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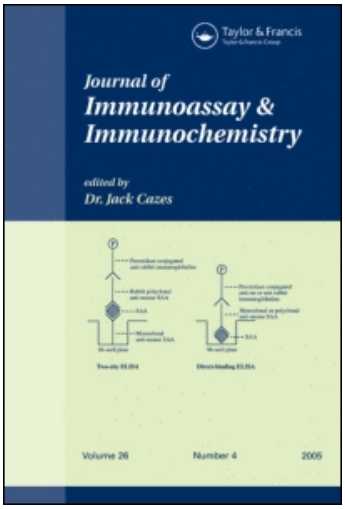

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Immunocapture Assay for Quantification of Human IgA Antibodies to Parasite Antigenic Enzymes. Application with the Alkaline Phosphatase of *Schistosoma mansoni*

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IMMUNOCAPTURE ASSAY FOR QUANTIFICATION OF HUMAN IGA ANTIBODIES TO PARASITE ANTIGENIC ENZYMES. APPLICATION WITH THE ALKALINE PHOSPHATASE OF *SCHISTOSOMA MANSONI* .

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ABSTRACT

Conditions are described for using solid phase adsorbed jacalins in an immunocapture assay for IgA antibodies to the alkaline phosphatase of *Schistosoma mansoni* . Microtiter plates were activated with polylysine and jacalins were covalently adsorbed by means of glutaraldehyde. From three different jacalins, the one purified from seeds of *Artocarpus tonkinensis* showed the lowest non-specific adsorption and was used for further studies. Comparing solutions of bovine serum albumin, ovalbumin and Tween 20, it was shown that the latter was most successful in blocking non-specific adsorption. Low serum dilutions resulted in a less efficient IgA capture by the adsorbed jacalin than higher dilutions. Under optimal working conditions, a high correlation could be shown between the presence of specific anti - alkaline phosphatase antibodies of IgA isotype and IgG isotype.

KEY WORDS : Antigenic enzymes, parasite, jacalin, *Artocarpus tonkinensis*

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INTRODUCTION

The serological detection of antibodies against parasitic antigens has now gained wide acceptance for the diagnosis and for epidemiological studies of *S. mansoni* infections (1). Recently, attention has been drawn to a subgroup of parasitic antigens with enzymatic activity as tools for immunodiagnosis (2,3) or as targets for experimental immunoprophylaxis studies (4,5). Using protein A - Sepharose to capture IgG and chromogenic substrates to reveal enzymatic activity, it could be shown that alkaline phosphatase, acid phosphatase, phosphodiesterase and Ca^{++} stimulated ATPase from adult worms and leucine aminopeptidase from eggs were all specifically recognizable in pooled sera from infected patients (6). In view of its high turnover number and specificity, the alkaline phosphatase obtained by butanolic extraction of an adult worm homogenate was shown to be the most promising of parasitic enzymes to use in a simple assay for epidemiological surveys (7,8).

To extend knowledge about the immune status of infected patients, we need the same enzyme marker to study the specific IgA response to the parasite. An immunocapture assay was set up using jacalin, a lectin from jackfruit with a specific affinity for IgA (9) and more especially for IgA1or secretory IgA1 (10). The optimal conditions for an immunocapture assay for quantitation of IgA1 anti-enzyme (alkaline phosphatase) antibodies are described here.

MATERIALS AND METHODS

Materials : Jacalins from three different species of *Artocarpus (tonkinensis, champeden* and *integrifolia)* were affinity-purified from saline extracts of jackfruit seeds on a desialylated mucin - CNBr-Sepharose 4B column as already described (11). Purification was assessed by SDS-PAGE and N-terminal sequencing.

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The source of alkaline phosphatase from *S. mansoni* was a butanolic extract of adult worm homogenates. Lyophilised worms were hydrated in sucrose 500 mmol/L, $MgCl_2$ 1 mmol/L, and phosphate buffer 10 mmol/L, pH 7.2. After homogenisation with a teflon pestle, the homogenate was centrifuged at 260000 g for 45 minutes at 4°C. The pellet was resuspended in $MgCl_2$ 1 mmol/L, and Tris buffer, 50 mmol/L, pH 8, recentrifuged, resuspended in the same buffer and recentrifuged under the same conditions. The membrane pellet was then extracted in water saturated n-butanol at 4°C and, after centrifugation for 5 minutes at 4°C, the lower aqueous phase was recovered. The proteinaceous interphase was extracted once again and the two recovered aqueous phases were combined and dialysed against the $MgCl_2$ Tris buffer as described above. To conserve the butanolic extract, glycerol was added to 20% v/v, Triton X-100 to 1 g/L, and NaN_3 to 200 mg/L, and the extract was stored at 4°C until use.

