Project: **Development of an in-vitro Pyrogen test kit: Evaluation of pyrogenicity using human whole blood**
(From March 2004 to June 2006)

Submitted to:
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Government of India, New Delhi
By
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OBJECTIVES:
The objective of the present project is, to develop an Enzyme Linked Immunosorbant Assay (ELISA) for pyrogen test using human whole blood.

DURATION : 2 years

MATERIALS AND METHODS
Development of polyclonal antibodies against pure IL 1ß in rabbits
Immunisation of rabbit
Healthy rabbit was selected for immunisation because it is the most common animal model for polyclonal antibody production. Animals care and husbandry was carried out as per Institutional Animal Ethics Committee (IAEC) and national regulations. Before giving immunisation, blood was collected and pre immune sera were separated. Interleukin 1ß antigen (Sigma) was reconstituted in phosphate buffer saline and mixed with freund's complete adjuvant was used for primary immunization and thereafter four booster doses were given to the same rabbit.

Blood collection: Blood was collected from the rabbit before and after immunization. Sera separated and were screened for the presence of antibody by double diffusion method and then purified.

Antigen binding and titre of antibodies using agarose immunodiffusion
Antibody identification (Double diffusion) by Ouchterlony method
1% Agarose (Biogene) gel was prepared and was used for Ouchterlony.
Antibody detection and titration: IL1β was reconstituted in borate buffered saline and pipetted into the central well (gel plate) and the diluted serum was added to 6 surrounding wells, incubated and stained with Coomosie brilliant blue stain. This technique was repeated for all the sera. The test were done in two ways;
1. Antibody was added in the middle well and serially diluted antigen was added to 6 peripheral wells
2. Antigen was added in the middle well and serially diluted antibody was added in the peripheral wells.

Purification of antibody (by Affinity Chromatography)

The chromatography system (Amersham Bio Sciences, AKTA Prime) was used for the purification of antibody by manual method. The pre packed ready to use column was used for preparative purification of antibodies. Sample was adjusted to the composition of the binding buffer by diluting the sample. The diluted sample was subjected to dialysis against binding buffer, centrifuged and thereafter the supernatant was filtered. The filtered samples were subjected to purification by affinity chromatography. The protein content of the collected sample was estimated (Lowry's method) and was subjected to check the purity by SDS-PAGE method.

SDS-Poly acrylamide gel electrophoresis for purity check.

SDS-PAGE electrophoresis was carried out and the sample was loaded along with marker at 100V for a period of 90 minutes. Gel was stained with Coomossie brilliant blue stain, de stained and dried.

Dot blot for Antibody detection

Indirect method

This was carried out on nitrocellulose papers to identify the specificity and sensitivity of the antibody to the antigen. Antigen was spotted on each of the nitrocellulose paper and allowed to dry. After blocking, diluted antibody was added to corresponding sheets and was incubated. At the end of incubation, blots were washed and developed using secondary antibodies. For detection of binding with labelled antibodies direct method is used without secondary antibodies. All antibodies purified by affinity chromatography, labelled antibodies, commercially available monoclonal antibodies and labelled monoclonal antibodies were subjected to dot blot assay.

Standardization of Enzyme Linked Immunosorbent Assay (ELISA)

Two-antibody Sandwich Assay (ELISA) for Antigen detection (Indirect method)

Two-antibody Sandwich Assay (ELISA) for antigen detection was carried out. Here the purified antibody was coated on a microplate, blocked and incubated. The coated plates were stored at 4^0C.

Two-antibody sandwich assay for antigen detection was carried out with coated plates. Serially diluted antigen was added to all the wells except the first well, which served as the blank. The plates were incubated overnight and antibody solution was added to each well and incubated again. Later, IgG HRP conjugate were added to all the wells. Again incubated at room temperature for 2 hours and diluted substrate were added to all the wells. The absorbance of the coloured solutions were read at 450nm using an ELISA reader.

Same steps were repeated with new antigen vial, increasing the washing steps, using plate wash for washing, adding Tween20 in wash buffer, secondary antibody HRP conjugate concentration was more reduced and incubated with antigen and thereafter after over night incubation.

