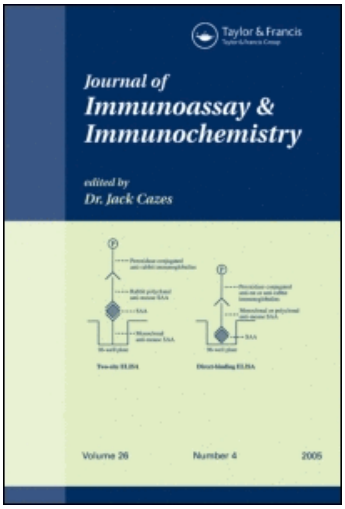

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Immunosorbent Binding Bioassay: A Solid Phase Biological Immunoassay for the Titration of Antisera to Alpha Interferons

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To cite this Article L'Haridon, René M.(1991) 'Immunosorbent Binding Bioassay: A Solid Phase Biological Immunoassay for the Titration of Antisera to Alpha Interferons', Journal of Immunoassay and Immunochemistry, 12: 1, 99 — 112

To link to this Article: DOI: 10.1080/01971529108055059

URL: http://dx.doi.org/10.1080/01971529108055059

JOURNAL OF IMMUNOASSAY, 12(1), 99-112 (1991)

IMMUNOSORBENT BINDING BIOASSAY: A SOLID PHASE BIOLOGICAL IMMUNOASSAY FOR THE TITRATION OF ANTISERA TO ALPHA INTERFERONS.

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SUMMARY

An Immuno Sorbent Binding Bio-Assay (ISBBA) for the detection and the titration of antisera to the highly biologically active proteins, alpha interferons, is described.

This method is similar to a classical solid phase immunoassay, except for the last step which uses the biological activity of the captured antigen. With specific serum antibodies the bound antigen prevents further virus induced cell lysis. On the contrary, with negative serum or preserum the antigen is washed out and virus induced cell lysis occurs, therefore no labelling is required.

ISBBA exhibits three main differences when compared to the reference method i.e. the neutralization assay (NA): *i)* ISBBA is 10 to 1000 fold more

Key words: Antisera to alpha interferons, titration of antisera to alpha interferons, ImmunoSorbent Binding BioAssay.

sensitive than NA; *ii)* in contrast to NA, ISBBA is based on the production of an antiviral effect; *iii)* ISBBA makes it possible to use unpurified antigen.

The applications of ISBBA to subtypes study in natural alpha interferon samples are discussed.

INTRODUCTION.

Solid Phase Immuno-Assay was first described for the titration of Human Growth Hormone (HGH) using the property of synthetic polymers to bind immunoglobulins. The antibody function of these proteins remained active. This assay was based on the competition between the binding of the radiolabelled standard antigen, and the added antigen to be titrated (1). Later this method was successfully applied to the titration of a large number of antigens using enzyme labelled antibodies. The resulting conjugate sharing both antibody and enzymatic functions was able to react specifically with a captured antigen (2). Thus solid phase immunoassays were largely developed (3). However to perform such assays it is necessary to have purified antigen or purified antibodies to avoid unspecific reactions with unexpected antigen present in the preparations.

Interferons (IFNs) are a group of proteins with antiviral antiproliferative and immunomodulating properties (4). Amongst these leukocyte interferons (IFNs-le), or alpha interferons (IFNs-α), are important systemic components of the IFN superfamily whose major property is the ability to inhibit viral replication. IFNs-α, are produced by white blood cells after viral infection, either *in vivo* or *in vitro* in cell culture. Recently, trophoblast proteins (TP) which are major conceptus secretion proteins of the ruminant embryo and which possess paracrine antiluteolytic properties, have been characterized as IFNs-α (5,6). Usually the antigenic characterization of IFN−α is based on the inhibition of the antiviral effect -Neutralization Assay- (NA, ref. 7). Other methods such as Solid Phase Antibody-Binding Assay (SABA, ref. 8), Enzyme Linked ImmunoSorbent Assay (ELISA, ref. 9,10), have been

described. However they have not been routinely used since they need either large quantities of highly purified IFN, or specific monoclonal antibodies.

The aim of this study is to describe an immunosorbent binding bioassay (ISBBA), which is based on antigen immunocapture, and which is more sensitive than the NA. The use of virus infected living cells in the final revealing step, avoids any labelling of both antigen and antibodies.

