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Monoclonal antibodies to human interferon-γ: production, affinity purification and radioimmunoassay

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Human interferon-γ (IFN-γ) purified to electrophoretic homogeneity by a cation exchange h.p.l.c., was used for the development of monoclonal antibodies. Following immunization, spleen lymphocytes of two mice showing the highest binding and neutralizing titers were isolated, fused with NSO mouse myeloma cells and cloned. The screening of hybridomas was based on precipitation of the immune complexes with a second antibody and recovery of the biological activity of IFN-γ from the precipitate. Twenty nine independent hybridomas secreting antibodies specific to IFN-γ were obtained. Twelve out of these 29 hybridomas produced antibodies that neutralized the antiviral activity of pure as well as crude IFN-γ. Moreover, IFN-γ obtained by various induction procedures was neutralized as well, indicating that these various IFN-γ subtypes are immunologically cross-reactive. Immune precipitation of partially purified ^{125}I-labelled IFN-γ by several monoclonal antibodies revealed two protein bands of 26 000 and 21 000 daltons. Immunoaffinity chromatography of IFN-γ gave a 50-fold purification to a specific activity ≥4 x 10^7 units/mg. Two of the monoclonal antibodies were found suitable for a sensitive and rapid double antibody solid-phase radioimmunoassay, allowing the detection of IFN-γ at concentrations of at least 4 ng/ml (150 units/ml) within 8 h.

Key words: hybridoma/immunoadsorbents/interferon-γ/solid-phase radioimmunoassay

Introduction

Monoclonal antibodies (mAb) are useful tools for purification, assay and monitoring of many biologically active molecules (Kohler and Milstein, 1975). Highly specific screening procedures based on a unique property of the antigen can be employed for selection of hybridomas even if the antigen is available only as a minor component in a complex mixture. Human interferon (IFN) (Stewart *et al.*, 1980) is a case in point. In most cases described so far, crude IFN preparations, containing <1% specific antigen, were used for immunization. The low specific activity was balanced by highly specific and sensitive screening procedures based on the antiviral activity of IFN (Secher and Burke, 1980; Hochkeppel *et al.*, 1981; Hochkeppel and de Lay, 1982). In spite of this possibility, immunization with a highly purified antigen increases the yield of specific hybridomas, thereby allowing selection of those that are most useful for a particular application.

We have recently developed hybridomas secreting mAb to human IFN-α (Novick *et al.*, 1982) and IFN-β (Novick *et al.*, 1983), which were selected by a new procedure based on formation of an IFN-anti-IFN complex, precipitation with a second antibody and recovery of the biological activity from the precipitate by acid dissociation. The last step mimics the elution conditions during affinity chromatography and therefore increases the chances of obtaining antibodies that are suitable for affinity chromatography. We now report production, screening and the use of mAb to IFN-γ. Since IFN-γ is acid-labile, a modified screening procedure was employed.

Results

Immunization of mice and cell fusion

The immune response in mice injected with IFN-γ was followed by the direct binding assay, by solid-phase radioimmunoassay (RIA) and by neutralization of IFN activity. After five injections, an average binding titer of 2 x 10^7 units/ml of serum and a neutralizing titer of 6 x 10^4 units/ml was obtained in BALB/c mice. The same sera were tested in the solid-phase RIA and at a dilution of 1:50 000 the average counts were five times higher than those of a control serum. A similar immunization schedule in C57Bl/6J mice did not elicit any immune response. Eleven days after fusion and cloning, hybridoma supernatants were screened for the presence of anti-IFN-γ antibodies by the direct binding assay and by neutralization of IFN-γ activity. Two hundred and ten samples (15%) were found positive by the direct binding assay and 24 of them were also neutralizing. All positive hybridomas were further grown and screened. After several passages, 29 stable hybridomas remained: they were all positive in both the direct binding assay and in solid-phase RIA, and 12 of them were also neutralizing. Seven hybridomas were then subcloned and further characterized. Their properties are summarized in Table I. Immunoprecipitation of ^{125}I-labelled IFN-γ by these antibodies revealed the presence of two antigens having apparent mol. wts. of 26 000 and 21 000 (Figure 1).

