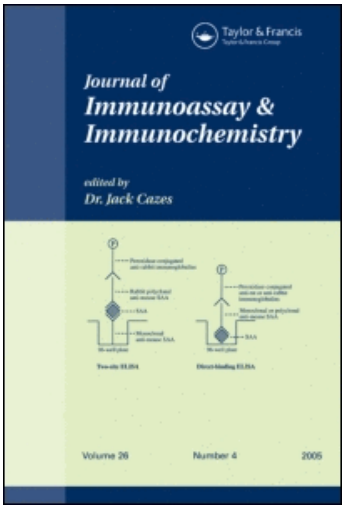

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A QUANTITATIVE CELL-ELISA FOR α - GALACTOSE SPECIFIC ANTIBODIES IN HUMAN MALARIA

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ABSTRACT

Naturally occurring antibodies to α-linked galactose (anti- gal) has been reported to be present in large quantities in normal human sera and they seem to play an important role in a variety of infectious as well as autoimmune diseases. A cell-ELISA using glutaraldehyde fixed normal rabbit erythrocytes was developed for quantification of anti-gal in human sera. This assay was compared with three other(commonly used) immunoassays viz. a) agglutination b) enhanced agglutination and c) lipid ELISA-assays for detection of anti-gal in human sera. The cell-ELISA was found to be the most sensitive assay followed by lipid-ELISA, enhanced agglutination and agglutination assay in decreasing order. Anti-gal affinity purified through a column of melibiose-agarose was tested by cell-ELISA. Monolayers of RRBC pre-treated with α-galactosidase was not reactive while in monolayers treated with ß-galactosidase, the anti-gal reactivity was comparable to those in untreated RRBC monolayer, thus indicating the high specificity of cell-ELISA for detection of antibodies to α-linked galactose.

(KEY WORDS : Cell-ELISA, Anti-gal, Immunoassay, Auto-antibody, Malaria)

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INTRODUCTION

A naturally occurring anti-galactosyl IgG antibody (anti-gal) constituting nearly 1% of total circulating IgG was reported a decade ago (1). These antibodies display a distinct specificity for the glycosidic structure, galactose α (1-3) galactose which is expressed in abundance on rabbit erythrocytes (2). A biological role for these antibodies has become evident in many diseases such as American cutaneous leishmaniasis and chronic Chagas disease (3 & 4), human *Plasmodium falciparum* malaria (5), rheumatoid arthritis, particularly in patients who have developed renal intolerance under administration of gold or D-penicillamine (6), sickle cell anaemia (7), thyroid disorders (8) and probably in a host of bacterial infections (9). Immunological methods employed by other workers to quantify anti-gal titres in human sera include i) an agglutination assay using rabbit erythrocytes (1), ii) enhancement of the agglutination reaction using anti-globulin reagents (10), iii) a rosette assay with the myeloid cell line K-562 (2) and iv) a lipid-ELISA using neutral glycospingolipid purified from rabbit erythrocyte membranes (3). We had developed a cell-ELISA to study the role of anti-gal in human *P.falciparum* infections (5). Since monitoring the anti-gal levels in human sera is becoming increasingly important in clinical immunology and is expected to be widely performed in the coming years for diagnostic or prognostic purposes in many diseases, the present study was undertaken to evaluate the four different immunoassays using a set of human sera. Although all the tests were specific for detecting antibodies to α-linked galactose, cell-ELISA was found to be by far the most sensitive and convenient assay for quantification of anti-gal in human sera.

MATERIALS AND METHODS

Collection of sera

Blood for sera were collected from areas highly endemic for *P.falciparum* malaria. Sera of adult individuals with or without peripheral parasitaemia (as shown by microscopic examination of Giemsa stained thick blood smears) were collected at the District Hqs. Hospital, Keonjhar, Orissa.