Positive sera were obtained from a Venezuelan endemic region.They were tested for parasitic charge with the Kato-Katz technique and/or for their seropositivity with the circumoval precipitin test (C.O.P.) and/or with an enzyme immunoassay on soluble egg antigen. Negative controls were determined by the same tests. All sera were stored at -20°C until use.

The chromogenic substrate p-nitrophenylphosphate and the fluorogenic substrate 4-methylumbelliferylphosphate were purchased from Sigma Chemical Company (St. Louis, USA). Poly-DL-lysine hydrobromide (M.W. 20,000) was also purchased from Sigma Chemical Company. All other reagents used were of analytical grade.

Adsorption of the jacalins on the microtiter plates : Immulon plates or Microfluor plates (Dynatech, Alexandria, Va) were coated for 1 hour at room temperature with 50 µL of a polylysine solution at 10 mg/L in water. After elimination of the supernatants, 50 µL of a jacalin solution

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at 10 mg/L in PBS (phosphate 10 mmol/L, NaCl 150 mmol/L, pH 7.4) was added together with the same volume of glutaraldehyde at 2.5 g/L in the same buffer. Coupling was allowed for 10 minutes, supernatants were decanted and the excess of glutaraldehyde neutralised with glycine 200 mmol/L in PBS.

Blocking of the non-specific binding sites : Saturation with 200 µL of blocking solution (Tween 20 5 g/L in PBS pH 7.4) was performed for 30 minutes at 37°C.

Binding of serum IgA1 : After washing the wells twice with TBS (Tris 10 mmol/L, NaCl 150 mmol/L, pH 7.4) supplemented with Tween 20 1 g/L, serum dilutions in the same buffer were added and the adsorption was allowed to proceed for 1 h at 37°C.

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Immunocapture of the alkaline phosphatase : After washing the wells thrice with TBS supplemented with Tween 20 1g/L, 50 µL of a butanolic extract containing alkaline phosphatase was added to each well and incubated at 37°C for 90 minutes. The extracts were recovered from the wells for reuse.

Determination of the enzymatic activity : Wells were washed thrice with TBS supplemented with Tween 20 1 g/L and 50 µL of substrate (p-nitrophenyl phosphate 1 mmol/L, or 4-methyl-umbelliferyl phosphate 100 µmol/L in diethanolamine buffer 50 mmol/L pH 9.5, supplemented with $MgCl_2$ 1 mmol/L) were added. For the chromogenic substrate, incubation was pursued for 6 hours at 37°C, the reaction volume was brought to 100 µL by adding 50 µL of water and the optical density was read on a Titertek (Flow, Rickmansworth, United Kingdom). For the fluorogenic substrate, the Microfluor plates were read every 15 minutes in a Microfluor reader (Dynatech, Alexandria, Va). Blank values were obtained by incubation without the IgA binding step.

To compare the anti-alkaline phosphatase IgA response with the anti-alkaline phosphatase IgG response, an alkaline phosphatase immunoassay previously described (Pujol et al., 1989) was used. Protein A was adsorbed directly on the Microfluor plates and the enzymatic activity determined with the fluorogenic substrate as described.

RESULTS

Optimisation of the IgA immunocapture assay:

The solution used for blocking the non-specific binding sites on the plastic wells and on the jacalins was studied. Four sera from negative controls and five sera from patients infected with *S. mansoni* were tested at a 1/20 dilution using p-nitrophenylphosphate as the chromogenic substrate. As shown in Figure 1, significant activity was observed in negative controls with bovine serum albumin 10g/L or ovalbumin 10g/L as blocking solutions. The non-ionic detergent Tween 20, 5g/L, although it decreased the activity of all the positive sera, completely blocked activity in the negative controls, and was used for further experimentation. The chromogenic substrate resulted in low optical densities, and further experiments were performed using the fluorogenic substrate.

In view of a recent report mentioning differential specificity for *Artocarpus* lectins of different species (12), three different jacalin preparations were tested in the immunocapture assay. Although the three lectins were equally efficient in the immunocapture assay, that of *A. tonkinensis* was preferred because, in contrast to the other lectins, non-specific binding was reduced (Figure 2).

