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LECTINS FOR THE DETECTION OF IgM ANTIBODIES TO T. GONDII IN THE DIAGNOSIS OF ACUTE TOXOPLASMOSIS BY IMMUNOFLUORESCENCE TEST.

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SUMMARY

Lectins were labeled with fluorescein and tried as conjugates in the immunofluorescence (IF) test for the detection of IgM antibodies to **T. gondii,** in the diagnosis of acute toxoplasmosis. This approach was an attempt to find alternative reagents for anti-human IgM fluorescent conjugates (AHIgMFC), which contain quite frequently anaibodies to toxoplasma, as contaminants, due to natural **T. gondii** infections among animals used for imunization. Lentil **(Lens culinaris)** lectin fluorescence conjugates (LcFC) provided most satisfactory results. The evaluation of LcFC carried out in a total of 179 sera from patients with acute and chronic toxoplasmosis, with non-related infections or healthy subjects, gave high values of relative efficiency, co-positivity and co-negativity indices, respectively 0.989, 0.969 and 1.000, in reference to the conventional AHIgMFC. Moreover, three batches of LcFC successively prepared gave reproducible test results. The advantage of LcFC as an alternative reagent for the serodiagnosis of acute toxoplasmosis is supported by practical aspects of its preparation.

KEY WORDS: Anti-IgM detection; Lectins; Acute toxoplasmosis; Lectin conjugate.

INTRODUCTION

One of the main serologic markers for the acute toxoplasmosis is the presence of IgM anti-toxoplasma antibodies in serum. To detect such antibodies, immunofluorescence tests are widely used because easy to perform. Although presenting limitations of sensitivity and specificity, these can be overcome by relatively simple procedures of removing the interfering factors, such as rheumatoid factor, with immunosorbents [3] and IgG antibodies to toxoplasma, with protein A [6], which afford a high reliability to the immunofluorescence assay.

A basic reagent for the IgM-immunofluorescence test is a specific anti-human IgM fluorescent conjugate (AHIgMFC), usually obtained by labeling with fluorochrome the IgG fraction of immunized animal serum, anti-human IgM. Quality of such conjugates differ considerably, not only in respect to the characteristics of specificity and affinity of the produced antibodies but also, by the usual presence of contaminating anti-toxoplasma antibodies. These are due to frequent infections by **Toxoplasma gondii** of animals as goats, sheeps or rabbits, commonly used for antiserum production. This problem is found even

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for products furnished by leading manufacturers.

Some lectins present a much higher affinity for IgM than to the other immunoglobulins [7,20]. Also, **T. gondii** tachyzoites do not present membrane sugar receptors for most known lectins [13]. In thsi way fluorescent conjugates of lectins were studied as alternative reagents to fluorochrome-labeled anti-IgM antibodies, in the detection of anti-toxoplasma IgM antibodies in serum. In spite of large applications in immunological, biological, chemical and even clinical researches [19], it seems that lectins have not been previously employed in the detection of antibodies for diagnostic purposes.

MATERIAL AND METHODS

Serum samples — The study was performed in a total of 179 serum samples, 64 of which presenting a serological patern of acute toxoplasmosis (pattern I), 26 with pattern II or III, corresponding to older toxoplasma infections, 40 from clinically healthy individuals and, 49 from patients with nonrelated infections, including 10 cases of Chagas'disease, 10 of malaria, 10 of mucocutaneous leishmaniasis, 11 of schistosomiasis mansoni and 8 of rheumatoid arthritis. Toxoplasmosis serologic patterns were established according to previous published criteria [4,5], on the basis of results of a battery of tests, including IgM and IgG-immunofluorescence (IgM-IF and IgG-IF), passive hemagglutination (HA) and complement fixation (CF) tests, performed as described [4].

Toxoplasma antigens — T. gondii tachyzoites were obtained from mouse peritoneal exsudates two days after inoculation of parasites and TG-180 sarcoma cells, as described [2]. After agglutinating the peritoneal cells with phytohemagglutining P and filtration in nylon wool, purified parasites were treated with formalin [13].

Lectin fluorescent conjugates (LFC) — Five commercially available lectins (Sigma) Chem. Co., St. Louis, USA) from **Lens culinaris, Phaseolus vulgaris, Glycine max, Arachis hypogea** and **Canavalia ensiformis,** were tested, for selecting the most adequate in detecting IgM antibodies.

For the study, three batches of lentil **(Lens culinaris)** lectins (I, II and III) were then prepared in large amounts, following the technique of HOWARD & SAGE [14,15] and one batch of Con A **(Canavalia ensiformis)** as described [1].

