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Research Institute for Health Sciences (RIHES)

Chiang Mai University

PROGRESS REPORT NO. 1

Development of an Immunodiagnostic "Spot" Field Test for Acute Reactions in Leprosy

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Project Profile

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Organization: Research Institute for Health Sciences (RIHES), Chiang Mai University.

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Authorized Officer: Kosin Amatayakul, MB, ChB, FRCOG, Acting Director, Research Institute for Health Sciences

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Budget Allocation for This Period: Bt 985,116

Contents

1. Background/Introduction:

Two general types of reactions are described in leprosy (Pfaltzgraff & Bryceson, 1985). The more frequently observed is Erytherma Nodosum Leprosum (ENL), occurring in patients with large numbers of leprosy bacilli in their lesions (ie, lepromatous or "multi-bacillary").

ENL is characterized by fever and malaise associated with crops of red, tender nodules which develop suddenly and simultaneously on several different parts of the body, in no apparent relationship to pre-existing skin lesions. Less frequently observed are so-called reversal reactions, characterized by gradual onset of erythema, swelling, and mild tenderness of pre-existing lesions and cause rapid nerve damage and loss of function. Reversal reactions develop only in the borderline types of leprosy (from BL-BT).

Approximately 50% of newly diagnosed leprosy patients in our series of patients in Thailand will experience one or more acute reactional episodes, usually within the first year or two after diagnosis. In the same series of patients, 50% of the patients first seek medical treatment because of a reaction which has aggravated symptoms of leprosy and which may have been present for months or years.

Recent data have suggested that immune complexes formed in the tissue are responsible for mechanisms of ENL reactions (Harboe, et al, 1987), but this has been difficult to confirm or pursue in further detail with conventional biopsy studies. Production of antibodies in vitro has been demonstrated by cells explanted from such skin biopsies (Lai, et al, 1980). Bjune and Barnetson (1976) reported an increase in the in vitro responsiveness of lymphocytes to M. leprae from patients with reversal reactions, and proposed that this increased responsivenes causes the reactions. They did not speculate on the mechanisms by which this increase was initiated, nor how it might be regulated.

2. Objectives:

1. To measure the intracutaneous levels of M. leprae antibodies and of soluble interleukin-2 receptors (IL-2R) in leprosy patients at high risk for the development of Erythema Nodosum Leprosum (ENL) and "Reversal" reactions, respectively.

2. To improve methods of predicting and monitoring reactions by measuring M. leprae antibodies and soluble IL-2R in intradermal fluid obtained routinely in leprosy patients from slit-skin smears.

3. Materials and Methods:

At the time of this writing, 39 patients have been enrolled in this study of which 20 are active patients and 19 inactive patients. Patients in the active group have been classified clinically as follow:-

1. Active patients without reaction 8 cases

   (LL = 3, BL = 3, BT = 1, TT = 1)

2. Active patients with ENL reaction 7 cases
   (LL = 4, BL = 3)

3. Active patients with "Reversal" reaction 5 cases

   (BL = 2, BB = 1, BT = 2)

An assay for antibody against antigen of M. leprae, phenolic glycolipid 1 (PGL-1) using a standard ensyme linked immunoassay (ELISA) technique with 23 known serum samples was employed in comparing methods of collecting skin smear fluid on pieces of glass slide sized 0.3 x 2.5 cm (the proper size convenient for transporting in appendorf) and filter paper disks.

4. Results and Discussion, Chiang Mai University :

Findings from checkerboard titration showed the appropriate concentration of PGL-1 at 2 ug/ml as shown in Fig 1 and proper conjugate and serum dilution were 1/4000 and 1/400, respectively as shown in Fig 2.

Considering serum sample collected in liquid form as method I, serum sample collected on filter paper disks as method II and serum sample collected on pieces of glass as method III, the percentage of optical density (reading at 492 nm.) of method II and method III as compared to method I are shown in TABLES I and II. The mean percentage of absolute and subtracted values in antibody recovery among 23 samples of method II as compared to method I were 55.66 ± 15.4 standard deviation and 52.87 ± 23.86 standard deviation, respectively, while those in the method III compared to method I was 80.04 ± 21.52 standard deviation and 88.61 ± 53.99 standard deviation, respectively. These results revealed higher sensitivity of antibody recovery in collecting slit-skin-smear fluid with pieces of glass as compared to the other using filter paper disks. We also studied only samples given high optical density value (10 cases) which showed mean percentages of absolute and subtracted values in antibody recovery 63 ± 17.40 and 62.2 ± 19.99, respectively with method II compared to those in method III, 80.5 ± 15.28 and 84.6 ± 14.95, respectively. These results assure us that we have less fluctuation in measuring antibody in ENL patients whose antibodies are increasing.

4.1. Results and Discussion, University of Hawaii:

Since the skin smear method does not permit accurate determination of fluid volume (estimated to total 5-15 microliter per patient), markers must be expressed relative to some "denomiator" values, among which total protein and albumin were suggested (see original proposal, section 6.10, "Standardization of Results").