Affinity chromatography

Affinity chromatography was performed with immuno-

Table I. Titers of several anti-IFN-γ mAbs

Hybridoma supernatant no.	Binding titer (units)[a]	Binding titer c.p.m.	Neutralizing titer (units)[a]	Ig class
3	600	1358	1000	IgG1
113	1600	1326	<30	IgG1
128	1200	1678	6000	IgG1
166	800	1486	6000	IgG1
186	1600	1666	1500	IgG1
192	1600	1748	750	IgG2
201	1200	950	<30	IgM
Negative hybridoma	35	200	<30	N.D.

[a]Units of IFN bound or neutralized by 1 ml of hybridoma supernatants.

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Table II. Affinity chromatography of IFN-γ on immunoadsorbent no. 3[a]

Sample	Total units (x 10^{-6})		Specific activity (units/mg)		Purification factor		Recovery (%)	
	Bioassay	RIA	Bioassay	RIA	Bioassay	RIA	Bioassay	RIA
Load	6.4	10.4	8×10^5	1.3×10^6	–	–	–	–
Effluent and wash	0.15	0.3	–	–	–	–	–	–
Eluate[b]	3.3	9.6	4×10^7	1.6×10^8	50	123	51	92

[a]A column of 0.9 ml agarose (6.2 mg antibody) was used. [b]Most of the activity (>90%) eluted in one fraction (1 ml).

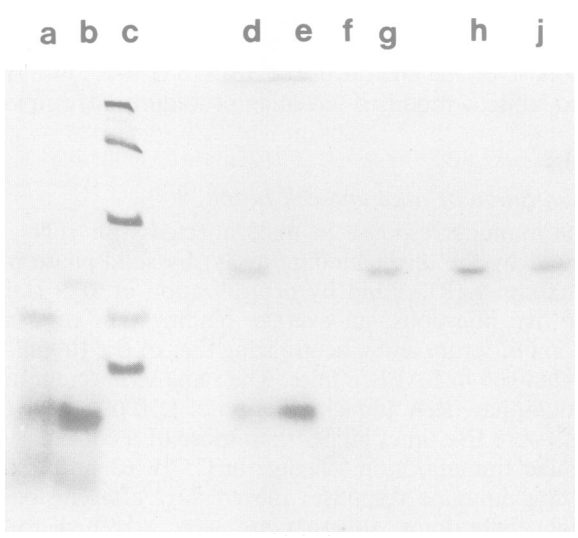

Fig. 1. Autoradiogram of [^{125}I]IFN-γ recovered from immunoprecipitates of various anti-IFN-γ monoclonal antibodies. **Lanes a** and **b**: partially purified [^{125}I]IFN-γ fractions; **lane c**: mol. wt. standards (from top: 69 000 bovine serum albumin; 46 000 ovalbumin, 30 000 carbonic anhydrase; 18 300 lactoglobulin A; 12 300 cytochrome c); **lane d**: mouse polyclonal anti-IFN-γ antiserum; **lane e**: serum of mouse injected with adjuvants only; **lane f**: negative hybridoma supernatant; **lanes g, h, i**: monoclonal antibody supernatants nos. 166, 192 and 128, respectively.