Two-antibody Sandwich Assay (ELISA) for Antigen detection (direct method)

In this assay the purified antibody was bound to microwell titration plates, and the antigen in a test solution was allowed to bind and the labeled second antibody was allowed to bind to the antigen. After washing, the assay was quantitated by measuring the amount of labeled second antibody that was bound to the matrix. This was achieved by adding substrate for the enzyme. Two-antibody sandwich assay for antigen detection was carried out using coated plates. Serially diluted antigen was added to all the wells except the first well, which served as the blank. The plates were incubated overnight. Labeled antibody solution was added and incubated at room temperature. Subsequently, substrate was added and incubated again. Absorbance was read at 450nm using an ELISA Reader.

Conjugation of antibodies with horse radish peroxidase (HRP) enzyme

Glutaraldehyde coupling method

Glutaraldehyde was coupled with pure horseradish peroxidase (HRP) and purified by gel filtration and brown coloured fractions were collected. The absorbance of the fractions was read at 405nm using UV Spectrophotometer. The first three portions were pooled and dialyzed against 100mM Sodium carbonate-Sodium bicarbonate buffer. Add 0.1ml antibody to the pooled fractions and incubated at 4^0C for 24 hours and thereafter the reaction was stopped by adding ethanolamine.

Periodate method

Periodate treatment of carbohydrates opens the ring structure and allows these moieties to bind to free amino groups. Coupling antibodies and horse radish peroxidase with periodate linkage is an efficient method. After completing conjugation their binding capacity is detected by dotblot assay.

Coupling Antibodies to Alkaline Phosphatase

Antibody mixed with alkaline phosphatase and dialyzed overnight. Add 1% solution of glutaraldehyde. After 2 hr incubation at room temperature and further dialyzed against PBS. Store the supernatant at 4^0C. After completing conjugation their binding capacity is detected by dotblot assay.

Protein estimation in coated plates

The quantity of proteins in the coated plates after washing is checked by Lowry's method. Graph plotted with standard and antibody coated wells are also subjected to protein estimation. The quantity of protein in the coated plates was found out. This is done in both monoclonal and polyclonal coated plates and standard graph preparation also done in ELISA plates.

Standardization of Enzyme Linked Immunosorbent Assay (ELISA)

ELISA standardization was started with new batch of antibody.

Ionic strength and pH of the buffer for obtaining optimal antibody binding in polysterene plate was identified using 4 different buffer compositions. ELISA (sandwich assay) experiments were conducted using various combinations of polyclonal and commercial monoclonal antibodies (Prospec, Cytolab). As the antibodies from same species could give non-specific binding, commercially available monoclonal antibodies were used for detection. Monoclonal antibodies (Prospec, Cytolab) were labeled with Horse radish peroxidase enzyme and used for direct ELISA. Labeled antibodies are used for ELISA after the detection of their antigen binding capacity using dot blot.

Method: Polyclonal antibody was purified by affinity chromatography, coated on to polystyrene plates. After washing with PBS antigen was added to all the columns except the one which serves as blank. Incubated for 2 hours and washed with PBS. Diluted monoclonal antibodies added to all the columns including blank. After incubation at room temperature and after washing secondary antibody added and reading was taken at 450nm in ELISA reader (Digi scan). Graph plotted with concentration against absorbance.

Stimulation of whole blood with known quantity of endotoxin and production of antigen.

Blood was collected from different persons in heparinised tubes and incubated with known quantity of endotoxin. After incubation the supernatant was lyophilized and used for the study. The stimulants are tested for the presence of IL 1β antigen using affinity purified antibody against IL 1β by dot blot assay. Stimulated plasma was subjected ELSA assay. Doubling dilution of stimulated plasma prepared and added to the wells. One column will serve as blank. The concentration of the interleukin 1 ß is found out with the help of standard graph.

Results and Discussions

Antigen binding and titre of antibodies using agarose immunodiffusion (Double diffusion by Ouchterlony) was carried out and only diffused lines of reactions were observed in all of the cases. Absence of clear arcs of precipitation is thought to be due to low molecular weight of the antigen. So the presence of specific antibody and it's binding with antigen is determined by dotblot assay. Later the antibodies were purified by affinity chromatography (Fig 1-4).