We have dilutions of human serum or plasma, and blister fluid samples from patients followed in Hawaii to test the accuracy and feasibility of different methods, comparing them to the buiret method of total protein and to electrophoretic separation of proteins. The bromphenol blue methods suggested in the proposal was found to be insufficiently sensitive and possibly susceptible to error in heparinized samples or those containing phenol in RPMI media used to dilute blister or skin smear fluids and was therefore rejected for the present objectives after preliminary studies (data not shown).

The Coomassie Brilliant Blue (CBB) method (Ramekers, 1984), however, has proved to be sensitive, accurate, reproducible and easily performed and standardized. With this method, total protein measured in blisters in our studies is 20-30% of that found in plasma (Fig 6), similar to results obtained in other laboratories using gas chromatography and other complex methods to analyze fluid from suction-induced blisters (Volden et al., 1980).

As we have modified it, this method requires only 50 ul or less of a diluted specimen and can be performed in microtiter plates and read in an automated ELISA reader to provide optimal speed and convenience in the field. The methods is also very inexpensive employing only a chemical reaction with the dye. One drawback with this method is its known underestimation of the globulin component of the fluid, but this should not be a major concern as total or individual globulins can be determined separately by immunoassays if desired.

In order to establish whether or not the production of potential markers of reactions in leprosy occurs in the skin lesions, it will be necessary in this project to demonstrate that elevated levels of antibody, Tac peptide etc., are found in higher relative concentrations in the skins than in the circulation. One suitable approach follows the example of the CSF/Serum Immunoglobulin Index, used to demonstrate increased synthesis of immunogloblulin in the central nervous system. Measurements of serum and CSF albumin are used as "normalizing" values, multiplied by values of CSF and serum immunoglobulin, respectively (Walsh & Tourtellotte, 1983). We have therefore begun efforts to quantitate albumin in blister fluid and serum or plasma.

Conventional electrophoresis techniques were not satisfactory with previously controlled blister fluids, because of the relatively low protein concentration in the diluted samples, and the resulting requirement for large volumes which produced broad "smeared" protein patterns rather than discrete bands (not shown). Various technical modifications might yet utilize electrophoresis; but because these appear unnecessary at this time,

and the resulting methods might not be easily adapted to field conditions, this work has been discontinued for the present.

A bromeresol blue method has been used with some success. Due to the small volumes, with a small quantity of protein present at relatively high dilution, some modifications of this method will be needed to use it for fluid blisters; and it is not yet certain that this method will suffice with the low concentrations in our specimens. Because of the simplicity and low cost of this method, however, we are continuing to evaluate this approach.

We have also begun work to establish a quantitative ELISA for human albumin, based on the sandwich method described by Schwerer and colleagues (1987) for CSF specimens. This method uses unlabelled goat anti-human albumin as a coating antibody in wells of a vinyl microtiter plate. A concentration of 100 ng/well of antibody has been found optimal with the reagents we have purchased (Fig 7). Peroxidase-labelled goat anti-human albumin of 1:24,000, based on standard curves obtained thus far (Fig 8). The method is sensitive to as little as 0.5.ng albumin per well and should therefore be eminently suited to the diluted skin-smear samples. Currently we are working to improve the reproducibility of the standardization curve for this method.

5. Conclusion/Remarks:

Even though these studies showed higher sensitivity in collecting fluid with pieces of glass which we are using for all slit-skin-smear fluid from subjects enrolled in this project, we also studied the degree of correlation between method I and II (Fig 3), I and III (Fig 4), II and III (Fig. 5) which revealed correlation coefficient (r) of 0.92, 0.93 and 0.95, respectively. In case of field study we can substitute filter paper disks for the same purposes which will give us correlated results.

6. Workplan for the Next Period:

1. Continued recruitment of new patients to study blister and slit-skin-smear fluids, as a means of increasing the basic patient population for long term follow-up.

2. Follow-up studies will also be continued.

3. The major technical step to be taken in the next few months concerns measuring of antibodies to M. leprae related antigens (phenolic glycolipid 1, disaccharide and trisaccharide antigens) in blister fluid together with reference standard in order to find the appropriate settings in applying these to tiny amounts of slit-skin-smear fluid.

4. During Dr Scollard's visit to Chiang Mai in February-March 1989, those methods which have been sufficiently tested in Hawaii will be transferred to the laboratory in Chiang Mai, together with reagents and standards.

Table I Comparison of percentages of antibody recovery among various O.D. samples in different methods of fluid collection.

	% OD 492 Compared to Method I *		
	Method II ** ± S D		Method III *** ± S D
Absolute value	56.56 ± 15.40	(P <0.05)	80.04 ± 21.52
Serum Control	62.48 ± 20.69	(P <0.05)	86.09 ± 51.36
Subtracted value	52.87 ± 23.86	(P <0.05)	88.61 ± 53.99

Table II Comparison of percentages of antibody recovery among high O.D. samples in different methods of fluid collection

	% OD 492 Compared to Method I *		
	Method II ** ± S D		Method III *** ± S D
Absolute value	63 ± 17.40	(P <0.05)	80.5 ± 15.28
Serum Control	66.1 ± 19.24	(P <0.05)	73.3 ± 37.74
Subtracted value	62.2 ± 19.99	(P <0.05)	84.6 ± 14.95

* Method I Known serum in fluid form.

