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Improvement of the indirect hemagglutination test for the detection of antibodies to *Streptococcus pyogenes*

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Abstract

An indirect hemagglutination test for a seroepidemiological survey of *Streptococcus pyogenes* infection was standardized. This is an improved modification of the indirect hemagglutination test which utilizes an unstable reagent prepared with fresh blood cells. Two types of bacterial antigens represented by extracellular products and purified streptolysin O were assayed, but only the former antigen gave good results. Pretreatment of the bacterial antigen with 0.15 M NaOH and neutralization to pH 5.5, as well as postfixation of sensitized red cells with 0.1% glutaraldehyde at 56ºC for 30 min were found to be essential to give long stability to the reagent in liquid suspension, at least 9 months at 4ºC. A total of 564 serum samples with high, moderate and low anti-streptolysin O antibodies as determined by the neutralization assay were studied by the indirect hemagglutination test using the new reagent. The sensitivity, specificity, efficiency, positive predictive value and negative predictive value of the test in relation to the neutralization assay were 0.950, 0.975, 0.963, 0.973, and 0.955, respectively. The kappa agreement index between the two techniques was high (0.926) and ranked as “almost perfect”. Antibody levels detected by both techniques also presented a high positive correlation (r_s = 0.726). Five reagent batches successively produced proved to be reproducible. Thus, the improved indirect hemagglutination test seems to be useful for public health laboratories.

Key words
- *Streptococcus pyogenes*
- Hemagglutination test
- Anti-streptolysin O antibodies

Introduction

The infection caused by *Streptococcus pyogenes* (group A, ß hemolytic streptococci) constitutes a public health problem in several countries (1), mainly due to nonsuppurative sequelae such as acute rheumatic fever (ARF) and acute glomerulonephritis. ARF is considered to be a more relevant sequela and also a severe form of the disease with the involvement of heart damage. This disease incapacitates many young adults who would otherwise have a productive life. In Brazil, cardiovascular lesions were found in 28% of young patients (2). The World Health Organization estimated that the prevalence of ARF associated with heart disease in school children is of 2.2/1,000 (3). Serologic methods have proved to be useful for the diagnosis of sequelae of group A streptococcal

infection, since infective microorganisms cannot be isolated at this stage of the disease.

Specific antibodies to bacterial cell components or extracellular products have been used in serologic tests. The streptolysin O (SLO) neutralization assay (4,5) has long been widely used. Recently, other assays such as latex agglutination, toraysphere agglutination and rapid hemagglutination, and immunoenzymatic, radiometric and nephelometric tests have been introduced. Among them, the immunoenzymatic, latex agglutination and nephelometric tests are preferred for use in routine laboratories (2,6-11).

Practical, reliable, and low cost assays are desirable for public health laboratories. The neutralization assay is a very complex and time-consuming technique (2). The latex agglutination test and rapid hemagglutination test were shown to provide nonreproducible results (6,12). Other assays require special equipment. The indirect hemagglutination test is one of the practical and economic tests, first described by Alouf et al. (13) for the diagnosis of streptococcal infections. Nevertheless, the need to use short-lived reagents makes this test inadequate for routine diagnosis. Thus, in the present study we improved the test by using a stable extracellular antigen of *Streptococcus pyogenes*, which also provides stable and reproducible reagent batches for the detection of antibodies to *S. pyogenes* in population surveys.

Material and Methods

The study was carried out in two steps. The first step involved the study of the factors influencing the indirect hemagglutination test such as type of antigen, stabilizer agents for red cells, pH, buffer solution, incubation time, temperature, tannic acid concentration, stabilizer agents for the reagent, and so on. Different types of reagents were prepared in a pilot study, in which the sensitivity, specificity, stability and reproducibility of the test were also determined. In the second step, the reagent showing the best diagnostic performance was selected and evaluated in the study of 546 serum samples from individuals showing high, moderate and low anti-streptolysin O antibodies.