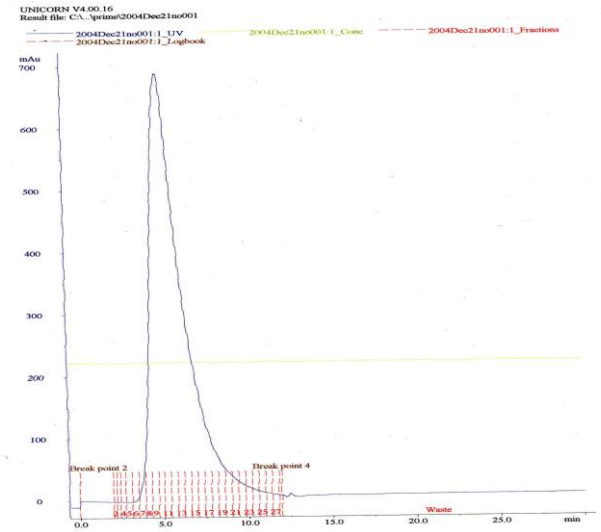


Fig : 1

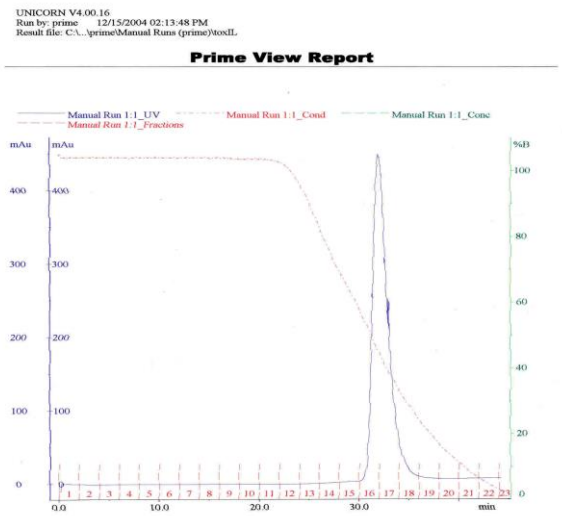
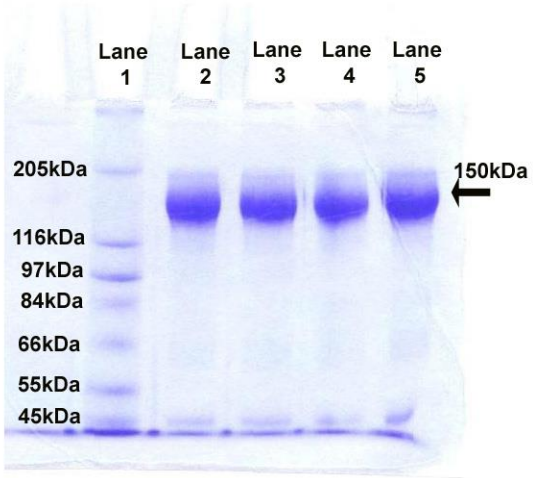


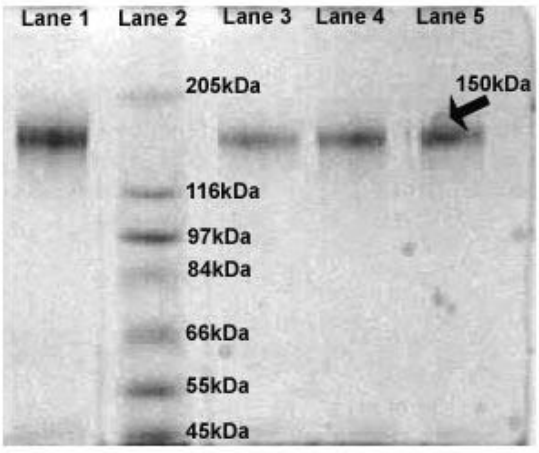
Fig : 2

All the fractions in the peak were pooled and the antibody purity was verified using SDS-PAGE analysis. The purity of the antibodies was demonstrated in Fig 2 - 3 by SDS-PAGE.