Finally, a checkerboard assay was performed to study optimal serum dilutions and enzyme concentration. As expected, increasing the enzyme concentration correlated with an increase in sensitivity.

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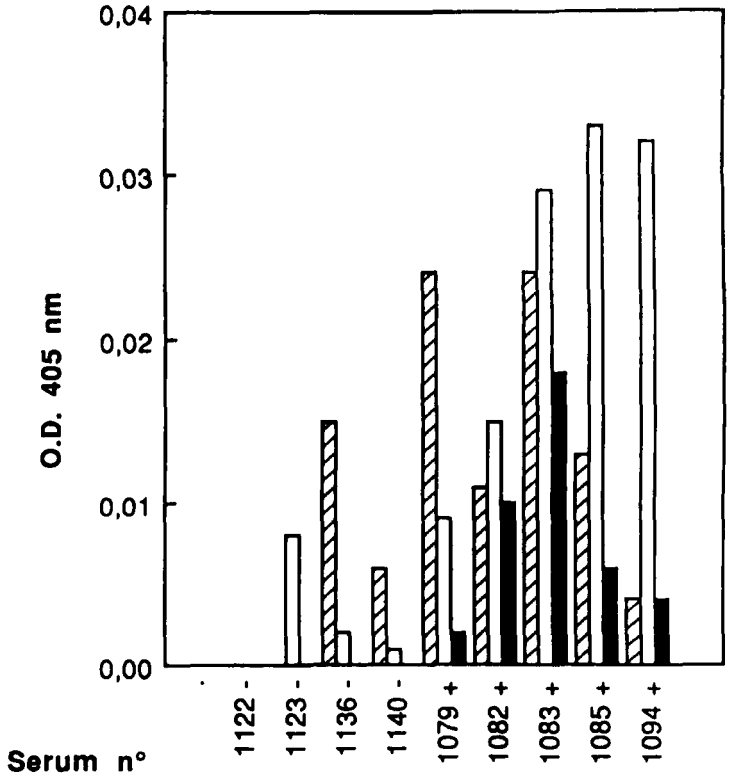

FIGURE 1 : Influence of blocking solutions on the specificity of the immunocapture assay. Four sera from negative controls (1122, 1123, 1136 and 1140) and five sera of patients infected with *S. mansoni* at a dilution of 1/20 were tested after saturation of the non-specific binding sites of *A. tonkinensis* jacalin with bovine serum albumin 1% (hatched bars), ovalbumin 1% (white bars) or 0.5% Tween 20 (black bars) in carbonate - bicarbonate buffer 100 mmol/L pH 9.5. Optical densities are shown after six hour incubation with the chromogenic substrate of alkaline phosphatase.

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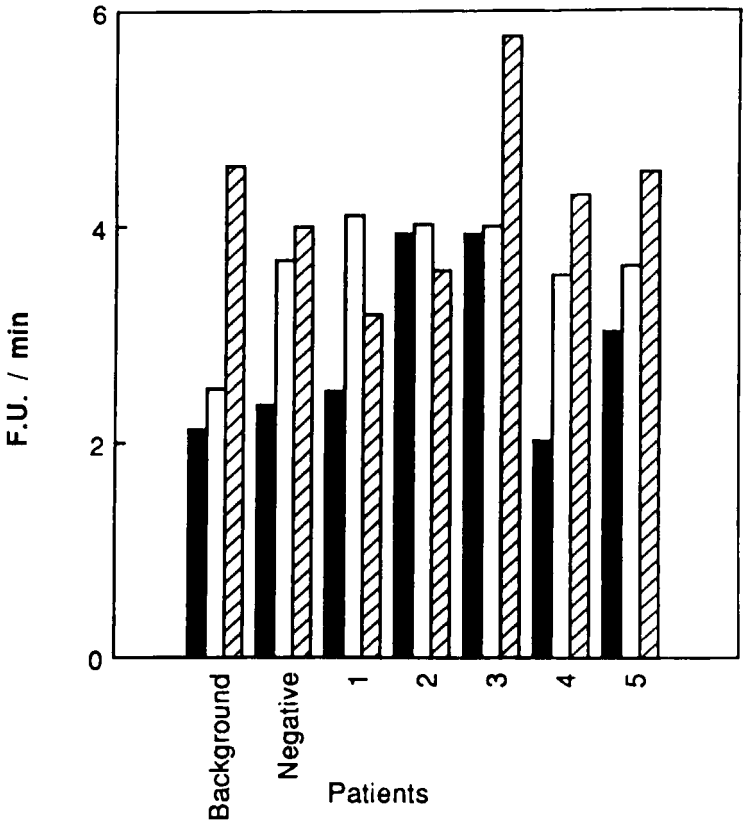

FIGURE 2 : Specificity of three *Artocarpus* species in the immunocapture assay. The assay was performed under standard conditions using the jacalins of *A. tonkinensis* (black bars), *A. champeden* (white bars) and *A. integrifolia* (hatched bars). The results are expressed as the rate of fluorescence increase. Background values were obtained by omission of serum in the standard procedure. One serum of a negative control and five sera of patients were used at a dilution of 1/20.