Serum samples

A total of 546 serum samples were studied. Blood was collected from outpatients with antibodies to *S. pyogenes* (N = 332) and clinically healthy individuals (N = 214) at the Serology Laboratory of the University Hospital and at the Clinical Analysis Laboratory, both laboratories belonging to the University of São Paulo, and in the Immunology Section of the Fleury Laboratory, São Paulo. Serum samples were divided into three groups. The first group comprised 263 serum samples from outpatients showing antibodies to streptolysin O equal or higher than 250 Todd units (TU) as determined by the neutralization assay. The second group consisted of 69 serum samples from patients with unrelated diseases: toxoplasmosis (N = 6), syphilis (N = 14), rubella (N = 2), rheumatoid arthritis (N = 13), nonspecific inflammatory processes with high levels of C-reactive proteins (N = 17), lupus erythematosus (N = 15) and salmonellosis (N = 2). These patients had no significant anti-SLO levels of antibodies (<250 TU). The third group consisted of 214 serum samples from clinically healthy individuals also showing low levels of anti-SLO antibodies (<250 TU).

Bacterial extracellular products

Extracellular products (ECP) of *S. pyogenes* were obtained in 400 ml of culture medium according to Alouf and Raynaud (14), with some modifications. Culture medium was centrifuged at 9,000 *g*, at 4ºC, for 10 min. In order to obtain a concentrated antigen, the supernatant was precipitated with 80% ammonium sulfate and kept at 4ºC for

24 h. The sediment was separated by centrifugation, resuspended in 40 ml of 0.15 M phosphate-buffered saline solution (PBS), pH 6.8, and dialyzed in the same buffer solution. This extracellular antigen was centrifuged again to remove all the insoluble particles and stored at -20°C, in 5-ml aliquots (13). The extracellular antigen was then titrated in the neutralization assay, to determine the hemolytic activity.

Also, a commercially available purified streptolysin O (PSLO) (Biolab S.A., São Paulo, Brazil) was assayed.

Reagent for the indirect hemagglutination test

Type O, Rh negative human red blood cells were treated with formaldehyde according to Camargo et al. (15). Prior to the sensitization of formaldehyde-preserved cells, the ECP was treated with 0.15 M NaOH at 4°C for 24 h, with pH adjusted to 5.5 with 3 M HCl. The sensitization process of cells with ECP was standardized based on a previously reported method (16) with several modifications. Briefly, a volume of 2.2 ml formaldehyde-preserved cells (6.5%) was twice washed in 0.15 M NaCl and resuspended in 10 ml of 1:15,000 tannic acid diluted in PBS, pH 6.4, and incubated at 56°C for 15 min with shaking. Two milliliters ECP (1:40 in PBS, pH 6.4) was added to the mixture and incubated at 37°C for 60 min with shaking. Then, 1.2 ml glutaraldehyde (1%) was mixed and incubated at 56°C for 30 min. Finally, 1.2 ml skim milk (Nestlé, Brazil) was added. The latter solution was prepared with 1 g skim milk dissolved in 10 ml 0.15 M NaCl, autoclaved at 121°C, at 1.5 atm. for 30 min and centrifuged at 6,000 *g* at 4°C for 30 min. Sodium azide was added to the supernatant to a final concentration of 0.01% and the prepared reagent was stored at 4°C.

A reagent was also prepared with purified SLO following the same sensitization process as described above, except that this antigen was used in its native form without pretreatment with 0.15 M NaOH.

Serologic tests

Neutralization assay. All serum samples were assessed in the neutralization assay (2,4) to confirm the results of neutralization assays previously obtained by the different laboratories mentioned above.

Indirect hemagglutination test. The test was carried out with the standardized reagent in V-shaped wells of plastic microplates using 0.1 M NaCl for serum sample dilution, as described (17). Twenty-five microliters of sensitized cell suspension was added to 50 µl of serial double dilutions of serum samples from 1/20 to 1/5,120. The red cell agglutination patterns were scored after incubating the microplates for 90 min at room temperature. Two control sera, one with <250 TU and the other with >625 TU, were always included in the test. The reagent was controlled by adding 25 µl of sensitized cell suspension to 50 µl 0.1 M NaCl.

Reproducibility and stability of the indirect hemagglutination reagent

Several batches of indirect hemagglutination reagent (N = 6) were produced successively in the study and their reproducibility was checked by a control chart method (18). Each reagent batch was assayed against a panel of 20 serum samples comprising 10 serum samples with positive anti-SLO titers (≥333 TU) and 10 with negative anti-SLO titers (<250 TU). The stability of the reagent was also studied at temperatures of 4°, 25° (room temperature) and 37°C. Tests were performed against 3 control sera (<250, 333 and >625 TU) for a period of 9 months.