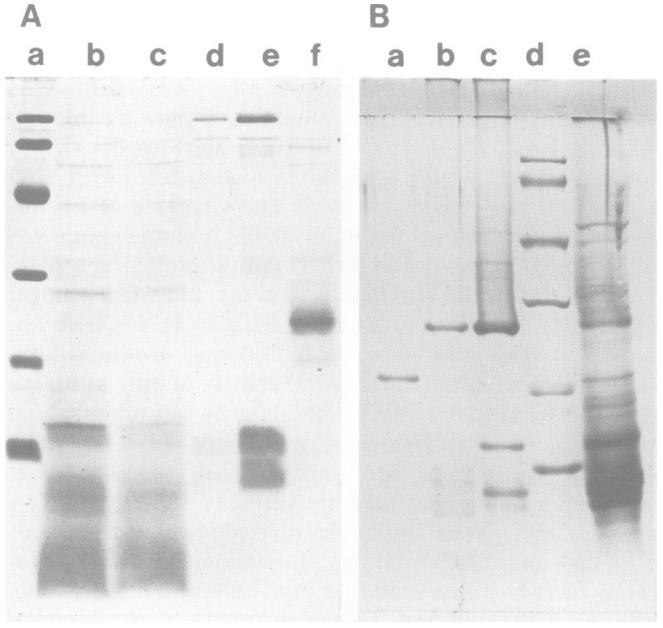

Fig. 2. **(A)** SDS-PAGE (15%) of IFN-γ after passage on immunoadsorbent. **Lane a**: mol. wt. standard (from top: 94 000, phosphorylase; 67 000, bovine serum albumin; 43 000, ovalbumin; 30 000, carbonic anhydrase; 20 100 soybean trypsin inhibitor, 14 400 lysozyme); **lane b**: partially purified IFN-γ loaded onto immunoadsorbent; **lane c**: effluent; **lanes d** and **e**: wash fractions with 20% propylene glycol and 1 M NaCl in PBS; **lane f**: elution fraction with 0.15 M NH_4OH in 3 M NaCl. **(B)** H.p.l.c. purified IFN-γ used for immunization of mice. **Lane a**: IFN-γ (mol. wt. 21 000) after h.p.l.c.; **lanes b** and **c**: IFN-γ (26 000) after h.p.l.c.; **lane d**: mol. wt. standards (see **A**); **lane e**: load fraction (CPG, IFN-γ). Fractions **a** and **b** were used for immunization.

adsorbents prepared from antibodies nos. 3, 113 and 166. Partially purified IFN-γ was loaded and in all three adsorbents a complete (≥98%) binding of IFN-γ activity was obtained. Washing with a solution of 20% propylene glycol and 1 M NaCl in phosphate buffered saline (PBS) prior to elution, had eliminated non-specifically bound proteins. Upon elution, the highest recovery of biological activity (50%) was obtained with hybridomas no. 3. The recovery was >90% when measured by a newly-developed solid-phase RIA of IFN-γ (see below). The specific activity of the purified IFN-γ was at least 4×10^7 units/mg and the degree of purification was 50-fold (Table II). Gel electrophoresis revealed the presence of a major protein band corresponding to mol. wt. 26 000 and a minor band of 21 000 (Figure 2).

Neutralization of IFN-γ from various preparations

The neutralizing titer of hybridoma supernatant no. 166 was measured with the aid of two purified IFN-γ subtypes (mol. wts. 26 000 and 21 000, separated by h.p.l.c.), as well as several crude IFN-γ preparations produced by three different inducers. In all cases a similar neutralizing titer was obtained while no cross-reaction with either IFN-α or IFN-β was detected (Table III).

Development of a solid-phase RIA for IFN-γ

To develop a double antibody radiometric assay for IFN-γ, we have analyzed biosynthetically labelled mAb for pairs that did not cross-inhibit the binding of each other to IFN-γ. One out of 30 posible pairs was found suitable for a solid-phase RIA of IFN-γ. The following assay procedure was then used: polyvinyl chloride (PVC) microtiter plates were coated with serum-free hybridoma supernatant no.3 (75 μl/well, 2 h at 37°C or overnight at 4°C). The plates were then washed three times and blocked (30 min at 37°C) with a diluent consisting of bovine serum albumin (5 mg/ml) and Tween-20 (0.05% by volume) in PBS. Serially-diluted aliquots (50 μl) of IFN-γ in the same diluent were added to the wells and incubated (2 h, 37°C). The plates were then washed three times with the diluent and ^{125}I-labelled antibody no. 166 (50 μl, 10^5 c.p.m.) was added to each well. Following incubation (4–16 h at 4°C), the plates were washed three times with the diluent, the wells were cut out and counted. A highly purified IFN-γ preparation (4×10^7 units/mg) was used as a laboratory standard. A typical titration curve is shown in Figure 3. The sensitivity of the assay was at least 150 units/ml (4 ng/ml).

Table III. Neutralization of various IFN preparations

Type of IFN	Inducer[a]	Purity of the IFN	Neutralizing titer units/ml
IFN-γ	Phytohaemagglutinin	Crude	7000
IFN-γ	Phytohaemagglutinin	Pure (mol. wt. 26 000)	6000
IFN-γ	Phytohaemagglutinin	Pure (mol. wt. 21 000)	6000
IFN-γ	Concanavalin A	Crude	9000
IFN-γ	Staphylococcal enterotoxin B	Crude	9000
IFN-α	Sendai virus	Crude	<20
IFN-β_1	Poly(rI):(rC), cycloheximide and actinomycin D	Crude	<20

[a]IFN-γ was produced by cultures of human mononuclear cells, IFN-α by leukocyte cultures and IFN-β by fibroblasts (FS11) cultures.