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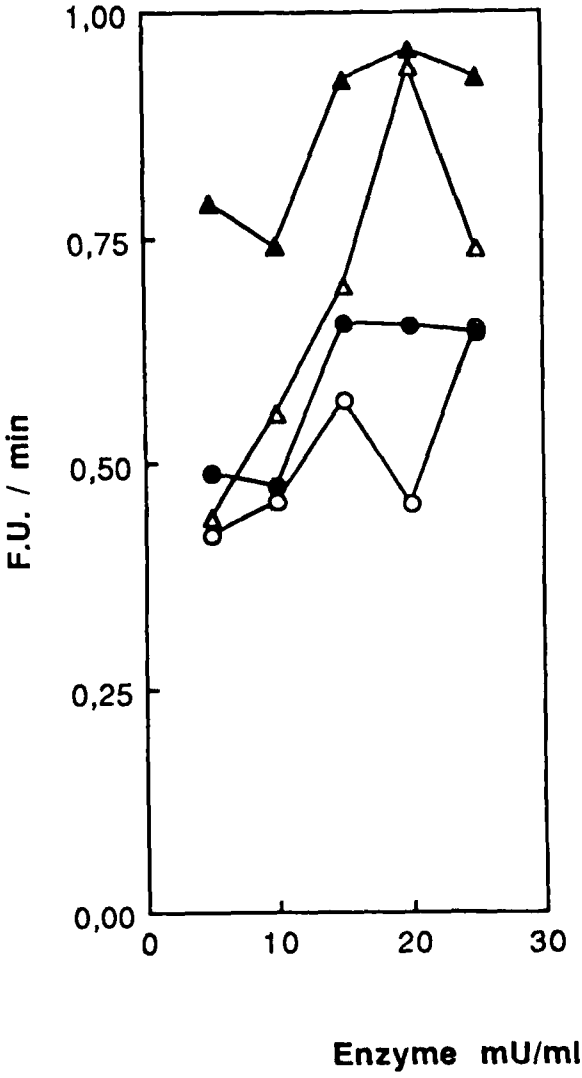

FIGURE 3 : Influence of serum dilutions and enzyme dilutions in the immunocapture assay. Serum of a patient infected with *S. mansoni* was diluted at 1/50 (O - O), 1/100 (● - ●), 1/200 (Δ - Δ) and 1/400 (▲ - ▲). Results are expressed as the rate of fluorescence increase. Blank values, obtained in the absence of serum, are substracted.

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TABLE 1

Synopsis of the Optimised Procedure

1. Sensitise the Microfluor (Dynatech) plates with 50 µL of 10 mg/L polylysine hydrobromide (Sigma P-8905) in water for 1 hour at room temperature
2. Empty the wells
2. Add 50 µL of a solution of *A. tonkinensis* lectin at 10 mg/L in PBS
3. Add the same volume of glutaraldehyde in PBS 2.5 g/L
4. Incubate 10 minutes at room temperature
4. Empty the wells
5. Add 200 µL of glycine 200 mmol/L in PBS for 15 minutes
6. Empty the wells
7. Add 200 µL of Tween 20 5 g/L in TBS
8. Incubate 30 minutes at 37°C
9. Empty the wells
10. Wash twice with Tween 20 1 g/L in TBS
11. Add 50 µL of serum diluted in the same buffer at 1/200
12. Incubate 1 hour at 37°C
13. Wash thrice in Tween 20 1 g/L in TBS
14. Add 50 µL of butanolic extract containing 15 U/L enzyme activity
15. Incubate 90 minutes at 37°C
16. Wash thrice in Tween 20 1 g/L in TBS
17. Add 100 µL of fluorogenic substrate (100 µmol/L) in diethanolamine buffer (50 mmol/L, pH 9.5) supplemented with $MgCl_2$ 1 mmol/L
18. Incubate at 37°C
19. Read fluorescence every 15 minutes for 120 minutes

More important, the sensitivity of the test was increased by using higher dilutions of the serum (Figure 3). This could be due to the fact that *Artocarpus* lectins seem to react with a few unidentified non-immunoglobulin serum proteins, which, at high serum concentrations could compete with IgA for the jacalin carbohydrate binding site (9,13).