Lectins were labeled with fluorescein isothiocyanate according to CLARK & SHEPARD [8] and lectin protein concentration determined by LOWRY et al method [17] with crystalline bovine albumin (Sigma Chem. Co., St. Louis, USA) as standard.

Activity of lectins — All the lectins were characterized by their ability in agglutinating formalinized, group 0, human red blood cells. Quantitative assays were performed in plastic microtiter plates with V-shaped wells. To 0.05 ml two-fold serial dilutions of lectins in 0.01M phosphate buffered saline solution at pH 7.2 (PBS), stating from 1:2, 0.025 ml 2% erytrrocytes suspension in PBS were added. Sedimentation patterns were read as usually, after 90 minutes, at room temperature.

Immunofluorescence (IF) test — Conventional assays for IgM-IF and IgG-IF were carried out as described [4] using commercial (Hyland Div. Travenol Lab., California, USA) fluorescent anti-human-IgM, μchain specific (AHIgMFC) and anti-human IgG, γchain specific (AHIgGFC) conjugates. Monospecificity of these conjugates was confirmed by immunoelectrophoresis, as well as absence of anti-**T. gondii** antibodies, as determined by immunofluorescence assays. Optimal conditions for tests with lectin fluorescent conjugate (LcFC) were standardized, as later referred. To verify the specificity of LFC, inhibition of IF tests was performed by previous addition of saccharide to the conjugate. Also, to verify the specificity of the interaction between LFC and IgM antibodies, sera showing positive results were treated with 2-mercaptoethanol [9] and retested. To avoid the interference of rheumatoid factors in assays for IgM antibodies, all sera were tested by RA latex agglutination test (Behringwerke AG, Germany) and those giving positive results were absorbed with heat-aggregated human gammaglabulin as recommended [3].

Statistical analysis — To compare results obtained with LFC and AHIgMFC in the IF tests, t-student test and coefficient correlation were utilized according to PAUL & WHITE and LUTZ [22,18]. For the evaluation of LFC tests, relative efficiency, co-positivity and co-negativity indices were estimated [10].

RESULTS

A total of 7 lectins were studied, 5 from commercial purchase and 2 prepared in our laboratory, for the standardization of the method.

Protein contents per milliliter and hemagglutinating titers were respectively: **L. culinaris** (10.8 mg, 1:512), **P. vulgaris** (14.8 mg, 1:16), **G. max** (10.8 mg, 1:8), **A. hypogea** (16.0 mg, 1:8), **C. ensiformis** 4.0 mg, 1:2), **L. culinaris** (I) (12.6 mg, 1:2,048), **C. ensiformis** (I) (5.5 mg, 1:16). The last two (I), locally prepared, presented higher titers in relation to those commercially acquired.

Different conditions of pH, temperature and time were required in the IF test with LFC to obtain comparable titers to those given by the conventional AHIgMFC [4]. Thus, serum samples were incubated with **T. gondii** antigen at 4°C, 25°C and 37°C for periods varying from 30 minutes to 27 hours. Best results were seen at 25°C (room temperature), from 4 to 27 hours. For technical convenience an incubation period of 24 hours was also Further incubation step with LFC was also assayed and most satisfactory results were obtained for 90 minutes at 37°C. Influence of pH was studied, best results being obtained by diluting sera in PBS at pH 8.0 and LFC at pH 7.5, whereas washing of slide preparation in PBS at pH 7.2.

As indicated in Table I, only lentil lectin fluorescent conjugate (LcFC), at the maximum reactivity, detected specifically IgM antibodies to **T. gondii** in a similar fashion to the reference AHIgMFC. For other lectins, although giving similar titers for IgM antibodies, much weaker fluorescence staining was observed, as well as non-specific reactions with negative sera diluted at 1:16.

T A B L E I

Comparative titrations with different lectins conjugates of sera from: a) recent **T. gondii** infection, pattern I; b) old **T. gondii** infection, pattern III; and c) non-infected individual

Fluorescent Conjugate and dilution used	Standard serum titer*			
	a	b	c	PBS**
Lens culinaris (1:20)***	256	0	0	0
Lens culinaris (I) (1:80)	256	0	0	0
Canavalia ensiformis (1:5)	256	16	16	0
Canavalia ensiformis (I) (1:10)	256	256	16	0
Glycine max (1:10)	256	256	16	0
Phaseolus vulgaris (1:5)	256	16	16	0
Arachis hypogea (1:1)	0	0	0	0

(*) = Standard serum with reference titer of 256 with anti-human IgM fluorescent conjugate.
(**) = PBS — 0.01M phosphate buffered saline solution, at pH 8.0, as diluent.
(***) = Dilution used
(I) = Lectins prepared in our laboratory

Table II depicts comparative results in the IgM-IF test with **Lens culinaris** and with anti-IgM antibodies, for 179 serum samples from patients with acute and chronic toxoplasmosis, other diseases and clinically normal individuals.