Agglutination

The anti-gal antibody titres were determined by passive agglutination assay using rabbit RBC (RRBC) that are known to express galactosyl residues on their surface (1). The rabbit blood sample collected in 0.15 M tri-sodium citrate was washed in phosphate-buffered saline and the upper layer containing leucocytes was removed. The erythrocyte pellet was washed three times and finally a 1% suspension of erythrocytes was made in PBS. Agglutination activity of human malarial sera was assessed by mixing two fold dilution of the sera with an equal volume of 1% RRBC suspension in the wells of V bottom microtitre plate. Agglutination titre was taken after the RBC settled at room temperature for 2 hr. This assay was repeatedly performed on five different days using the same set of sera and the highest mean serum dilution was expressed as the anti-gal titre.

Enhanced agglutination

Enhanced agglutination activity of the various malarial sera was performed as described earlier(10) with minor modifications. In brief, 50 µl of two fold serial diluted sera were mixed with an equal volume of washed 1% rabbit red blood cell suspension in V-shaped wells of a microtitre plate. After 30 min. incubation at

37°C, the red cells were resuspended by gentle shaking after which 50 µl of 1:500 diluted rabbit anti-human IgG antibodies (Dako, Denmark) was added to each well. Agglutination was assessed after the red cells settled at R.T. for 2 hr. The titres were expressed as the highest dilution of sera that caused complete agglutination. A compact button was taken as a negative reaction. The same sera were tested five times and the mean titre was taken.

Extraction of lipids from NRRBC membrane

Normal rabbit red blood cells (NRRBC) collected in 0.15 M tri-sodium citrate were washed twice with PBS and subsequently washed with distilled water thrice to remove haemoglobin from erythrocytes. Lipids were extracted from normal rabbit erythrocyte membranes as described elsewhere (3). Membrane glycolipids were successively extracted by adding 50 ml chloroform : methanol (2:1) to the membrane suspension. The mixture was incubated for 1-2 hrs with intermittent shaking at 4°C. The extracted membranes were then sedimented by centrifugation at 2000 rpm for 20 min. The supernatant was collected and the residue was re-extracted with chloroform : methanol (1:2) solution. The two extracts were combined and evaporated to dryness. The total lipids collected were subsequently used as antigen in lipid-ELISA.

Lipid-ELISA

The method essentially described by Avila *et al* (3) was followed. Briefly, the antigen was coated onto 96 well microtitre plate by evaporating 100 µl per well of extracted membrane lipids (40 µg/ml) in ethanol. After washing two fold diluted test sera (100µl) in PBS containing 0.1% Tween20 were added in duplicate. After 90 min incubation at 37° C the plates were washed with PBS-T and incubated for

60 min with 100 µl of 500 fold diluted anti-human immunoglobulin -peroxidase conjugate (Dakopatts,Denmark). After washing the plate extensively with PBS-T, the bound enzyme activity was measured using O-phenylene-diamine in citrate phosphate buffer, pH 5.6 (1 mg/ml). The reaction was stopped by the addition of 25 µl of 8 N H_2SO_4 and the absorbance at 492 nm was measured in an ELISA reader (Bio-Rad, Richmond, VA).

Preparation of rabbit RBC monolayer

Normal rabbit erythrocyte monolayers were prepared as described previously (5). Briefly, 96 wells flat bottomed polystyrene plates (Costar, Cambridge) were activated at 4° C overnight with 0.1% glutaraldehyde in PBS (pH 7.2). After washing the plates thrice with PBS, 100 µl of 0.1% rabbit erythrocytes in PBS was added to the wells. The plates were centrifuged at 1000 rpm for 1 min in an IEC CENTRA 7R, USA and 100 µl of 0.1% glutaraldehyde in PBS was added to each of the wells. After incubation at room temperature for 1 hr, the plates were washed extensively with PBS to remove unbound cells and glutaraldehyde. The monolayers were then treated sequentially, 20 min each, with 70% ethanol, methanol containing 0.5% H_2O_2 and again with 70% ethanol after which the plates were washed thrice with PBS. Plates were allowed to dry and used immediately for the assay or stored desiccated at -20°C until further use.

Cell-ELISA

For the assay, 100 µl of human serum (two fold diluted) in PBS containing 0.1% Tween 20 was tested in duplicate. After 90 min. at 37° C, the plates were washed with PBS-T and incubated for 60 min with 500 fold diluted anti-human immunoglobulin-peroxidase conjugate (Dako, Denmark). After washing the plate

extensively with PBS-T, the bound enzyme activity was measured using O-phenylene-diamine in citrate phosphate buffer, pH 5.6 (1 mg/ml). The reaction was stopped by the addition of 25 µl of 8 N H_2SO_4 and the absorbance at 492 nm was measured in an ELISA reader (Bio-Rad, Richmond,VA).