MATERIAL AND METHODS

1. - Antigens

1.1 - Interferons (IFNs):

Bovine, ovine, and porcine leukocyte IFNs were obtained from virus induced peripheric blood lymphocytes:

Blood from adult pigs, cows, or sheep were collected aseptically on sodium heparinate (SERVA/TEBU, Le Perray en Yvelines, France.) 20 units/ml final concentration). Lymphocytes were separated by centrifugation on a ficoll cushion, d=1.08 (ref.: "MSL", Eurobio, Paris, France.) 15 mn at 1500g. The resulting ringed lymphocytes were washed twice with Phosphate Buffered Saline (PBS) and resuspended in RPMI 1640 medium containing penicillin 100 IU/ml, streptomycin 0.1mg/ml at the final concentration of 10^7 cells/ml. The lymphocyte cultures were induced with purified bovine coronavirus (11) 10μg/ml and incubated 15-18 hours at 37°C. Cells were then pelleted at 2000g/10mn, and the supernatants assayed for antiviral activity. If the antiviral activity was higher than 1000 I.U. IFN (12), the samples were centrifuged 2 hours at 100,000g to remove the pelleted virus, and the supernatants were stored at -20°C.

For rat immunization, Porcine leukocyte IFN was concentrated by precipitation at 80% saturation of ammonium sulfate: 36% w/w (13). The

precipitated proteins were pelleted at 100,000g for 30mn, and after solubilization in a minimum of PBS, they were extensively dialyzed against PBS, titrated for antiviral activity, and stored at -20°C for using them as immunogens.

Human leukocyte IFN, Cantell type (14) was a gift of Dr Adamowitz* (Institut Pasteur, Garches, France).

1.2-Trophoblast Proteins:

Ovine Trophoblast Protein (oTP) and Bovine Trophoblast Protein (bTP) were from culture supernatants of respectively 16 day old ovine embryos and 18 day old bovine embryos and were kindly provided by Dr. J. Martal, (Embryo Endocrinology Research Unit, Department of Physiology, this institute).

2. - Antisera.

2.1 - Anti Porcine leukocyte IFN antiserum:

Ten week old female Louvain inbred rats (C.S.E.A.L., Orleans, France) were inoculated intraperitoneally with increasing doses up to $4x10^6$ IU Porcine leukocyte IFN in 0.6 ml PBS, homogenized with 0.6 ml Freund Complete Adjuvant. Three, five, and eleven weeks later the animals were boosted with the same dose of antigen without adjuvant, and blood was collected from orbital sinus, at regular intervals, and the sera assayed for anti interferon activity.

2.2 - Sheep anti Human leukocyte IFN antiserum

Anti Human leukocyte IFN antiserum (Sheep 166), was a gift of Dr. Adamowitz*. (Institut Pasteur, Garches, France).

2.3 - Rabbit anti oTP antiserum

Anti Ovine Trophoblast Protein (Rabbit 2), was kindly provided by Dr. J.

*: Dr. Adamowitz: Present address, CNTS, Les Ulis, France.

Martal, (Embryo Endocrinology Research Unit, Department of Physiology, this institute).

3. - Assays

3.1 - IFN titration:

The biological activity of IFN was determined using the inhibition of the cytopathic effect induced by Vesicular Stomatitis Virus (VSV -Indiana Strain- kindly provided by Dr. C. La Bonnardiere, this laboratory) on MDBK cells (15) monolayers cultured in 96 well plates (Falcon 3072, Becton Dickinson, Lincoln Park, N.J.) as described (12,16)

3.2 - Neutralization Assay (N.A.):

The NA was performed as indicated (7, 16). Briefly, serial dilutions of the antiserum to be titrated were prepared in Minimum Eagle Medium (Eurobio, Paris, France) supplemented with 2% Foetal calf serum penicillin 100 IU/ml and Streptomycin 0.1mg/ml (MEM2%). The diluting ratio was 1:3.17, and the starting dilution was 1:100, and then each of these dilutions (1:100, 1:317, 1:1000, 1:3170,...) were distributed in 16 wells (two columns), of a 96 well plate containing MDBK cell confluent monolayers. Next the challenge IFN sample to be neutralized was serially diluted (ratio 1:3) in the serum dilutions. The following steps were performed as for an IFN titration. The anti IFN titer of the antiserum was expressed as the last dilution of this serum that reduced 9-fold (3^2-fold) the titer of the IFN challenge. This method permitted the use of non pretitrated challenge IFN.

3.3 - ImmunoSorbant Binding Bio-Assay (ISBBA):

The assay was carried out as preliminary described (17) using M 129B plates (Dynatech, Marnes La Coquette, France.). These plates with good immunobinding properties, allowed monolayer formation when MDBK cell suspensions were seeded in their wells.