** Method II Serum collection on filter paper disks.

*** Method III Serum collection on pieces of glass.

7. References:

Ramekers J M (1984) "Coomassie Blue: an alternative method for proteins". Clin. Chem. 30: 1433-1434.

Schwerer B, Bach M, and Bernheimer H (1987) "ELISA for determination of albumin in the manograme range: assay in cerebral fluid and comparison with radial immunodiffusion". Clinica Chimica Acts 163: 237-244.

Volden G, Thorsrud A K, Bjornson T, and Jellum E (1980). "Biochemical composition of suction blister fluid determined by high resolution multicomponent analysis (capillary gas chromatography -- mass spectrometry and two-dimensional electrophoresis)". J. Invest. Dermatol. 75: 421-424.

Walsh M J and Tourtellotte W W (1983). "The cerebralspinal fluid in multiple sclerosis". In Multiple Sclerosis, J F Hallpike, C W M Adams, and W W Tourtellotte, Eds. London, Chapman and Hall, pp 275-378.

8. Publications and presentations arising from this work:

Presentation: Scollard D M, Rangdaeng S, Suriyanon V, Thamprasert K, and Theetranont C. "Changes in mononuclear cell (MNC) populations in vivo in human leprosy lesions". XVII International Academy of Pathology, International Academy of Pathology, Dublin, Ireland, September 1988.

Presentation: Scollard D M, Suriyanon V, Wagner D K, Thamprasert K, Bhoopat L, Smith T, and Theetranont C. "Intralesional cellular and soluble immunoregulatory events in type T ("Reversal") reactions". XIII International Leprosy Congress, The Hague, The Netherlands, September 1988.

Scollard D M, Theetranont C, Suriyanon V, Bhoopat L, Rangdaeng S, Thamprasert K, and Smith T. "Measurement of cellular and soluble immunologic changes in vivo in human leprosy lesions." Acta Leprologica (Pasteur), in press. Proceedings of the Pasteur Institute's 4th International Colloquium on the Mycobacteria, Paris, September 1988.

Rangdaeng S, Scollard D M, Suriyanon V, Smith T, Thamprasert K, and Theetranont C. "Studies in human leprosy lesions in situ using suction-induced blisters. 1. Cellular components of new, uncomplicated lesions". Int. J. Lepr., in press (June 1989).

Presentation: Bhoopat L, Thamprasert K, Theetranont C, et al. "Evolution od in situ immune responses in clinical subgroups of erythema nodosum leprosum reactions in leprosy: An immunoperoxidase study using suction-induced skin blisters." His Majesty's Fifth Cycle Commemorative Conference of USAID Science Research Awardees, 24-26 July 1987.

Presentation: Thamprasert K, Bhoopat L, Theetranont C et al. "Application of pressure induced suction blisters for immunohistochemical study of leprosy." His Majesty's Fifth Cycle Commemorative Conference of USAID Science Research Awardees, 24-26 July 1987.

9. Figures:

Fig. 1. Titration curve, conjugate 1/4000 showed the appropriate PGL-1 Ag concentration at 2 ug/ml.

Fig. 2. Conjugate titration using PGL-1 Ag = 2 ug/ml showed the most suitable serum dilution at 1/400.

Fig. 3. Correlation curve between method I and method II.

Fig. 4. Correlation curve between method I and method III.

Fig. 5. Correlation curve between method II and method III.