In comparison with the anti-IgM fluorescent conjugate test, the test carried out with lentil conjugate furnished a co-positivity index of 0.969, a co-negativity index of 1.000 and a relative efficiency index of 0.989. In 64 cases total agreement of titers was seen in 42 (65.6%), differences of one dilution in 18 (28.1%) and of two dilutions in 4 cases (6.3%). The geometric mean titers for AHIgMFC was 8.37 and for LcFC, 7.79. A positive correlation, $r = 0.800$, was observed between the IgM antibody titers obtained by both fluorescent conjugates and, accordingly, an equation of regression line could be estimated: $Y = 1.1340 + 0.8394$ (fig. 1). The significance of this value was confirmed by the finding of t-test value of 12.66, in comparison to the critical value of 2.66, (62 d.f at 1% level).

Three different batches of LcFC (I, II and III) were prepared in the laboratory for the study of the reproducibility of the reagents. They showed respectively the hemagglutinating titers of 1,024, 2,0448 and 4,096 and dilution of use in the IF test of 1:40, 1:80 and

TABLE II

Comparative results of **Lens culinaris** and anti-human IgM fluorescent conjugates in IgM-IF tests, performed in 179 serum samples

Diagnosis	N.º of serum	Fluorescent conjugate			
		Lectin		Anti-human IgM	
		Positive	Negative	Positive	Negative
Acute toxoplasmosis	64	62	2	64	0
Chronic toxoplasmosis	26	0	26	0	26
Chagas'disease	10	0	10	0	10
Malaria	10	0	10	0	10
Schistosomiasis mansoni	11	0	11	0	11
Mucocutaneous leishmaniasis	10	0	10	0	10
Rheumatoid arthritis	8	0	8	0	8
Clinically normal individuals	40	0	40	0	40

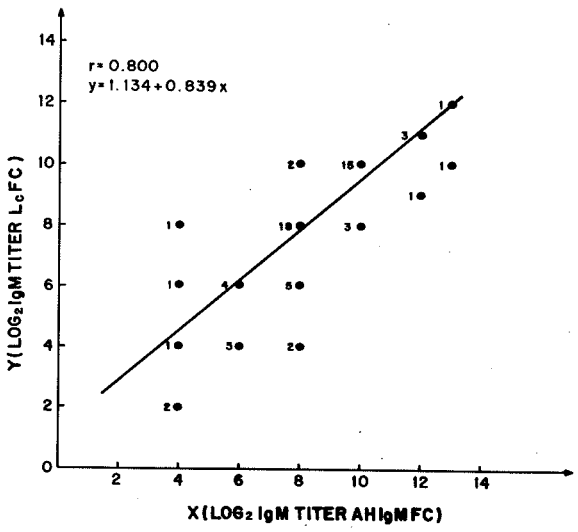

Fig. 1 — Regression of IgM antibody titers detected by lentil lectin fluorescent conjugate (LcFC) against titers obtained by anti-human IgM fluorescent conjugate (AHIgMFC). Numbers along dots correspond to the frequency of titers.

1:160. These results demonstrate possibility of producing fluorescent reagents of good quality with easy in relation to the conventional AHIgMFC.

DISCUSSION

Results here presented show that lentil lectins labeled with fluorescent isothiocyanate can constitute a satisfactory alternative reagent for the anti-IgM antibody conjugate, in IF test for the detection of anti-toxoplasma IgM antibodies. As sensitive as the antibody conjugate, lectin conjugates have the advantage of maintaining closely reproducible reactive characteristics among different batches and of beeing free of contaminating anti-toxoplasma antibodies.

Isolation of lentil lectins here processed by gel filtration chromatography on Sephadex G-100, as described by HOWARD et al [14,15], gives a mixture of two active components, A and B, with similar biological properties [14,15,23]. Both show a molecular weight about 49.000, their hemagglutinating activities can be equally inhibited by d-mannose and d-glucose and, in gel precipitating systems, a line of immunochemical identity is presented by the anti-A and the anti-B immunosera raised in animals. Because of this close relationship, in the present work the A and B lectin components were not further separated in a carboxymethyl cellulose chromatography as recommended [15].