Statistical analyses

The diagnostic features of the indirect hemagglutination test were evaluated in re-

lation to the neutralization assay as described by Galen and Gambino (19). The 95% confidence intervals were also calculated for different diagnostic parameters. The kappa index of agreement was determined for the comparison of two tests (20,21). The antibody levels (Todd units or titer) were transformed to log for statistical analysis (22). The Spearman correlation coefficient (23) was calculated for antibody levels expressed as titers and as Todd units.

Results

Factors influencing the indirect hemagglutination test

Factors influencing the indirect hemagglutination test were determined in the first step of the present study. A total of about 60 different types of reagents were prepared and assayed because of the many influencing factors investigated.

Four types of antigens were tested: PSLO and ECP in their native forms, and the same antigens submitted to previous treatment with 0.15 M NaOH and then neutralized. The first and fourth types of antigens were more sensitive and also gave more reproducible results than the other two. PSLO and ECP were neutralized at different pH values ranging from 5.0 to 9.0; however, the best results were obtained when the antigens were kept at pH 5.5.

Red cell sensitization was carried out in two different processes. The first process, known as the direct sensitization process, consisted of incubating the PSLO or ECP antigen directly with formaldehyde-preserved red cells, whereas in the second process red cells were previously treated with tannic acid and then incubated with the antigen. In the first process of direct sensitization, the incubation period ranged from 3 to 24 h at room temperature or at 37°C, but the results obtained under these conditions were poor. In the second process, which yielded the best results, tannic acid was tested at concentrations ranging from 1:10,000 to 1:30,000 and the concentration of 1:15,000 was chosen because of its sensitivity. Also, tannic acid provided better results when diluted in PBS at pH 6.4 than when diluted in PBS at pH 7.2. Red cells were incubated with tannic acid, at 37° and 56°C for periods ranging from 10 to 60 min. Best results were obtained with incubation at 56°C for 10 min or at 37°C for 50 min. However, incubation at 56°C for 10 min was preferred because of its practical aspects.

In the sensitization process, tanned red cells were incubated with PSLO and ECP in their native form and after alkaline treatment. Buffer solutions were tested at pH ranging from 6.4 to 8.2, and 0.15 M PBS, pH 6.4, was chosen for this process. Different incubation times (10 to 60 min) and temperatures (37° and 56°C) were assayed for the sensitization process, and the optimal conditions were found to be 37°C for 50 min.

Glutaraldehyde at concentrations ranging from 0.04 to 0.8% was tested in order to enhance antigen binding to the red cell surface. Glutaraldehyde was incubated with sensitized cells at different temperatures (37° to 100°C). The glutaraldehyde concentration and the conditions selected to keep the antigen steadily fixed onto the cell surface were 0.1% and 56°C for 30 min, respectively.

The reagent prepared with PSLO in its native form gave better results than that prepared with PSLO pre-treated with 0.15 M NaOH. Conversely, the reagent prepared with ECP submitted to alkaline treatment gave more sensitive results than that prepared with the same antigen in its native form. Thus, these two types of reagents were produced in large amounts in order to assess their diagnostic features in the second step of the study.

Different stabilizing agents were investigated for storage of sensitized red cells in suspension. Substances such as skim milk, ethylenediaminetetraacetic acid (EDTA),

bovine serum albumin (BSA), and sodium thioglycolate were assayed at different concentrations and pH values but an autoclaved skim milk solution in 0.9% NaCl was found to provide the best stability.

Evaluation of the indirect hemagglutination test

The neutralization assay was taken as a reference technique to evaluate the diagnostic features of the indirect hemagglutination test with the use of two types of reagents. Serum samples with ≥250 Todd units were considered to be positive and those with lower values were considered to be negative.

The serologic performance of the indirect hemagglutination test using two different reagents was assessed in terms of sensitivity, specificity, efficiency and positive and negative predictive values. The values obtained in the indirect hemagglutination test with the ECP reagent were high, as shown in Table 1.

