Comparing epitopes A and B, we observed that mAb CA1A3, having a ten-fold higher affinity than CB27H2, showed a slightly lower neutralizing activity (16- and 9-fold excess, correspondingly). Therefore, the binding to epitope group B by mAb CB27H2 would generate a more efficient mechanism to impair IFN-receptor recognition.

On the other hand, none of the antibodies from groups C and D were able to neutralize the IFN biological activity (Table 1). This might be due to affinity issues (mAbs CA8B7, CA9C3 and CB24D5 showed the lowest affinity constants) or to the binding to IFN biological activity non-related areas since mAb CB13A7, having the same K_A than the neutralizing mAb CA2G7 (group B), was not able to inhibit the cytokine biological effect.

3.4. Isolation of single-chain antibodies from mAbs

Considering the affinity constant, the inhibition of rhIFN-α_{2b} biological activity and the recognition of different molecular areas of the cytokine, four mAbs were selected to produce scFv fragments (CA1A3, CA5E6, CB15D7 and CB27H2). Total mRNA was isolated from hybridomas and converted to cDNA. Immunoglobulin genes (V_H and V_L) were PCR amplified, assembled with the linker DNA fragment, and the resulting scFv antibody DNA was ligated into the pCANTAB 5E expression vector and used to transform *E. coli* TG1 cells. In the presence of the helper phage M13KO7, scFvs were displayed on recombinant phage tips allowing for specificity and affinity selection.

It is well known that truncations and mutations can be generated due to PCR and cloning processes. Therefore, the cloned library was enriched for functional scFvs by biopanning over antigen coated ELISA plates. After 3 rounds of selection on wells coated with 1 μg ml^{-1} rhIFN-α_{2b}, only scFv phages CA5E6 and CB27H2 were able to recognize the immobilized antigen. Further experiments (data not shown) determined that the mAb CA1A3 secreting hybridoma preferably produced an aberrant mRNA, which is transcribed from rearranged but non-functional heavy-chain genes. The aberrant chains (in this case a V_H chain) may greatly dilute the appropriate antibody sequences, which are the only that bind to the antigen in a pool of non-productive antibody-like sequences (Krebber et al., 1997). With regard to mAb CB15D7, the oligonucleotide set used to assemble V_H and V_L chains into a scFv fragment always produced a smaller product (650 bp instead of 750 bp of the correct fragment). Moreover, as the nucleotide sequence of its V_H and V_L chains (data not shown) were very similar to those from mAb CB27H2 (which binds to the same epitope), mAb CB15D7 was discarded.

The corresponding scFv phage was used to infect the non-supressor strain *E. coli* HB2151 for the soluble expression of clones CA5E6 and CB27H2 clones. The synthesis of scFv CA5E6 was analyzed in whole-cell lysates, in periplasmic lysates and in culture medium. Soluble scFv was detected mainly in the periplasmic fraction by SDS-PAGE followed by western blot, employing an anti-E-tag murine antibody (data not shown). The specific reactivity of soluble fragments (in the different fractions) was evaluated by ELISA in plates coated with rhIFN-α_{2b}. The specific binding was confirmed with scFvs secreted into culture medium and periplasmic fraction, being the intensity of the signal a function of the incubation time. During short-term cultures, the scFvs were mainly secreted into the bacterial periplasm but after a longer incubation time, the scFv concentration was higher in the culture supernatant. Otherwise, those fragments obtained in the whole-cell fraction were not able to recognize the antigen, probably due to folding issues in the cellular cytoplasm (Fig. 3).

Soluble scFvs were purified from bacterial periplasm by affinity chromatography and their purity was evaluated by SDS-PAGE followed by silver staining. The antibodies were visualized as a 30 kDa band (Fig. 4) showing a purity of 80%.

3.5. scFv characterization

The association constants of the complexes produced between the rhIFN-α_{2b} and the scFvs were determined as outlined before. scFv CA5E6 and the parental mAb