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Validation of the IgA immunocapture assay:

Using the optimal conditions summarised in Table 1, four sera of negative controls were compared to four sera of patients infected with *S. mansoni* . The linearity of the enzymatic product formation was checked as a function of time. As shown in Figure 4, the minimal incubation time at 37°C necessary to discriminate between negative and positive sera was 60 minutes. A more quantitative expression of the results could be given by expressing the rate of fluorescence increase since this parameter is directly related to the amount of enzyme captured. This corresponded to a mean value of 0.46 pmol 4-methyl-umbelliferone/min (S.D. 0.04) for 30 infected patients, and 0.18 pmol/min (S.D. 0.03) for 4 negative controls.

As shown in Figure 5, a highly significant correlation was found between IgA and IgG anti-alkaline phosphatase antibodies ($p = 0.0001$) suggesting that the immunological response against this marker enzyme for *S. mansoni* infection probably ranges over the different immunoglobulin isotypes.

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DISCUSSION

Immunodiagnostic tests are important in the diagnosis of parasitic diseases. Especially in diseases in which the detection of the parasite is tedious as e.g. the detection of eggs in stools of patients infected with helminth parasites, immunodiagnosis is being increasingly used for epidemiological surveys. In several parasitic diseases, an important part of the immune response seems to be directed against antigenic enzymes (14-17). Immunocapture assays using enzymatic activity to reveal the presence of parasite specific anti-enzyme antibodies have therefore been set-up (3).

When parasites are in contact with the intestinal mucosa during their life cycle, a knowledge of the IgA immune response could be of

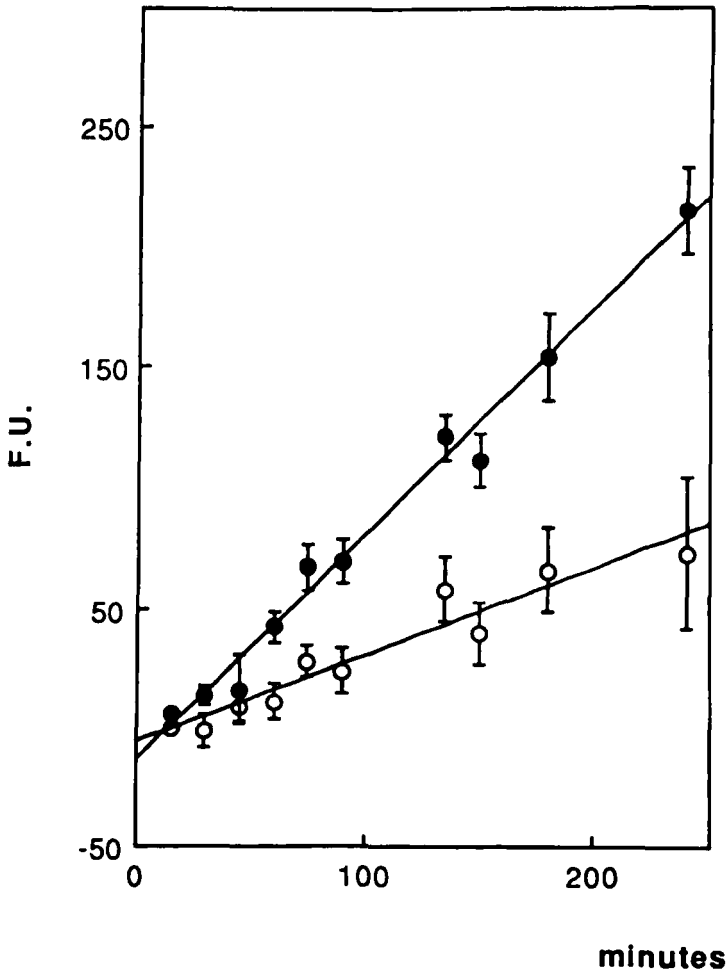

FIGURE 4 : Increase of fluorescence as a function of time. Four sera from negative controls (O - O) and four sera from infected patients (● - ●) were tested under the standard procedure (Table 1). Fluorescence increase was read every 15 minutes for 4 hours. Mean values and standard errors are given. The reaction rates were calculated by regression as 0.36 F.U./min for the negative sera(r^2 = 0.922) and 0.94 F.U./min for the sera of infected patients (r^2 = 0.985).