Activities of the fluorescent lectin conjugates differed somewhat from those reported for native lectins. For instance, SPENGLER & WEBER [20] have found a selective binding of phytohemagglutinin (PHA), a lectin from **Phaseolus vulgaris**, to IgM of human normal serum. However, we had poor results with this lectin, possibly because we have dealt with IgM antibody combined with antigen. In consequence of modifications in molecular configuration of the immunoglobulin when forming immunocomplexes some sugar components could become less exposed.

Carbohydrates were estimated as 12.8% of the IgM molecules, comprising 7.6 parts of mannose and glucose, 1.4 parts of fucose and sialic acid, and 1.0 part of galactose, in con-

trast to 2.9% of saccharides in the IgG molecule [11,12,21]. It should then be expected that lectins with affinity to mannose and glucose such as Con A, a lectin from **Canavalia ensiformis** and lentil lectins, could provide good reagents. However, Con A showed to be less effective than lentil lectins, probably because of factors as molecular size, spacial configuration, pH and temperature, which might be limiting its interaction with the IgM molecules bound to antigen fixed on microscope slides. For instance, HUET [16] observed maximal reactivity of Con A at pH 7.0 and 37°C, conditions that differed from those standardized for our tests.

A previous addition of mannose or glucose to the LcFC inhibited its activity in the IF test. Also, specificity of the conjugate could be indicated by negative results when testing serum samples presenting serologic patterns II or III, corresponding to chronic or pregressive toxoplasma infections, and samples from clinically healthy individuals or cases of non-related diseases. Moreover, positive sera from patients with toxoplasmosis became completly negative after serum treatment with 2-mercaptoethanol.

To obtain sensitive results with lentil lectin conjugates, different factors had to be carefully ascertained, such as pH, temperatures and periods of incubation. For diluents, better results were seen for pH values above 7.2. Incubation periods required were in general longer than for the reference system, and 24 hours at room temperature was chosen for incubating sera and, 90 minutes at 37°C for the conjugate. Besides different physicochemical properties, lentil lectin conjugates interact with fewer sugar receptors found on IgM molecules than the number of epitopes reacting with labeled anti-IgM antibodies. By lengthening the time of serum incubation more residual antibodies could bind on the antigens, thus rendering a large number of sugar receptors to react with the lentil conjugate.

Reliability of the lentil lectin conjugate in the test for anti-toxoplasma antibodies was indicated by the relative efficiency index of 0.989, derived from high co-negativity (1.000) and co-positivity (0.969) indices in relation to the classical IF test. Similarity of results between both kind of conjugates was shown by the close geometric mean titers (8.37 for AHIgMFC and 7.79 for LcFC) and by a positive titer correlation of $r = 0.800$.

It is interesting to note also the similarity between staining patterns of both conjugates, including even the non-specific polar staining eventually observed for negative sera.

In this way we conclude on the advantages in using lentil lectins conjugates in the IgM-IF test for toxoplasmosis, instead of animal immune sera derived conjugates, in view of the economical aspects, technical ease in obtaining quite reproducible fluorescent conjugate batches and reliability of test results. Our preliminary results also indicate the possibility of applying the lectins in simlar fashion for enzyme immunoassays for the diagnostic purposes.

RESUMO

Lectinas para a detecção de anticorpos IgM anti-T. gondii no diagnóstico da toxoplasmose aguda pela reação de imunofluorescência

Lectinas foram marcadas com fluoresceína e testadas na reação de imunofluorescência para a detecção de anticorpos anti-**T. gondii**, no diagnóstico da toxoplasmose aguda. Neste estudo tentou-se encontrar reagentes alternativos para conjugados fluorescentes anti-IgM humano (CFAIgMH), que com freqüência contém anticorpos antitoxoplasma, como contaminantes, devido às infecções naturais por **T. gondii**, entre os animais utilizados para imunização.

Conjugados fluorescentes de lectina de lentilha (CFL) **(Lens culinaris)** forneceram resultados satisfatórios.

Avaliação do CFL efetuada em total de 179 amostras de soros de pacientes com toxoplasmose aguda e crônica, com infecções não relacionadas e de indivíduos sadios, mostrou valores altos nos índices de eficiência relativa, co-positividade e co-negatividade, respectivamente de 0,989, 0,969 e 1,000, em relação ao CFAIgMH convencional. Além disso, três partidas de CFL preparadas sucessivamente forneceram resultados reprodutíveis. A vantagem

de se empregar o LFC como reagente alternativo no diagnóstico sorológico da toxoplasmose aguda consiste nos aspectos práticos da sua preparação.

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