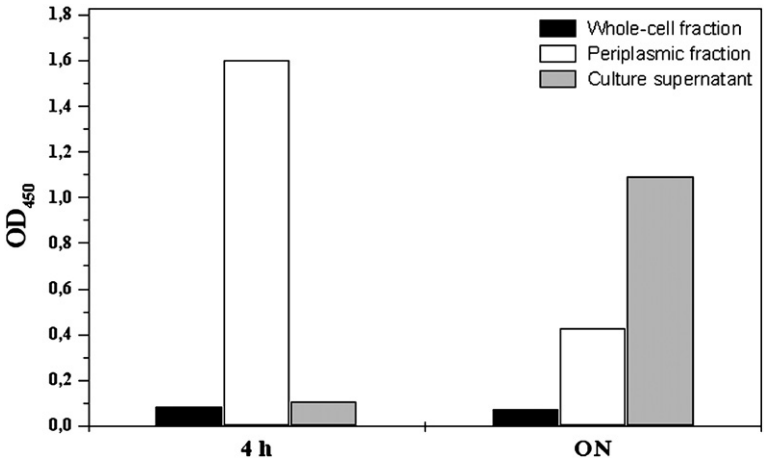

Fig. 3. Indirect specific ELISA of soluble scFv antibodies. IPTG induced cultures were grown at 30 °C either for 4 h or overnight with gentle agitation. Supernatant, periplasmic and whole-cell-derived fragments were assayed using a horseradish peroxidase-labelled anti-E-tag mAb.

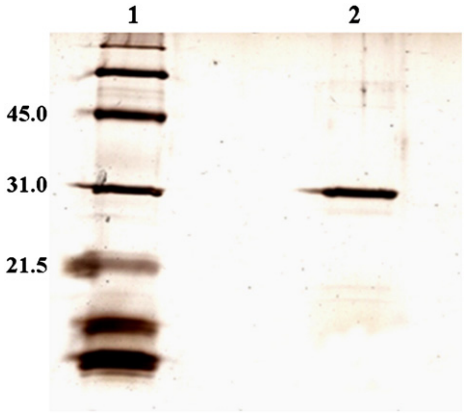

Fig. 4. SDS-PAGE analysis of the scFv CA5E6 after affinity purification. Lane 1: molecular mass standards (Bio-Rad), lane 2: concentrated eluate from anti-E-tag affinity chromatography.

showed very similar K_A (Table 2). In contrast, scFv CB27H2 showed a considerably lower affinity for rhIFN-α_{2b} compared to its corresponding mAb (a 29-fold decrease in the K_A was observed). It is likely that the epitope binding site of scFv CB27H2 was not able to achieve the proper conformation due to the lack of constant regions. The variable regions could probably be stabilized by the flexible linker in the case of scFv CA5E6, thus preserving the affinity of the original paratope.

Taking into account that several quite divergent IFN-αs are detected in diseases where IFN-α is over-expressed (Huang et al., 1995), the selected antibody would have to neutralize most of the IFN-α's variants. However, this is not the case of most IFN-α-neutralizing antibodies previously described (Noll et al., 1989; Viscomi et al., 1999) Therefore, we evaluated the ability of scFv CA5E6 and CB27H2 to block the bioactivity of IFN-αs produced by activated leukocytes and Namalwa cells (leukocytary and lymphoblastoid IFNs, respectively) as well as the biological activity of rhIFN-α_{2a} and rhIFN-α_{2b}, using MDBK cells challenged with VSV virus. By this approach, it was demonstrated that scFv CA5E6 was able to neutralize the wide spectrum of IFN-αs produced by activated leukocytes and Namalwa cells (Table 3). This was quite an unexpected result considering that the original mAb was developed by

Table 2
scFv and mAb association constants

	mAb (10^8 M^{-1})	scFv (10^8 M^{-1})
CA5E6	2.2±0.2	1.50±0.40
CB27H2	11.0±2.0	0.37±0.07

Table 3
scFv neutralizing activity against different IFN-αs

	CA5E6 (nM)	CB27H2 (nM)
IFN-α_{2a}	0.21±0.10	3.12±1.25
IFN-α_{2b}	0.40±0.07	3.75±0.75
Leukocytary IFN	25±8	–
Lymphoblastoid IFN	12±8	–

immunizing mice with rhIFN-α_{2b}. scFv CA5E6 would probably recognise a molecular area shared by the group of divergent IFN-αs tested. On the other hand, scFv CB27H2, whose parental mAb bound to the same epitope as mAb CA5E6, was not able to neutralize the activity of the diverse set of IFN-αs. This result can be explained considering the drastic drop of the fragment association constant in comparison with the original mAb. Furthermore, the anti-viral activity of rhIFN-α_{2a} and rhIFN-α_{2b} was considerably less affected by the scFv CB27H2 than by the scFv CA5E6 (a 15-fold and 9-fold decrease in the neutralizing activities was observed for cytokines), supporting the idea of a strong correlation between antibody affinity and neutralizing activity.