Comparison of the serologic assays

Data obtained by the indirect hemagglutination test using the ECP reagent and the PSLO reagent were compared to data obtained by the neutralization assay. Serum samples were divided into three groups according to their antibody levels (TU) as detected by the neutralization assay (Figure 1). The first group had antibody levels ≥625 TU (N = 184), the second had 333 TU (N = 79), and the third <250 TU (N = 283). The indirect hemagglutination test using the ECP reagent showed that about 91% of the serum samples from the first group presented titers ≥640 (2.81 in log), 90% of the second group had titers between 320 and 640 (from 2.50 in log to 2.81 in log), and 98% of the third group showed titers <160 (<2.20 in log). The indirect hemagglutination test with the use of the PSLO reagent showed that 83% of the serum samples from the first group presented titers ≥640, 54% of the samples from the second group had titers between 320 and 640, and 54% of the samples from the third group had titers <160.

The geometric mean titers (GMT) and 95% confidence intervals obtained in the indirect hemagglutination test with the use of ECP were 2.84 (2.64-3.04) for the first group of serum samples, 2.51 (2.36-2.66) for the second group, and 2.21 (2.13-2.29) for the third group (Figure 1), and the values obtained in the indirect hemagglutination test with the PSLO reagent were 3.03 (2.73-3.34), 2.50 (2.21-2.80) and 2.43 (2.13-2.74) for the first, second and third groups of serum samples, respectively.

The kappa agreement index between the indirect hemagglutination test and neutralization assay was 0.926 ($z_{obtained}$ = 16.85; $z_{critical}$ = 3.89, P<0.001), a value ranked as "almost perfect". The indirect hemaggluti-

Table 1 - Diagnostic features of the indirect hemagglutination test using two different antigens for the detection of anti-SLO antibodies in the study of 546 serum samples.

PSLO, Purified streptolysin O with no pretreatment; ECP, extracellular products of alkali-pretreated S. pyogenes. The 95% confidence interval is indicated within parentheses.

Type of antigen	Sensitivity	Specificity	Efficiency	Positive predictive value	Negative predictive value
PSLO	0.826 (0.693-0.909)	0.857 (0.685-0.943)	0.838 (0.738-0.905)	0.905 (0.779-0.962)	0.750 (0.579-0.868)
ECP	0.951 (0.917-0.971)	0.975 (0.950-0.988)	0.963 (0.944-0.976)	0.973 (0.945-0.987)	0.955 (0.924-0.974)

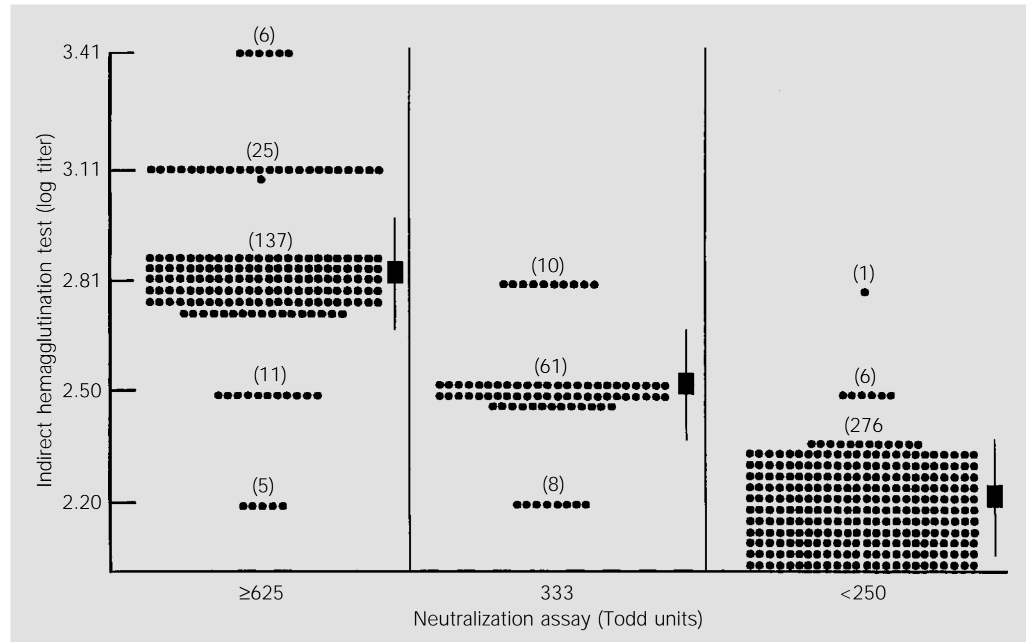

Figure 1 - Antibodies detected by the indirect hemagglutination test in the study of 546 serum samples with high (≥625), moderate (333) and low (<250) Todd units of anti-streptolysin O antibodies as detected by the neutralization assay.