Affinity purification of anti-gal

Anti-gal was isolated from a pool of malaria sera by using a column of melibiose-agarose (M-5889, Sigma Chemicals Co., USA). One ml of malarial serum pooled was passed through a column of melibiose-agarose and washed extensively with PBS, pH 7.2. The bound antibodies were eluted with gly-HCl buffer, pH 2.8. The pH of the fractions was adjusted to pH 7.0 using about 15-20 ul of 0.5% NaOH, diluted 1:1 with PBS-T and tested by ELISA as described in the results.

RESULTS AND DISCUSSION

Anti-gal levels in twenty malarial sera were determined and compared by four different tests as shown in Fig.1. Agglutination assay with rabbit erythrocytes could yield only low titres of anti-gal in sera - lowest detectable titre being 1/20. The sensitivity of the enhanced agglutination assay was appreciably more in comparison to simple agglutination assay - the lowest detectable titre being 1/160. The sensitivity of lipid-ELISA was comparable to that of enhanced agglutination assay. The cell-ELISA was found to be the most sensitive assay for detection of anti-gal antibodies - the lowest detectable titre being 1/5120. The order of sensitivity of the different assays was found to be cell-ELISA >lipid-ELISA> enhanced agglutination assay > agglutination assay.

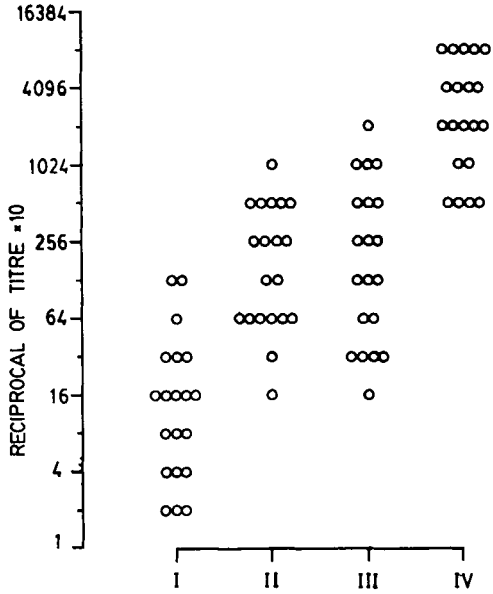

FIGURE-1 Comparison of four different immuno-assays for quantification of anti-gal titres in twenty malarial sera:I-Agglutination assay, II-Enhanced agglutination assay, III-Lipid-ELISA and IV- Cell-ELISA.

The ELISA absorbance values of anti-gal titration at a dilution of 1/160 by lipid-ELISA was comparable to that of a dilution of 1/5120 in cell-ELISA (Fig.2). Further, a significant correlation was found (correlation coefficient $r=0.86$, $p<0.001$) between cell-ELISA and lipid-ELISA, thereby clearly indicating that (a) cell-ELISA and lipid-ELISA detect similar determinants and (b) cell-ELISA is about 40 times more sensitive than lipid-ELISA taking into consideration the dilution of serum and absorbance in ELISA. This could be due to the fact that in cell-ELISA a particulate antigen is used as a monolayer resulting in better exposure of α-gal determinants while in lipid-ELISA the glycolipid antigen is taken in the organic phase resulting in inferior coating efficiency on polystyrene plates.