Fig. 6. Total protein: CBB method - plasma vs blister fluid

Fig. 7. Albumin ELISA: Titration of coating Ab. and conjugate

Fig. 8. Albumin ELISA: Standard curves/conjuagte dilutions

RIHES Chiang Mai
8 February 1989
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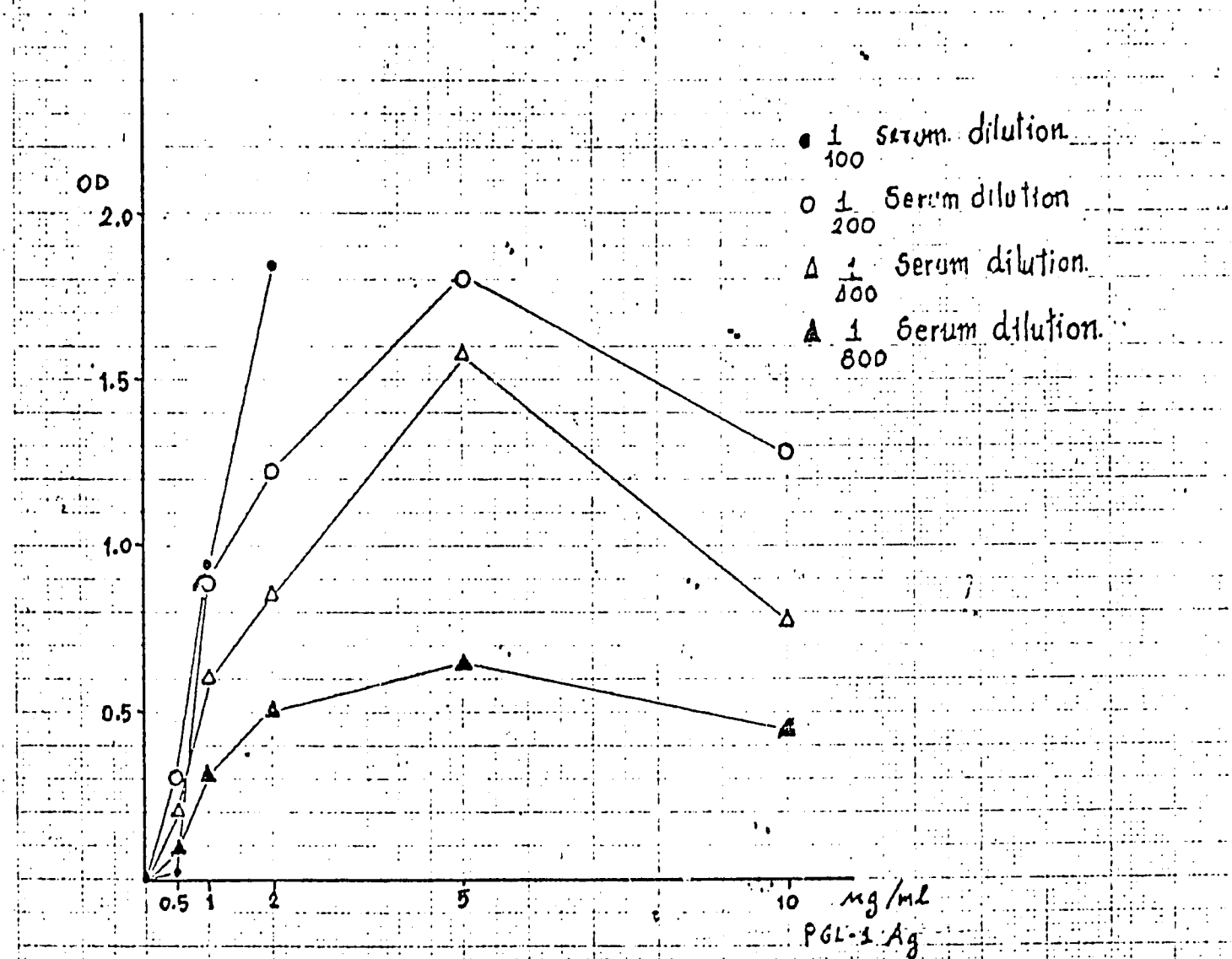
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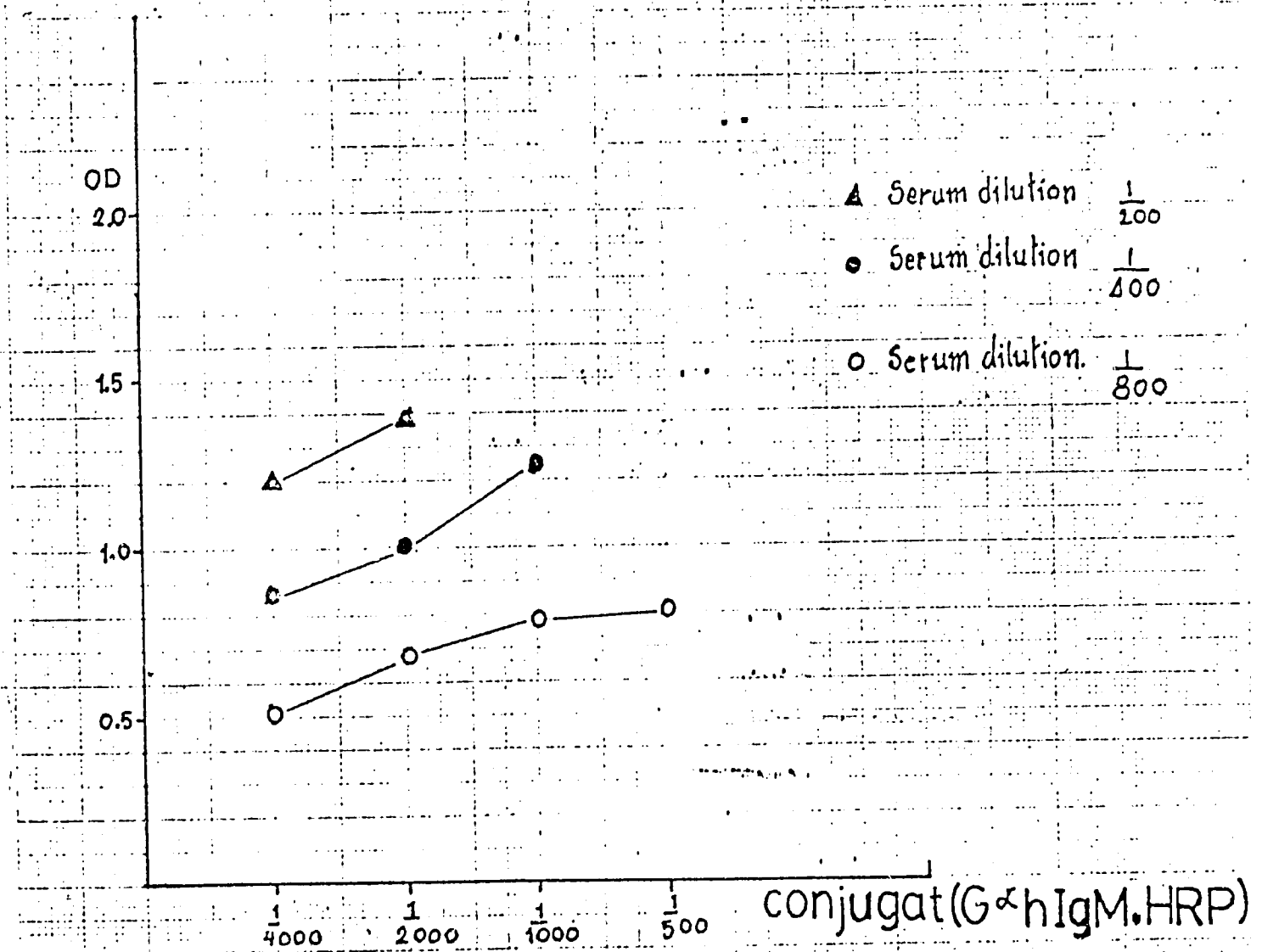
1/200 Serum dilution
1/400 Serum dilution
1/800 Serum dilution