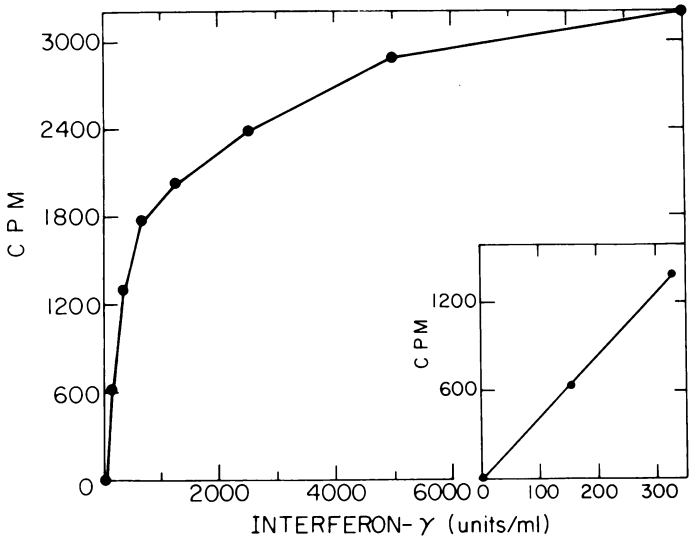

Fig. 3. Titration curve of pure IFN-γ by a double antibody radiometric assay. A background of 500 c.p.m. was subtracted.

Discussion

The mAbs described in this study have allowed affinity purification and radioimunoassay of IFN-γ. Although the preparation of mAb to IFN-γ has already been reported (Hochkeppel and de Lay, 1982) their use for affinity purification and/or radioimmunoassay has not yet been described. This situation is common because a large number of different antibodies directed against the various epitopes of an antigen can be produced, while only a few of these antibodies will be suitable for a specific application. Therefore, it is desirable to produce many different mAbs to a given antigen and, in addition, to use screening procedures that will rapidly select the useful antibodies. The approach taken in this study combined both of these requirements. By using highly purified IFN-γ as an antigen, a large number of specific hybridomas was generated. A screening procedure for selecting hybridomas suitable for affinity purification (Novick *et al.*, 1982) was then successfully applied. However, acid dissociation of the immune precipitate was replaced by treatment with NH_4OH (75 mM pH 11) because IFN-γ was acid labile. Preliminary studies have shown that, under basic conditions, IFN-γ was stable (at 4°C) for at least 1 h.

Interestingly, the conditions required for elution of IFN-γ from the immunoadsorbent were more stringent than those needed for dissociation of the immune precipitate. Studies on the mol. wt. of IFN-γ indicated that it is probably a dimer (Yip *et al.*, 1982). It is likely that the release of one IFN-γ subunit from the antibody by high pH was sufficient for recovery of biological activity, whereas, more stringent conditions (high ionic strength in addition to high pH) were needed for the dissociation of both subunits from the immunoadsorbent in order to elute it successfully. In spite of these stringent conditions, the specific activity of IFN-γ eluted from the immunoadsorbent [4 x 10^7 units/mg, as measured on human WISH cells with vesicular stomatitis virus (VSV)] was the highest reported so far and is probably close to the theoretical specific activity of pure, intact IFN-γ.

The double antibody radiometric assay is one of the most convenient and sensitive forms of solid-phase RIA. It requires the selection of two high affinity antibodies which interact with two distinct epitopes on a given antigen and in our case the IFN-γ molecule. In fact, when six different mAbs were tested only one out of 30 possible pairs of antibodies was found suitable for this application.

These studies have generated additional information on the structure of native IFN-γ. The specific immunoprecipitation of both the 26 K and 21 K polypeptides by a variety of mAbs indicated that these two peptides are structurally related. Furthermore, crude IFN-γ produced by three different inducers, as well as the two isolated subtypes of IFN-γ were completely neutralized by one of the monoclonal antibodies. These results confirm the finding of a single gene sequence for IFN-γ (Gray *et al.*, 1982). The two different IFN-γ subtypes resulted, most probably, by post-translational modifications. No cross-neutralization of either IFN-α or IFN-β was seen.