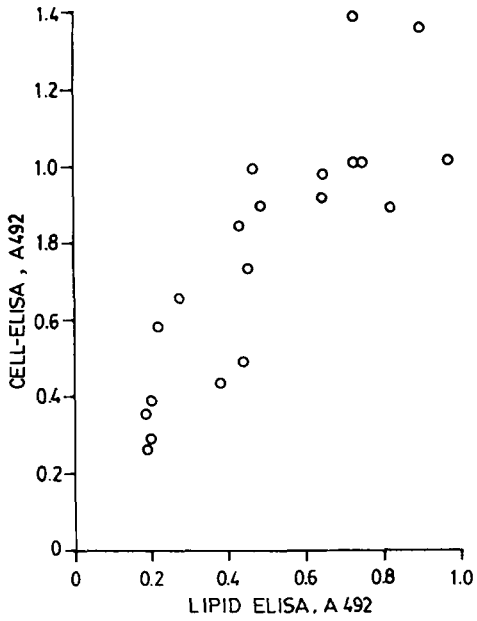

FIGURE-2 Correlation between lipid-ELISA and cell-ELISA for quantification of anti-gal in twenty malarial sera. The absorbance values in 1/160 diluted sera by lipid-ELISA were compared with the absorbance in 1/5120 diluted sera by cell-ELISA.

The specific binding of anti-gal to RRBC monolayer was analyzed by using affinity purified anti-gal antibodies. Pooled malarial sera were passed through a column of melibiose-agarose. The affinity eluted fractions were tested against untreated, or NRRBC monolayers treated with α-galactosidase or ß-galactosidase (Fig. 3). Treatment of RRBC monolayer with α-galactosidase (at a concentration of 1U/ml) decreased the reactivity of affinity purified anti-gal antibody while ß-galactosidase treatment (at a concentration of 10 U/ml) did not reduce the binding of anti-gal antibody. We had earlier demonstrated the high specificity of cell-ELISA for detection of anti-gal in sera by inhibition with melibiose, a disaccharide containing

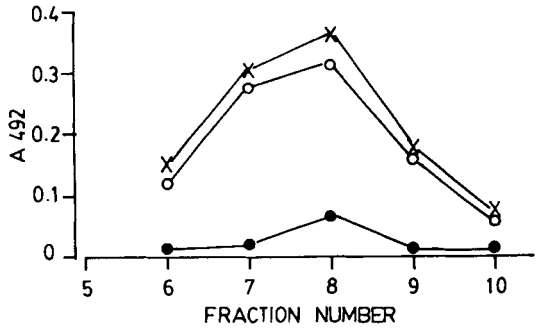

FIGURE-3 Cell-ELISA: Reactivity of affinity purified anti-gal to rabbit erythrocyte monolayer: Pools of malarial sera were passed through a column of melibiose-agarose and the bound immunoglobulins were eluted and reactivity tested against monolayers treated with α-galactosidase (● — ●), ß-galactosidase (X— X) or untreated controls (O — O).

α-linked galactose (5). Similar observations on specificity of immunoassays using rabbit erythrocytes for detection of anti-gal in human sera have been made by other investigators also (1) indicating thereby that human sera do not contain antibodies to determinants other than α-galactose on rabbit erythrocytes.

Antibodies to α-linked galactose have been shown to be present in healthy individuals(1). About 1% of human B-lymphocytes are capable of producing these anti-gal antibodies(11). Since the anti-gal levels have been reported to be markedly elevated in patients with a variety of autoimmune diseases like Graves disease (8), Scleroderma(12), Henoch-Schonlein parpur(13), some parasitic diseases such as Chagas disease, leishmaniasis(3) and malaria (5), the determination of its titre may have clinical application for diagnostic or prognostic purposes in many diseases. In the present study, an attempt has been made to evaluate anti-gal titres in human malaria sera by using four different assays such as agglutination, enhanced

TABLE-1

Comparison of the immunoassays for detection of anti-gal.

Assays	Sensitivity	Specificity	Reproducibility	Reactivity on a linear scale	Antigen preparation	Ig6 subgroup typing
Agglutination	Poor	good	poor	NP	easy	NP
Enhanced agglutination	moderate	good	moderate	NP	easy	NP
Lipid- ELISA	Moderate	good	good	possible	cumbersome	possible
Cell-ELISA	very good	good	good	possible	easy	possible