Fig.1.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0.5
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conjugat(Gαh IgM.HRP)

Fig. 2

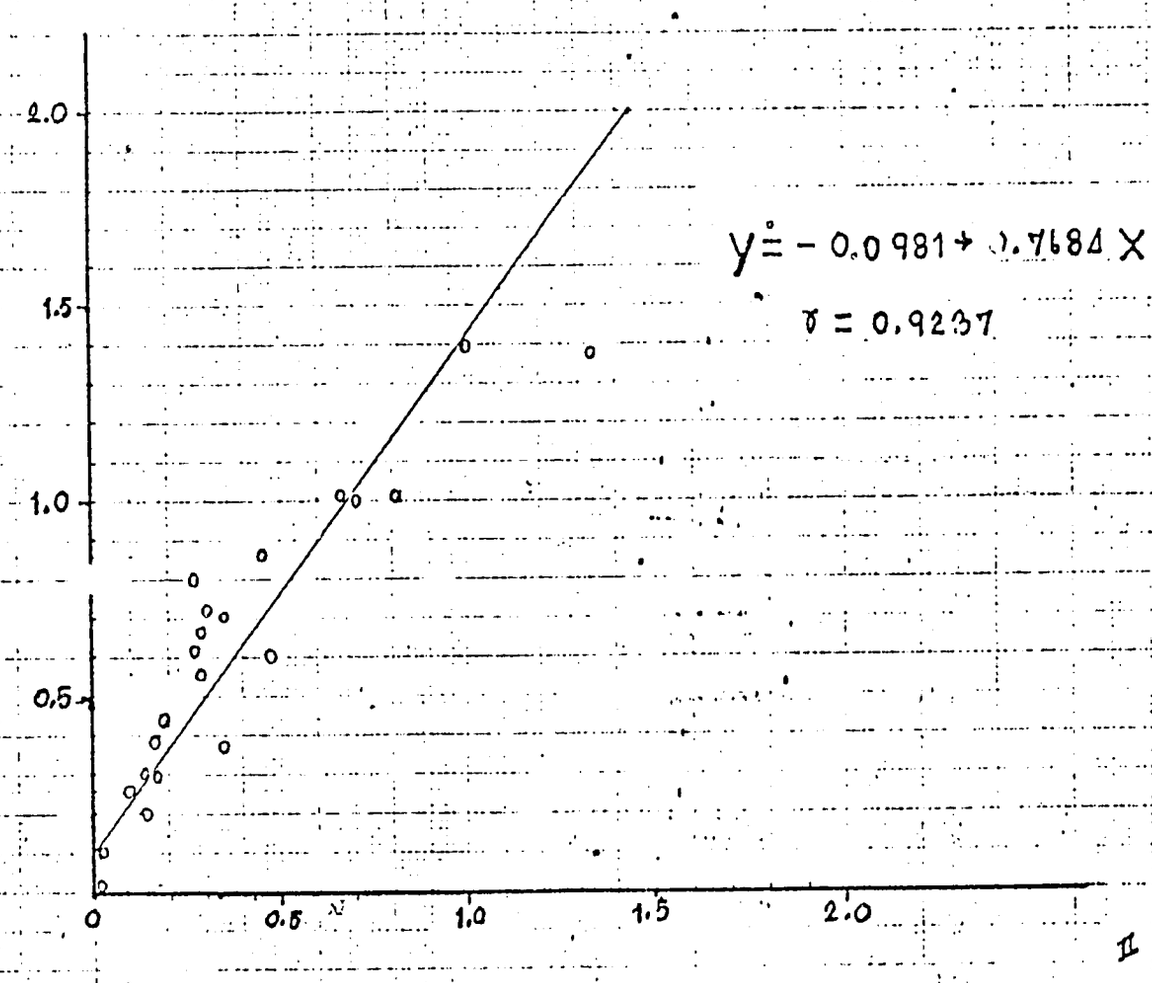
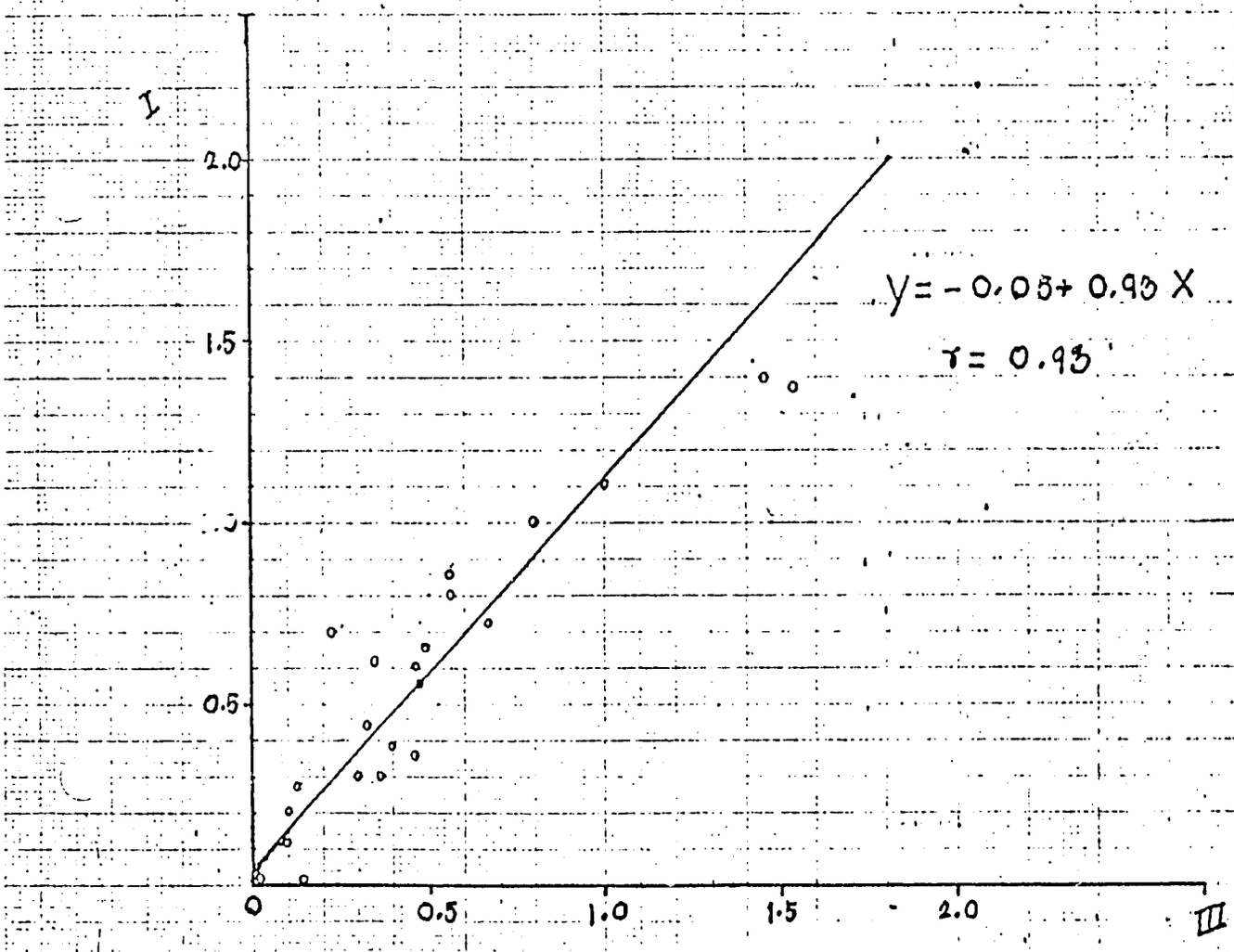