To dilute the samples and to wash the wells, "modified Earle's salt solution" (MESS) was used. This salt solution was Earle's solution (18) - usually used in cell culture laboratories-, minus glucose to limit yeast and fungi contamination, with sodium bicarbonate 0.350g/l, and plus PIPES 50 mM, pH 7.1, that permitted incubation in the absence of CO_2.

The assay was performed in four steps:

i - Coating with the first antibody: The wells were filled with 100μl of either Rabbit anti Rat IgG (Gammaglobulins), 2μg/ml in MESS, or Rabbit Antisheep IgG (Gammaglobulins), 2μg/ml (Respectively references BI 1011 and BI 1008, Biosys, Compiegne, France), or Goat Anti-Rabbit IgG (Affinity Purified Antibodies: reference 61 610, ICN/Miles, Paris, France), 1μg/ml in MESS, and incubated overnight at 37°C. The wells were washed three times with MESS containing Tween 20 (Prolabo, Paris, France.), one per thousand in MESST.

ii - The antisera to be tested were serially diluted in MESST, and 100μl of each sample was seeded in duplicate wells and incubated 90mn at 37°C. The wells were washed three times.

iii - The antigen to be captured (Challenge Interferon: 1000 IU diluted in MESST) was placed in the wells, 100μl per well, and incubated 90 mn at 37°C. The wells were washed four times.

iiii - Each well received $5x10^4$ MDBK cells infected with $5x10^2$ Tissue Culture Infectious Doses ($TCID_{50}$, ref. 19) of VSV (i.e. a mixture of cells and virus) in 100μl MEM2%. They were incubated overnight at 37°C in 5% CO_2 atmosphere. During this incubation time both virus multiplied in cells (i.e. control cells), and cell monolayers formed. After a 18-22 hour incubation period, monolayers were stained with crystal violet 2% (w/v) in an aqueous alcohol solution (90% v/v distilled water, 10% v/v ethanol) during 5 minutes, and washed extensively with tap water.

Results were scored as positive when full cell protection occurred with the antibody captured IFN, or negative when cells were destroyed by the virus if specific antibodies to IFN were absent (Fig. 1).

TABLE 1.- **NA and ISBBA titers of three anti IFN-α sera:**

Titers have been indicated as the reciprocal $\log_{10}$ of the last serum dilution giving a 3^2 (9-fold) lower IFN titer in NA, or at least a 50% protective effect on cell layers in ISBBA. The * indicates a lower cell layers protection in standard procedure (1000 IU Bovine leukocyte IFN as challenge), when the assay was performed using 10^4 IU Bovine leukocyte IFN, full cell layer protection was observed.

	Antisera to :					
	Po. IFN-le. (Rat 10)		Hu. IFN-le. (Sheep 166)		oTP (Rabbit 2)	
Assay	NA	ISBBA	NA	ISBBA	NA	ISBBA
Po. IFN-le.	2	5	4	5.5	<2	<2
Hu. IFN-le.	<2	<2	4	7	<2	<2
oTP	<2	<2	<2	<2	4	5
Bo. IFN-le.	<2	<2	<2	5*	2	4
Ov. IFN-le.	<2	<2	<2	<2	<2	<2
bTP	<2	<2	<2	<2	<2	<2

RESULTS

Rats were inoculated with increasing doses of concentrated antigen, until a neutralizing activity to Porcine leukocyte IFN was found in their serum. Injections with doses lower than $4x10^6$ antiviral units had no immunizing effect. A weakly positive antiserum (neutralizing titer: 1:100) was obtained after 3 inoculations with $4x10^6$ IU IFN. This serum was used to develop an assay more sensitive than the Neutralization Assay.