NP = Not possible

agglutination, lipid-ELISA and cell-ELISA reported in literature (1,3,5 & 10). The relative merits and demerits of the four different assays as revealed by the present as well as earlier investigations are summarized in Table.1. The cell-ELISA clearly offers an easy and convenient system with very high sensitivity and specificity for anti-gal. The reproducibility of cell-ELISA and lipid-ELISA was evaluated by assessing the inter-assay and intra-assay variability of the tests performed at different time intervals. We have found the shelf life of RRBC monolayer coated microtitre plates to be more than six months (unpublished observations) indicating the possibility of development of a kit for anti-gal determination for routine clinical use. Although high levels of anti-gal antibodies have been reported in various diseases (3,4,5,6,7,8 & 9), the biological relevance of these antibodies in disease processes is not yet clearly understood - protective as well as pathological roles have been attributed to them (reviewed in 14). The biological function of auto-antibodies is believed to be related to their isotype and subclass. Thus, the additional advantage of isotype typing offered by cell-ELISA may help to elucidate the specific role of anti-gal in various diseases.

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REFERENCES

1. Galili, U., Rachmilewitz, E.A., Peleg, A. and Flechner, I. A unique natural human IgG antibody with anti-α-galactosyl specificity. J. Exp. Med. 1984; 160:1519-31.

2. Galili, U., Macher, B.A., Buehler, J. and Shohet, S.B. Human natural anti-α-galactosyl IgG. ii. The specific recognition of α (1-3) linked galactose residues. J. Exp. Med. 1985; 162: 573-82.

3. Avila, J.L., Rojas, M. and Galili, U. Immunogenic Gal α1-3 Gal carbohydrate epitopes are present on pathogenic American Trypanosoma and Leishmania. J. Immunol. 1989; 142:2828-34.

4. Towbin, H., Rosenfelder, G., Weislander, J., *et al.* Circulating antibodies to mouse laminin in chagas disease, American cutaneous leishmaniasis and normal individuals recognize terminal galactosyl (α 1-3) galactose epitopes. J. Exp. Med. 1987; 166: 419-32.

5. Ravindran, B., Satapathy, A.K. and Das, M.K. Naturally occurring anti-α-galactosyl antibodies in human *Plasmodium falciparum* infections: a possible role for autoantibodies in malaria. Immunol Lett.1988;19:137-42.

6. Malaise, M.G., Davin, J.C., Mahieu, P.R. and Franchimont, P. Elevated anti-galactosyl antibody titres reflect renal injury after Gold or D-pencillamine in Rheumatoid Arthritis. *Clin. Immunol. Immunopathol.* 1986; 40:356-64.

7. Galili, U., Clark, M.R. and Shohet, S.B. Excessive binding of the natural anti-α-galactosyl Ig G to sickle red cells may contribute to extravascular cell destruction. J. Clin. Invest. 1986; 77:27-33.

8. Etienne-Decerf, J., Malaise, P., Mahieu, M. and Winand, R. Elevated anti-α-galactosyl titre. A marker of progression in autoimmune thyroid disorders and in endocrine ophthalmology? Acta Endocrinologica. 1987; 115:67-74.

9. Galili, U., Mandrell, R.E., Hamadeh, R.M., Shohet, S.B. and Griffis, J.M. Interaction between human natural anti-α-galactosyl immunoglobulin G and bacteria of the human flora. Infect Immun. 1988; 57: 1730-7.

10. Galili, U., Bushler, IJ., Shohet, S.B. and Macher, B.A. The human natural anti-gal IgG III. The subtlety of immune tolerance in man as demonstrated by cross-reactivity between natural anti-gal and anti-B antibodies. J. Exp. Med. 1987; 165:693-704.

11. Galili, U., Anaraki, F., Thall, A., Hill-Black, C. and Radic, M. One percent of human circulating B lymphocytes are capable of producing the natural anti-gal antibody. Blood 1993; 82:2485-93.

12. Gabrielli, A., Leoni, P., Davieli, G., Herrmann, K., Krieg, T. and Wieslander, J. Antibodies against galactosyl α(1-3) galactose in connective tissue diseases. Arthritis Rheum. 1991; 34:375.

13. Mahieu, P. Anti-α-galactosyl antibodies and immune complexes in children with Henoch-Schonlein Purpura or IgA nephropathy. Kidney Int. 1987; 31:1132-9.

14. Galili, U. Evolution and pathophysiology of the human natural anti-α-galactosyl IgG (anti-gal) antibody. Springer Semin Immunopathol. 1993; 15:155-71.