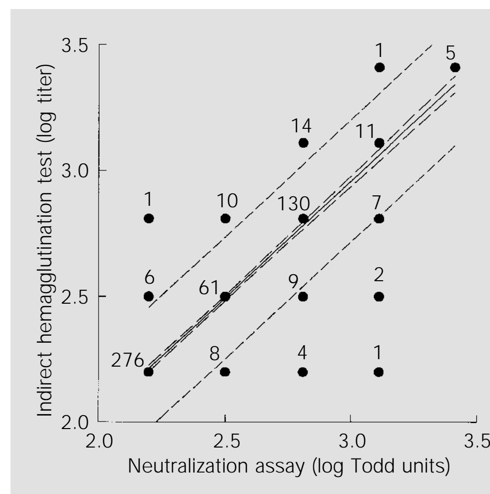

Figure 2 - Relationship between antibodies detected by the indirect hemagglutination test and by the neutralization assay (r_S = 0.726).

nation test using the PSLO reagent gave, as expected, a lower kappa agreement index, 0.665 ($z_{obtained}$ = 5.64; P<0.001) and ranked as "substantial".

The antibody titers detected by the indirect hemagglutination test using the ECP reagent were compared with the corresponding Todd units determined by the neutralization assay, as shown in Figure 2. The Spearman correlation coefficient (r_s) was 0.726. On the other hand, the r_s obtained in the indirect hemagglutination test with PSLO was 0.440.

Reproducibility and stability of hemagglutination reagent batches

In this study 6 reagent batches were successively produced with ECP antigen and tested against a reference panel of 20 serum samples. Standard deviations (SD) were calculated and compared with a stipulated control limit corresponding to 0.20 SD. Five reagent batches were approved by this control method because the SD values were 0.184, 0.069, 0.138, 0.140 and 0.155, i.e., lower than the control limit. However, one reagent batch was rejected because its SD was 0.296 i.e., higher than the control limit.

The hemagglutination reagent was stored at 4°C, at room temperature and at 37°C. The reagent kept at 4°C showed no change in reactivity even after 9 months, when the present study was concluded. At room temperature, the reagent showed no change in reactivity for a period of 6 months, whereas

the reactivity of the reagent stored at 37°C lasted only 45 days.

Discussion

Antibodies to *Streptococcus pyogenes* ECP have been reported to occur in streptococcal infections (4). Alouf et al. (13) found an excellent correlation between the anti-ECP antibodies detected by the indirect hemagglutination test and the conventional anti-SLO antibodies detected by the neutralization assay. In population surveys the indirect hemagglutination test is thought to be suitable because of its practical and easy execution aspects, requiring no special equipment.

In the present study, two types of reagents, one of them prepared with PSLO antigen without any pretreatment and the other with an alkali-pretreated ECP antigen, gave sensitive results in the standardization step. Nevertheless, a significant difference between these two reagents was observed thereafter when large numbers of serum samples were tested, with a clear superiority of the ECP reagent. This difference may be explained by the fact that the commercially supplied PSLO antigen has some protein contaminants which are not antigenic and which attach to the tanned red cells during reagent preparation. These contaminants, however, did not interfere with the neutralization assay. Thus, the diagnostic features of the indirect hemagglutination test using the ECP reagent were shown to be significantly better than those of the test using the PSLO reagent.

The reagent prepared with alkali-pretreated ECP can be considered as an improvement of the reagent previously described by Alouf et al. (13) who used an unstable and short-lived reagent in liquid solution.