Fig.3



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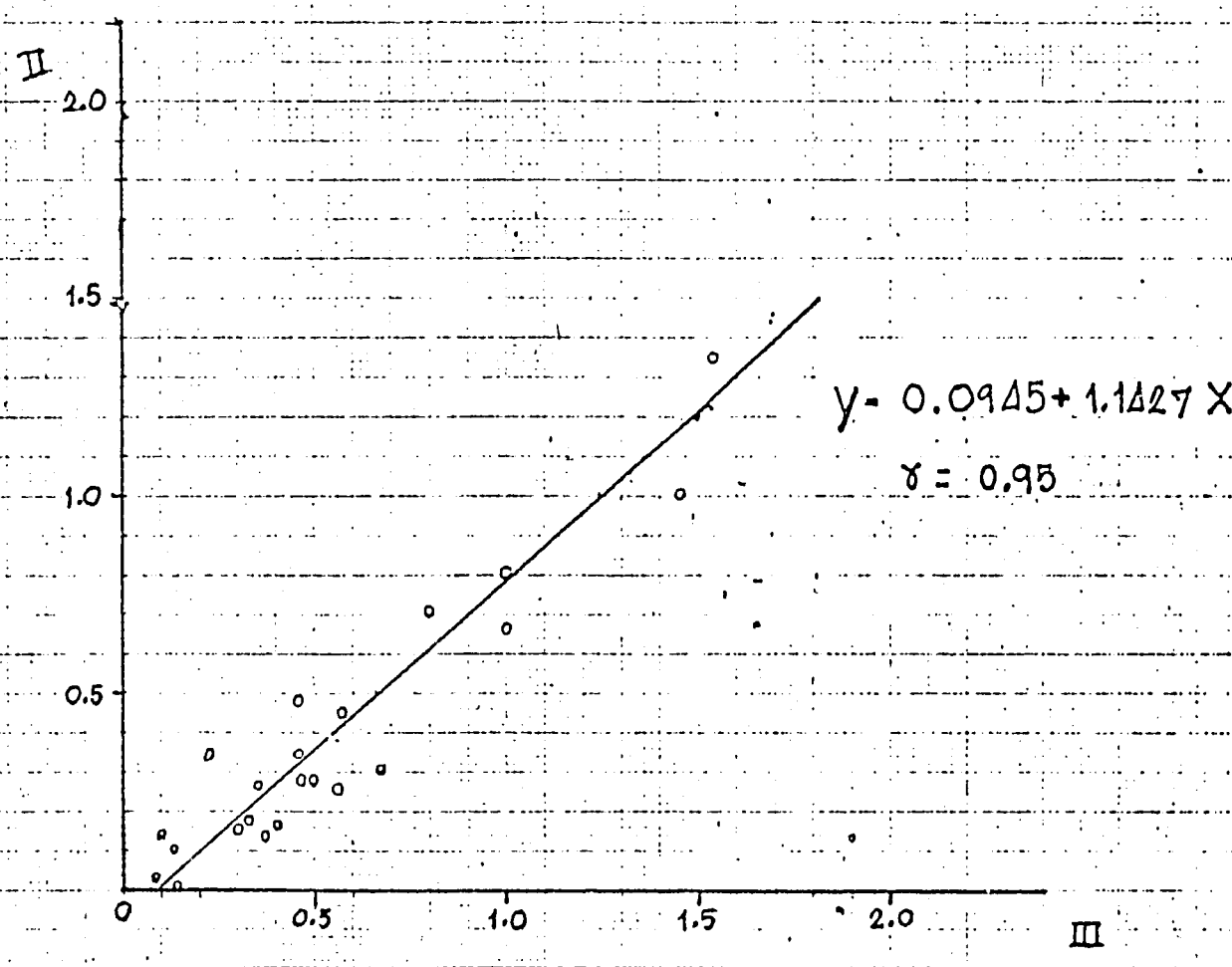
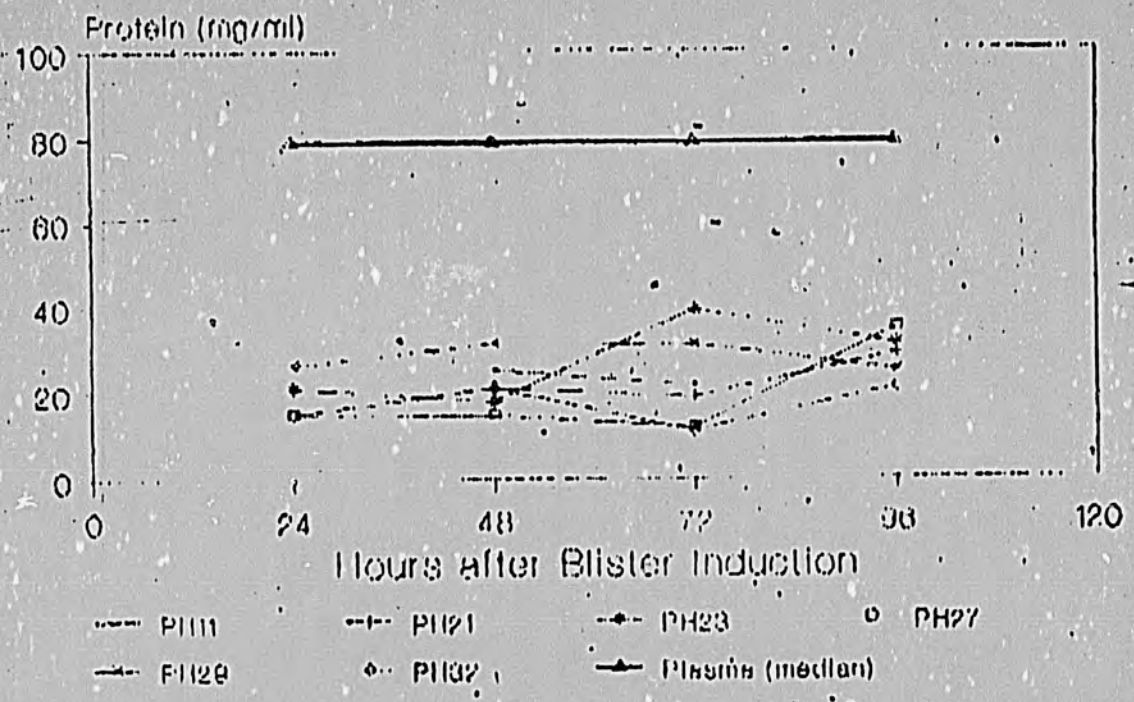