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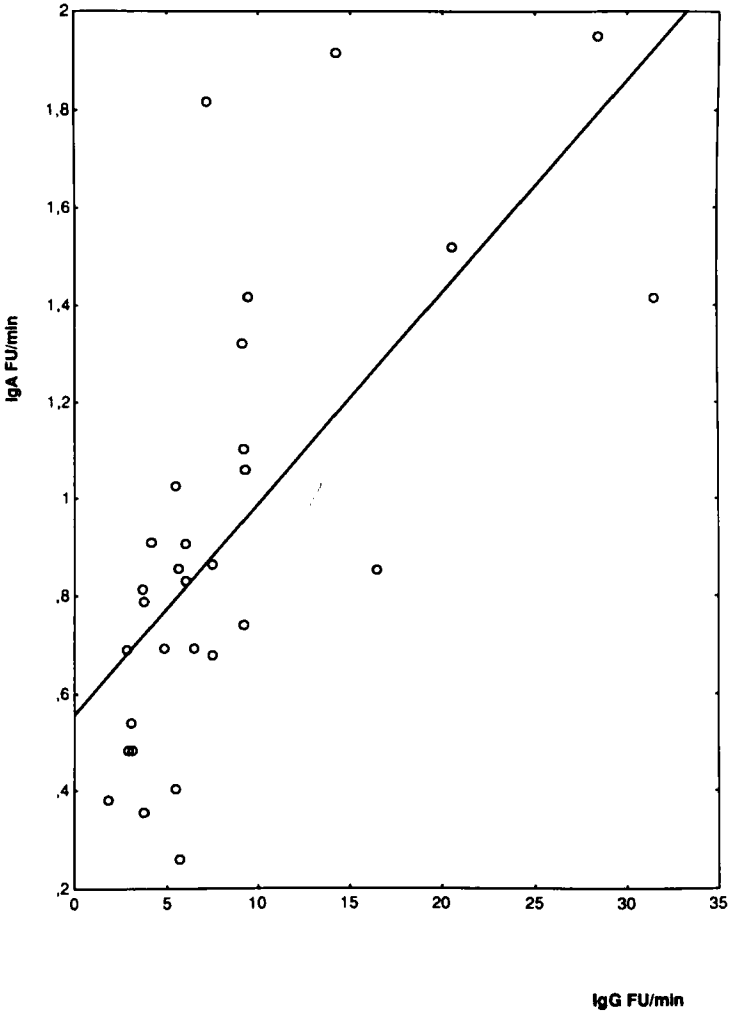

FIGURE 5 : Correlation between the protein A immunocapture assay (IgG) and the jacalin immunocapture assay (IgA). Thirty sera of infected patients were tested by the protein A immunocapture assay (3) and the jacalin immunocapture assay described here. Values of the immunocapture assays are expressed as rate of fluorescence increase.

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interest for the study of the local immune status. For this purpose, an IgG immunocapture assay for alkaline phosphatase of *S. mansoni* was used as a model and succesfully adapted to study the specific IgA1 response, using the jacalin from *A. tonkinensis* .The test presented here could also be used to study immune complexes with enzymatic activity (18) and could be extended to the study of secretory IgA by using streptococcal protein B (19).

Conflicting results have been reported on the affinity of jacalins for other serum proteins and more particularly for IgD (13;20-22). Results presented here, show that at low serum dilutions there is indeed competition between jacalin-binding proteins and IgA1. In view of the relative amounts of IgA1 and IgD present in serum, it is however doubtful that the immunocapture assay could detect antigen specific IgD in the serum of patients. Indeed, all studies implying that IgD can bind to jacalin have been performed in the presence of high concentrations of monoclonal IgD. This a situation which is far from that found in serum where IgD concentrations are several orders of magnitude lower than IgA1 immunoglobulins. A more likely candidate for competition with the IgA is the C1-inhibitor.

Although preliminary results were obtained using a chromogenic substrate for the alkaline phosphatase, the low absorbance of p-nitrophenol obtained after six hours limited the sensitivity of the test. The use of a fluorogenic substrate not only increased the sensitivity of the test but allowed kinetic measurements under initial velocity conditions which are better correlated with the amount of enzyme captured than endpoint values obtained after long incubations.

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