The mAbs described in this study will facilitate purification and detection of IFN-γ and thereby help to establish its structure, its role in nature and its potential clinical value.

Materials and methods

Production and purification of human IFN-γ

IFN-γ was produced as previously described (Yip *et al.*, 1981). Briefly, cultures of mononuclear cells (5 x 10^6/ml) in serum-free RPMI-1640 medium were induced by a combination of phorbol-12-O-myristate 13 acetate (Sigma and phytohemagglutinin (Burroughs Wellcome). Culture supernatants were collected after a 24 h incubation (37°C, 5% CO_2) and stored at 4°C. Concentration and initial purification were achieved by chromatography on controlled-pore glass (CPG, Yip *et al.*, 1981; Georgiades, 1982). Following concentration by ultrafiltration, a step of cation exchange h.p.l.c. was performed. One fraction contained highly purified IFN-γ (sp. act. 7 x 10^6 units/mg) and exhibited a major protein band (mol. wt. 26 000) corresponding to IFN-γ (Figure 2B). This preparation was used for immunization of mice. Details of this purification procedure will be published separately.

Assay of IFN-γ

IFN activity was assayed in 96-well microculture plates by the inhibition of cytopathic effect of VSV in human WISH (ATCC CCL-25) cells. In the absence of an IFN-γ standard, the assay was calibrated against IFN-α reference standard G-023-901-527, kindly provided by the National Institute of Health (Bethesda, MD), and a laboratory standard of IFN-γ. The assay system was similar to a previously published procedure (Rubinstein *et al.*, 1981).

SDS-polyacrylamide gel electrophoresis

Aliquots of the various fractions containing IFN-γ in sample buffer with 2% β-mercaptoethanol were heated for 5 min at 100°C and electrophoresis

was performed on a polyacrylamide (15%) gel slab (Laemmli, 1970), After electrophoresis, protein bands were visualized either with Coomassie blue or by silver stain (Sammons *et al.*, 1981).

Protein determination

Protein concentration was determined by the dye binding assay (Bradford, 1976) and by an assay based on reaction with fluorescamine (Stein and Moschera, 1981). Crystalline bovine serum albumin (Miles) was used as a standard.

Immunization, cell fusion and cloning

Three month old female BALB/c mice were first injected s.c. with partially purified IFN-γ from CPG chromatography (20 000 units/mouse, 8 x 10^5 units/mg) emulsified in complete Freund's adjuvant. Three weeks later the mice were boosted with the same preparation of IFN-γ mixed with 0.4 mg alumina gel (0.25 ml/mouse). Three more injections were given s.c. at 10 day intervals with h.p.l.c. purified IFN-γ (7 μg/mouse, mixed with alumina gel). Four weeks later, two mice showing the highest binding and neutralizing titers received an i.p. injection of h.p.l.c. purified IFN-γ (10 μg/mouse). Three days later their splenic lymphocytes (180 x 10^6 cells) were fused with 30 x 10^6 NSO/1 myeloma variant (NSO cells, kindly provided by C. Milstein, MRC, Cambridge, UK) in a method described before (Eshhar *et al.*, 1980). The fused cells were distributed into 1311 wells of microculture plates (96-wells) and selection for hybridoma growth was in Dulbecco's modified Eagle's medium (high glucose, Gibco), supplemented with 1 mM pyruvate, 2 mM glutamine, penicillin (10 units/ml), streptomycin (20 μg/ml), 15% heat-activated horse serum and containing HAT (Littlefield, 1964). Hybridomas that were found to secrete anti-IFN-γ antibodies were cloned and recloned by the soft agar procedure (Pluznik and Sachs, 1965).

Screening for IFN-specific hybridomas

Hybridoma supernatants were tested for the presence of anti-IFN-γ antibodies by three different methods: (i) direct binding assay; (ii) neutralization of IFN-γ antiviral activity and (iii) solid-phase RIA.