The antigen binding capacity of the purified antibody was verified using dot blot.



Lane 1 - High Molecular Weight Markers
Lane 2-5 - Purified IgG



Lane 2 - High Molecular Weight Markers
Lane 1, 3-5 - Purified IgG

Fig 3 & 4. The single band (arrow mark indicated) represents the purified immunoglobulin against the high molecular weight marker

Dot blot assay

The details of Figure 7 are; 1. First antibody: Dot blot done with antibody purified after first bleeding, 2. Second antibody: Dot blot with antibody purified after second bleeding, 3. Third antibody: Dot blot with antibody purified after third bleeding (more intense dot) 4.Control: Dot blot of control serum.

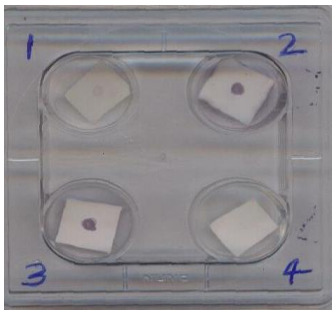


Fig 5

Fig 5: Dot blot direct method using antibody conjugated with HRP was carried out and colored dot was observed in the sheet (direct) treated with antibody-enzyme conjugate. Dot blot indirect method using secondary antibody enzyme conjugate was also carried out with the unlabelled antibody and control. It was seen that, there was no colour developed in the sheet with control serum, where as dark dot was obtained in the sheet with unlabelled antibody (indirect). This indicated that the antibody enzyme conjugate may be used for further ELISA tests (Fig 5).

Control

Indirect

Direct

Fig 6. Control: Dot blot with control serum (no dot), indirect: Dotblot with antiserum using indirect method with Anti Rabbit HRP conjugate (dot obtained), Direct: Dotblot using antiserum with antibody HRP conjugate (dot obtained)

Stimulation of whole blood with known quantity of endotoxin for the production of IL1β antigen and detection using dotblot.

Fig 7: The dot blot assay with doubling dilution of stimulants conducted on single strip of nitrocellulose paper and results are as follows. Ag indicated IL1β antigen (commercial). 1-7 are doubling dilutions of stimulated plasma.

Fig 7

Protein estimation in coated plates:

After coating the ELISA plates with primary antibodies, the coating efficiency was analyzed by assay of the attached protein content by Lowry's method. When 20µg of purified polyclonal

antibody was added in each weel, the protein content after thorough washing was found to be 0.18±0.01 µg (n=8). Whereas when commercial monoclonal antibody was coated

ELISA standardization

Standardization of ELISA by Indirect two-antibody sandwich assay was carried out earlier. High background was seen in all the cases indicating the nonspecific binding. There was a slight reduction in background on increasing the dilution of HRP-Secondary antibody conjugate. This may be due to the cross reaction between the secondary antibody and the primary antibody coated on the wells

In order to eliminate the non specific binding, the antibody was labeled with Horse Radish Peroxidase enzyme and a preliminary step for standardizing ELISA was carried out by the direct method of two-antibody sandwich assay. The conjugation with HRP enzymes could not yield consistent result neither on dot blot nor on ELISA.

Commercially available monoclonal antibodies (Cytolab, Prospec) are procured and standardization of ELISA was carried out. This was carried out by standardizing with monoclonal antibodies and antimouse IgG HRP. This could yield positivity and reducing the concentration of secondary antibody could yield reduction in background. Dynamic range could be established.