The new reagent differed from the previous one in the following ways: a) tannic acid was used instead of glutaraldehyde to bind antigen to the red cells; b) the antigen was treated with 0.15 M NaOH which conferred stability, requiring no protease inhibitors; c) the time for red cell sensitization was reduced to 50 min instead of the previous 24 h; d) antigens coating red cells were postfixed with 1% glutaraldehyde at 37°C for 30 min for long reagent stability. The previous reagent was found to be stable only for 15 days at 4°C, in contrast to the new reagent which lasted at least 9 months at 4°C, 6 months at room temperature and 6 weeks at 37°C. The processes to keep a reagent in liquid solutions without changing its reactivity are usually manufacturer's patents or secret formulation. e) Serum dilutions with hypotonic saline solution (0.1 M NaCl) gave better results than the isotonic saline solution (0.15 M NaCl) used for the previous reagent, confirming the findings of Yamamoto et al. (17) for the toxoplasma system; f) V-shaped plastic microplates showed clear-cut results within 90 min compared to U-shaped plates which gave results within 2 or 3 h. These conditions for the preparation of the ECP reagent were also optimal for obtaining the PSLO reagent.

In the second step of the present study, the indirect hemagglutination test using the ECP and PSLO reagents was applied to a large number of serum samples from individuals clinically suspected to have streptococal infections showing high, medium and low levels of anti-SLO antibodies. The cutoff titer for the indirect hemagglutination test using two types of reagents was 320 or 2.50 in log. This cutoff was stipulated based on data provided by the NA which corresponded approximately to 333 TU (Figures 1 and 2) or 2.52 in log. The cutoff adopted for the neutralization assay by different investigators ranges from 250 to 333 TU, and is influenced by factors such as bacterial strains, geographical localization of the population, age group, socioeconomic conditions and so on (24-27). In the present study, the

cutoff for the NA was based on the value reported by Pereira et al. (27) which is similar to that adopted in the United States (28).

The correlation coefficient found between antibody titers obtained by the indirect hemagglutination test and Todd units obtained by the neutralization assay was high (r = 0.726), as illustrated in Figure 2, showing that titers and Todd units are closely related. This value was similar to those observed for the commercial slide agglutination test (Streptozyme) (29), and for the immunoenzymatic ELISA (9,30), both assays in comparison to the neutralization assay.

The sensitivity obtained for the indirect hemagglutination test with the use of PSLO was significantly lower (0.826) than that of the indirect hemagglutination test with the use of the ECP reagent, as also was its specificity (0.857). Therefore, the indirect hemagglutination test with the use of PSLO in this test was considered unsuitable for diagnostic purposes.

The sensitivity (0.978) and specificity (0.689) of the indirect hemagglutination test obtained by Alouf et al. (13) were calculated based on their reported data. The original indirect hemagglutination test had a sensitivity similar to that obtained by us (0.951) for the same test with the use of the new reagent. The specificity of the original test, however, was significantly lower in comparison to the improved indirect hemagglutination test (0.975). The difference in specificity may be explained by the fact that those authors used native *S. pyogenes* extracellular products which probably crossreacted with other unrelated infections, whereas in the present study alkaline treatment of the same antigen resulted in high specificity, probably because many small nonspecific fragments derived from this treatment did not attach to the red cell surface.

Also, the sensitivity of the indirect hemagglutination test using the new reagent was slightly higher than that of the latex agglutination test (7), but much higher than that of immunoenzymatic ELISA (30).

In turn, the specificity of the indirect hemagglutination test with the new reagent was superior to those of the latex agglutination test and of ELISA.

The new reagent will also be tested against sera from patients with defined diagnoses of acute rheumatic fever, acute glomerulonephritis and pyoderma. The aspects related to the kinetics of antibody production in such patients during the acute and chronic stages of the disease will also be investigated. The improvement of the indirect hemagglutination test with the use of a stable reagent seems useful for epidemiological serum surveys in developing countries.

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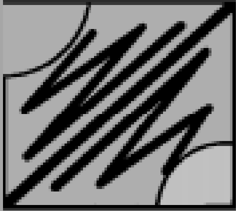

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- *Cell Cryopreservation*
- *Development and Distribution of Human and Animal Primary Cell Cultures*
- *Development of New Monoclonal Antibody Secreting Hybridomas*
- *Monoclonal Antibodies, in Cell Culture Supernatants or Purified*
- *Identification of Microbiological Contaminants*
- *Decontamination of Cell Lines*
- *Cytotoxicity Tests*
- *Characterization of Cell Lines*
- *Training in "in vitro" Culture of Human and Animal Cells*
- *Consultation Assistance in Cell Culture*

Contact us for specific services not listed above

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