Direct binding assay. Hybridoma supernatants (50 μl) were placed in conical bottom microtiter (96-wells) plates and partially purified IFN-γ (30 μl 10 000 units/ml, 8 x 10^5 units/mg) was added. The mixture was incubated for 4 h at 37°C. Normal mouse serum (20 μl, 1:40 in PBS) was added followed by a calibrated amount of goat anti-mouse $(Fab')_2$ serum. The mixture was incubated for 30 min at 37°C and then overnight at 4°C. The plates were spun (1200 *g*, 5 min, 4°C) and the supernatant was removed by inverting the plates. Cold PBS (150 μl) was added, the plates were gently shaken and then spun as above. This washing was repeated once more. Immediately before bioasay the precipitate was dissolved in 75 mM NH_4OH (50 μl) and assayed for IFN activity. Each sample was assayed by four 2-fold dilutions, allowing for 24 individual tests per one microtiter plates. Samples which gave full protection, at least along three 2-fold dilutions (equivalent to 100 units/ml) were considered positive.

Neutralization of interferon activity. Serial 2-fold dilutions of hybridoma supernatant in minimal essential medium containing 10% fetal calf serum (50 μl) were incubated with IFN-γ (20 – 50 units/ml, 50 μl) in microtiter plates for 4 h at 37°C, and then overnight at 4°C. The mixtures were then added to cultures of WISH cells in microculture plates followed by virus challenge, incubation and staining. One neutralizing unit is defined as the amount of antibody sufficient for neutralization of one unit of IFN-γ.

Solid-phase RIA. PVC microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with affinity-purified goat anti-mouse $(Fab')_2$ immunoglobulins (80 μg/ml, 80 μl) for 2 h at 37°C. The plates were washed three times with 0.05% Tween-20 and 5% fetal calf serum (FCS) (washing solution), hybridoma culture supernatants (50 μl) were added and the plates were incubated for 4 h at 37°C. The plates were then washed three times with the washing solution, ^{125}I-labelled IFN-γ (50 μl, 10^5 c.p.m., purified by h.p.l.c., and iodinated by the chloramine-T method; Hunter, 1973) was added and incubation was carried out for 4 – 18 h at 4°C. The plates were washed four times with the washing buffer and individual wells were cut and counted in a gamma counter. Samples giving counts that were at least four times higher than the negative control value were considered positive.

Immune precipitation of ^{125}I-labelled IFN-γ

Partially purified IFN-γ (10 μg, 5 x 10^6 units/mg) was radio-iodinated by the chloramine-T method (Hunter, 1973). Various hybridoma supernatants (50 μl) or diluted sera, were incubated with ^{125}I-labelled IFN-γ (10^5 c.p.m., 20 μl) for 1 h at 37°C followed by 3 h at 4°C. Normal mouse serum (20 μl of a 1:20 dilution in PBS) was then added, followed by a calibrated amount of goat anti-mouse immunoglobulin serum. Following incubation (30 min at 37°C and overnight at 4°C) the immune precipitate was collected by centrifugation (14 000 *g*, 2 min) and washed twice (1 ml, 5% FCS and 0.05% Tween-20 in PBS). The immune precipitate was then analyzed by SDS-polyacrylamide (15%) gel electrophoresis followed by autoradiography.

Preparation of immunoadsorbents and affinity chromatography

Serum-free hybridoma supernatants were concentrated by ultrafiltration on a YM-10 membrane (Amicon) and washed with PBS. The concentrated solution (5 – 10 mg/ml, 1 ml) was coupled to 1 ml of agarose-polyacryl hydrazide (Wilchek and Miron, 1974). Partially purified IFN-γ (3 – 8 x 10^6 units in 6 ml of 0.5 M tetramethylammonium chloride) obtained by CPG chromatography was loaded on a 1 ml column at 4°C at a flow rate of 0.5 ml/min. The column was washed with cold PBS (10 ml), a mixture of propylene glycol (20% by vol) and 1 M NaCl in PBS (10 ml) and PBS (10 ml). IFN-γ was eluted by 0.15 M NH_4OH in 3 M NaCl (8 x 1 ml fractions). Eluted fractions were neutralized with acetic acid (1 M, 75 μl) within 15 min. Residual proteins still retained on the immunoadsorbent were eluted with a citrate-HCl buffer (50 mM, pH 2), and neutralized immediately with $NaHCO_3$ (1 M, 150 μl per 1 ml of eluate). The immunoadsorbent was then washed with PBS and stored at 4°C.

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