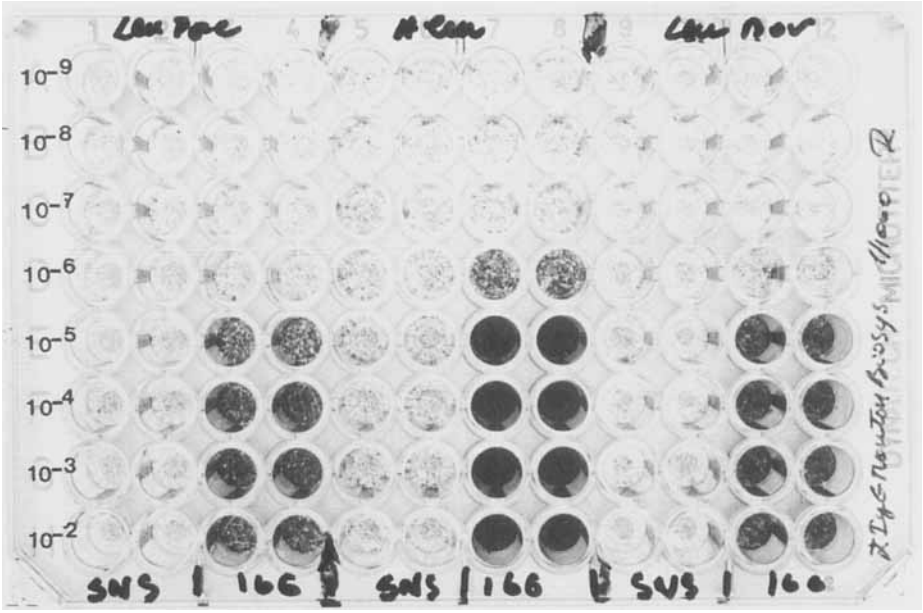

FIGURE 1.- **ISBBA titration of an antiserum to Human leukocyte IFN.**

Titration of sheep 166 serum (anti Human leukocyte IFN), versus three different animal species leukocyte IFNs .

Columns 1-2; 5-6; 9-10: Sheep Normal Serum.

Columns 3-4 ; 7-8; 11-12: Sheep 166 Hyperimmune antiserum to Human Leukocyte IFN.

Sera were serially diluted from 10^{-2} to 10^{-9}.

Challenge antigens were: Porcine leukocyte IFN (columns 1 to 4), Human leukocyte IFN (columns 5 to 8), Bovine leukocyte IFN (columns 9 to 12).

Some properties of IFNs such as high specific activity (20), and ability to remain active when covalently bound to a solid matrix (21), led us to examine whether the IFN remains biologically active after capture on a solid phase. The difficulty encountered in the improvement of the method was due to the choice of the solid phase. The plates used as solid phase must have after coating procedures the following characteristics: absence of nonspecific

binding of IFN to the plastic surface, but anchorage of the cells on the bottom of dishes commonly used for Solid Phase Enzymo-Immunoassays. Cobalt 60 sterilized 96 wells plates offered good properties for solid phase immunobinding and for cell layer formation. This type of plates made the method feasible. The microwell antibody coating step was also critical and had to be checked.

At the beginning of this work the last phase of the assay was performed in 2 different steps: after seeding the MDBK cells in the microwells, the plates were incubated overnight (to allow monolayer formation), then challenged with 0.01 $TCID_{50}$ VSV per cell. This procedure resulted in high sensitivity of antisera titration but in most cases the cell layer became detached in many wells, so only partial results were obtained. The use of infected cells (i.e. MDBK cells plus VSV) in the last step gave reproducible results. This cell and virus suspension, which is called *"infected cells"*, could be stored one or two hours on ice before distributing in plate wells, without any change in the results. The method has been successfully extended to the titration of sheep anti Human leukocyte IFN antiserum, and to rabbit anti oTP antiserum. The main condition for this adaptation was the need for a convenient first antibody (against IgG of the animal species in which anti-IFN has been raised). The use of heavy chain (i.e. τ chain) specific antibodies can improve this first step of the method (data not shown).

Figure 1 shows the results of an ISBBA test. Different dilutions of a reference serum (anti Human leukocyte IFN) were allowed to react with different leukocyte IFNs. The last serum dilution binding IFN activity can be easily read by the naked eye, after crystal violet staining.

ISBBA and NA respective titers of antisera to Human leukocyte IFN, to Porcine leukocyte IFN, and to oTP assayed with five different IFNs-α as antigens are shown in table 1. All sera positive in NA were also positive in ISBBA. All sera negative in NA were also negative in ISBBA except in one

case: Anti Human leukocyte IFN reacted weakly with Bovine leukocyte IFN in ISBBA but was negative with this IFN in NA.

When compared each other, ISBBA titers were always higher than NA titers. Ratio ISBBA: NA was from 1 $\log_{10}$ (i.e. 1/10) for antiserum to oTP-1 to 3 $\log_{10}$ (i.e. 1/1000) for antisera to Human and Porcine leukocyte IFNs with their homologous antigens. In all other cases, when antisera were checked with heterologous antigens, ISBBA was also more sensitive than NA. It must be noted that the anti oTP antiserum which had been used in this work did not react with bTP, although it reacted with Bovine leukocyte IFN. Ovine leukocyte IFN was not cross reactive with Human and Porcine leukocyte IFNs in both assays NA and ISBBA.