Standard graph for interleukin 1 beta (Fig 11)

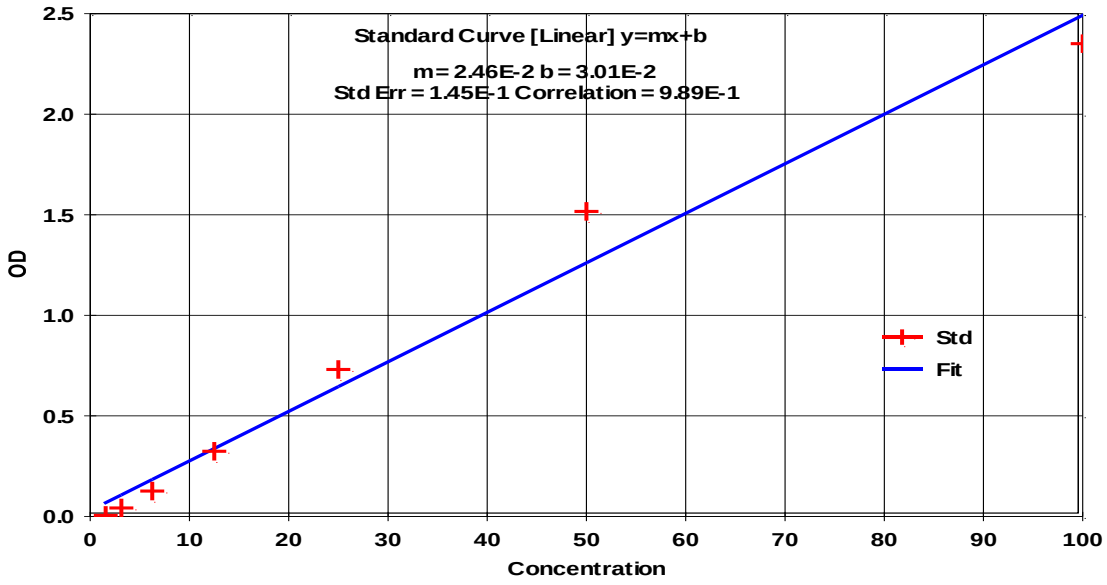


Fig 11 shows the standard graph obtained with doubling dilution of antigen. The graph plotted indicated good correlation coefficient of 0.989. This will be used to find out the concentration of unknown antigen present in the stimulated plasma.

The stimulated plasma is diluted in doubling concentration and added to wells shown to have linearity. The amount of antigen present in the supernatant plasma will be calculated by the following equation Y= mx + b (see fig 11)

Summary:

1. Antibody was raised using purified antigen obtained from commercial source.
2. The antibody was purified to homogeneity.

3. Labeling of antibody with enzymes (HRP) was standardized.
4. Demonstrated the binding of purified antibodies with antigen using dot blot assay.
5. Even after purification and labeling antibody is stable and reacts well in the dot blot assay.
6. ELISA has not given any significant positivity with the labeled antibody.
7. ELISA standardization started with monoclonal antibodies and antimouse IgG HRP. A standard graph with linear correlation of 0.989 was obtained.
8. Whole blood stimulated with endotoxin and production of IL1β detected using dot blot method.
9. The concentration of IL1β produced by stimulation of endotoxin was found out with the help of standard graph for IL1β and it has shown good linearity.

Conclusion:

Though the antibody is developed, purified and tested, the titre of specific antibody in serum seems to be low during the initial period. Later, more boosters were given to increase the antibody titre, so that ELISA was carried out with required sensitivity. Blank values were reduced by adjusting the dilution of secondary antibody. Whole blood sensitized with known concentration of endotoxin and the amount of Interleukin 1ß produced was found out from the standard graph prepared with antigen. The validation part of the experiment is not carried out during the period because; the standardization part took long time. The parent Institute promised the support to complete the validation part

Patent

1. Mohanan PV, Siddharth Banerjee, Muralidharan CV, Lissy K Krishnan, Bhuvaneshwar GS. Development of an 'A Kit for the evaluation of pyrogenecity and a methodology of preparing the same. Indian Patent No. 314849/27/06/2019.

Publications:

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- Mohanan PV, Siddharth Banerjee, CS. Geetha (2011). Detection of pyrogenicity on medical grade polymer materials using rabbit pyrogen, LAL and ELISA method. J. of Pharmaceutical and Biomedical Analysis. 55,1170-1174
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- Megha KB, Siddharth Banerjee, Mohanan. PV (2011) Investigation of interleukin - 1β release from cryopreserved blood stimulated with endotoxin.Cryobiology,63(3):273-278.