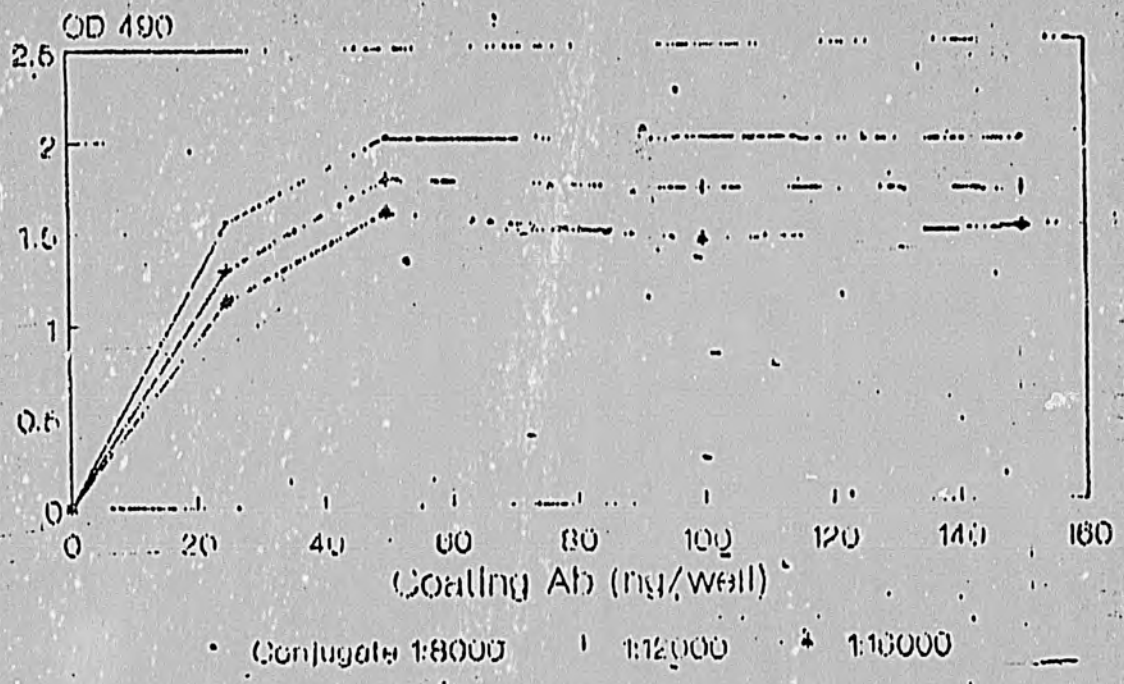
Fig. 5.

Fig. 6. Total Protein: CBB Method
Plasma vs Blister Fluid



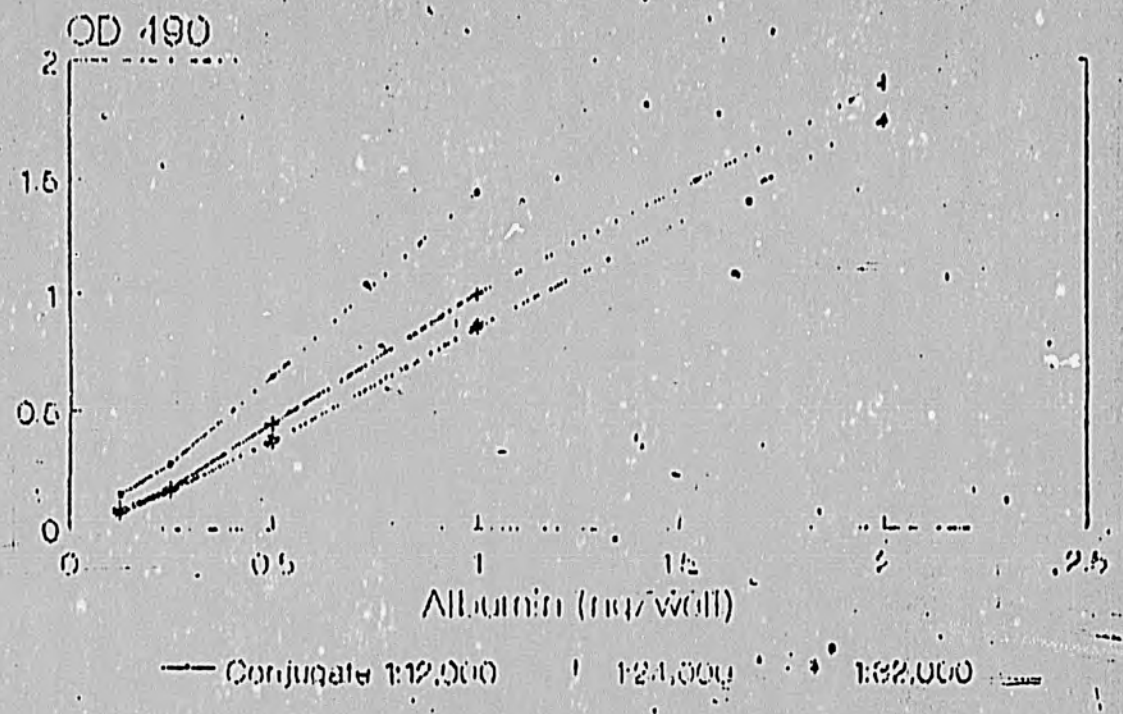
14

Fig. 7. Albumin ELISA
Titration of Coating Ab. and Conjugate



1 ng Albumin/ well

Fig 8. Albumin ELISA
Standard Curves / Conjugate Dilutions



1ng Albumin/well; Coating Ab-100 ng/well