DISCUSSION

An immunosorbent binding bioassay has been developed according to the classical pattern of solid phase immunoassays as an alternative assay to NA for anti IFN-α antisera titration. This new assay has been devised as an alternative to enzymo-immuno assay (ELISA, ref. 3,9,10). When applied to the detection of IFNs, or to the detection of polyclonal antibodies against IFN, ELISA may give false positive results and needs large quantities of antigen: about 100 ng per well. Since the specific activity of IFN-α is $5x10^8$ IU/mg, the amount of IFN needed per plate is about $5x10^6$ antiviral units of IFN-α, that which would correspond to a 5000 ml lymphocyte cell culture production of crude 10^3 IU Porcine leukocyte IFN! -This illustrates the need for a method using relatively small amounts of antigen and which can detect either non neutralizing antibodies or whole antibodies to IFN-α.

In other words, until now, NA was the only method available for the detection of antibodies raised against IFNs-α following the injection of this antigen in experimental animals, its sensitivity was low, probably due to multiple subtypes in natural IFN-α (22,23,24). The production of antibodies

against a soluble antigen such as IFN in experimental animals, needs micrograms of antigen (25). One microgram of IFN-α corresponds to $5x10^5$ antiviral units (20), therefore to prepare neutralizing sera, immunizing amounts of IFN must be higher than 10^6 antiviral units (i.e. 5μg) . However this is correct if only one IFN subtype predominates (or if several subtypes share antigenic cross reactivity). The results reported here show that the lower Porcine leukocyte IFN immunizing dose was about $4x10^6$IU IFN (i.e. about 10μg). This result is in agreement with the hypothesis that one dominant IFN subtype can induce the production of specific antibodies in an immunized animal.

The low sensitivity of the NA method may be due to interference of some residual minor IFN-α subtypes that differed antigenically (on their active site) from the dominant subtype, and which would be in too small amounts to be immunogenic. The presence, in the sample used in NA, of this minor IFN-α subtype may be able to induce an antiviral effect. In these conditions IFN neutralization would be hidden. Indeed we observed weak neutralization of Porcine leukocyte IFN by specific antiserum.

The ability of anti Human leukocyte antiserum to react with Porcine leukocyte IFN, previously described (16) could be observed both in NA and ISBBA. In all assays performed with IFNs and their homologous antisera, NA and ISBBA were very well correlated, ISBBA being more sensitive than NA. When assays were performed with IFN and heterologous antiserum, ISBBA in contrast with NA, permitted to detect antibody to Bovine leukocyte IFN in antiserum against Human leukocyte IFN. These observations could be due to the fact that ISBBA needs specific binding of only a single IFN-α subtype .

No binding occurred between Ovine leukocyte IFN and sheep anti Human leukocyte IFN. This result could be due to high differences between Ovine leukocyte IFN and IFNs-α from other animal species, but more probably to the non responsiveness of the sheep to its own Ovine leukocyte IFN.

Until now, most of the comparisons between IFNs were based on gene sequences and on the deduced amino-acid sequences. However even with large sequence homology antigens could be different. This hypothesis is supported by our data. Bovine trophoblast protein (bTP) and Ovine trophoblast protein (oTP) share high gene sequence homology (26) but, in ISBBA, bTP did not react with antiserum against oTP.

The same immunochemical reactivities were probably involved in ISBBA and in a previous experiment performed using mouse IFN bound to sepharose beads as solid phase (21). These beads were able to protect L cells from viral propagation, as soluble IFNs do. Further investigations would provide explanation on the mode of action of such bound IFNs.

This new solid phase immunoassay uses living cells, it can be related to an immunoassay in heterogeneous phase, even though NA can be compared to an immunoassay in homogeneous phase (3). This assay may become a useful tool in screening procedures for the production of monoclonal antibodies to IFN-α. In addition this method may have some applications for the evaluation of resistance of patients treated with recombinant IFNs-α which could be related to an immune response (27).

Acknowledgments: The author is grateful to Dr. J. Laporte for his encouragements in this work, to Dr. C. La Bonnardiere for fruitful discussions, and to Jacqueline BONNAL and Ginette YONNET for technical assistance. He is indebted to Dr. G. Corthier and to Dr. Helen Hayes for their help in writing this manuscript.

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