3.6. ScFv CA5E6 affinity maturation

Having established that CA5E6 was able to neutralize a wide diversity of IFN-αs, the scFv CA5E6 DNA

Table 4
Description of the selected mutants

Clone	Number of mutations	Substituted residue [a]	Region [b]	K_a (10^8 M^{-1})
EP5	2	S88P	Fw3 V_H	0.92±0,09
		T51A	CDR2 V_L	
EP6	1	R40G	Fw2 V_H	1.01±0.01
EP11	2	V34M	CDR3 V_H	1.43±0.07
		S56G	CDR2 V_L	
EP12	4	I78V	Fw3 V_H	0.37±0.09
		T107A	CDR3 V_H	
		S122P	Fw4 V_H	
		D70E	Fw3 V_L	
EP18	1	T58A	CDR2 V_H	3.50±0.10
EP20	1	I48V	Fw2 V_L	1.40±0.10
EP33	2	E42G	Fw2 V_H	3.20±0.30
		M11V	Fw1 V_L	
EP34	3	V104L	CDR3 V_H	1.13±0.04
		G129D	Linker	
		S12T	Fw1 V_L	
EP35	1	E10V	Fw1 V_H	1.23±0.02
EP36	2	R40G	Fw2 V_H	1.50±0.10
		S56R	CDR2 V_L	
EP49	1	K24R	Fw1 V_H	1.70±0.01

[a] Individual numbering of variable chains according to Chotia and Lesk (1987).
[b] CDR, complementary determining regions; Fw, framework.

segment was used as template to generate a random library using an error-prone PCR approach with the aim of improving the antibody's affinity. The analysis of the DNA sequences from a library showed that mutations were all different and randomly distributed throughout the frameworks, CDRs and linker (data not shown).

In order to isolate a scFv fragment with an improved binding capacity, a mutagenic library was panned against decreasing concentrations of rhIFN-α_{2b} using stringent conditions. After the fourth round of panning, the soluble scFvs from 94 colonies were analyzed by specific ELISA. Then, rhIFN-α_{2b} specific scFv fragments were produced and purified from the periplasmic fraction. Finally, a new panel of 11 scFv fragments bearing 1 to 4 aminoacidic substitutions was conformed (Table 4).

The association constants of the mutated scFv fragments were determined as outlined before. Although all scFvs with association constants equal to or lower than the parental scFv CA5E6 should have been eluted at pH 2, a broad range of affinities was obtained (Table 4). While EP18 and EP33 clones showed an increase in the association constant (3.50×10^8 and 3.20×10^8 M^{-1}, respectively), other clones suffered a significant affinity decrease, such as EP12 (carrying four point mutations). It is likely that some mutated aminoacid could improve the complex stability at pH 2 (during the biopanning) but conversely, the same change in the aminoacid sequence or another aminoacid change could be harmful to the EP12-IFN-α binding energy in the conditions used for the measurement of the affinity constant.

The scFv fragments EP18 and EP33 were analyzed in their ability to neutralize the biological activity of rhIFN-α_{2b}. In spite of its increased binding capacity, scFv EP33 showed a drop in its neutralizing activity comparing to scFv CA5E6 (1.00 nM and 0.40 nM, respectively). In contrast, 2-fold less amount of scFv EP18 (0.20 nM) was needed to block the 50% of the cytokine anti-viral activity in comparison with the parental scFv CA5E6. Additionally, scFvs EP18 and CA5E6 showed a comparable *in vitro* neutralization ability when leukocytary and lymphoblastoid IFNs were assayed (data not shown).

4. Conclusions

From a well-characterized panel of eleven mAbs developed using rhIFN-α_{2b} as immunogen, two scFv antibody fragments were obtained. One of these fragments (identified as CA5E6) could retain the binding properties of the parental mAb and could also efficiently neutralize the *in vitro* anti-viral activity of the rhIFN-α_{2b}. Most notably, this scFv fragment was able to block the activity of a wide spectrum of IFNs, including IFN-α_{2a}, IFN-α_{2b}, leukocytary and lymphoblastoid IFN. Then, an error-prone PCR approach was developed generating a scFv-mutated library, which was panned against rhIFN-α_{2b} using stringent conditions. As a result, a new antibody fragment (EP18) with increased affinity constant and improved neutralizing activity was generated.

The possibility to produce an antibody fragment like scFv capable of neutralizing a soluble cytokine is a valuable alternative in the field of therapeutic antibodies since, having a reduced size and being less immunogenic in humans, they constitute the main scaffold to obtain humanized antibodies. Moreover, the broad IFN-α specificity and proper affinity of scFv EP18 would support a suitable platform to be used in those diseases where the production of this cytokine is exacerbated, such as systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis and psoriasis.

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