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SIGNIFICATIVE AND PROBLEM BASED LEARNING IN IMMUNOLOGY: OBTAINING A KIT TO TYPE *NEISSERIA GONORRHOEAE*

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ABSTRACT

Neisseria gonorrhoeae (Ng) have been classified serologically on the basis of the antigenicity of the major porin (Por). PorI occurs in two immunochemically distinct serogroups: PorIA and PorIB. Because the diagnostic, therapeutic, social, and legal consequences of misidentification of a nongonococcal *Neisseria* isolate as Ng can be substantial, the accurate and rapid identification of this organism is important. Typifying of Ng is done by techniques based on phenotypic characteristics and plasmidic content that individually don't reach an adequate discrimination, and so combination of techniques must be used. The aim of this work is to obtain polyclonal specific Ab that discriminate Ng types PorIA and PorIB. For this purpose, we immunized two rabbits with sonicated PorIA and PorIB strains of Ng (isolated from clinical samples and serologically classified). The Ab response was analyzed along the protocol by ELISA and by direct agglutination with latex coated with sonicated Ng. With these data, we selected the bleeding providing the serum with maximum specific Ab titer to prepare the typing reagents. Unwanted Ab directed against shared epitopes were removed by adsorption with Ng latex. The typing reagents were obtained by coating latex with each depleted sera. Our results suggest that high titers of specific Ab be obtained for both strains of Ng and the depleted sera be discriminated between both strains. These results suggest that these diagnostic reagents could be useful to confirm presumptive identification by a simple and rapid method.

The present work was proposed to be done in the Immunology practical course of the School of Chemistry.

RESUMEN

Neisseria gonorrhoeae (Ng) ha sido clasificada serológicamente sobre la base de la antigenicidad de su Porina principal (Por). PorI presenta dos serogrupos distintos desde el punto de vista inmunológico: PorIA y PorIB. A causa de las consecuencias diagnósticas, terapéuticas, sociales y legales que apareja la incorrecta identificación de cepas de Neisserias no gonocóccicas como Ng, es imprescindible la identificación rápida y precisa de este organismo. La tipificación de Ng se realiza mediante técnicas basadas en sus características fenotípicas y contenido de plásmidos que, individualmente, no alcanzan una discriminación adecuada y, por lo tanto, debe utilizarse una combinación de técnicas. El objetivo del presente trabajo es obtener anticuerpos (Ac) policlonales específicos que discriminen Ng tipo PorIA y PorIB. Para ello se inmunizaron 2 conejos con sonicado de Ng PorIA y PorIB (cepas aisladas de casos clínicos y clasificadas serológicamente). El seguimiento de la respuesta humoral se realizó mediante las técnicas de ELISA y aglutinación directa (látex recubierto con sonicado de Ng). Con estos datos se seleccionó la extracción óptima para obtener los Ac de máxima especificidad para preparar los reactivos de tipificación. Los Ac dirigidos contra epítopes compartidos fueron removidos por adsorción con látex Ng. Los reactivos para tipificar fueron obtenidos recubriendo látex con los sueros depletados. Nuestros resultados muestran que pueden obtenerse altos títulos de Ac específicos contra ambas cepas de Ng y, que los sueros depletados discriminan ambos tipos de Ng. Estos resultados sugieren que estos reactivos diagnósticos serían útiles para confirmar identificación presuntiva por medio de un método simple y rápido.

El presente trabajo fue propuesto en el curso práctico de Inmunología de la Facultad de Química.

Palabras clave: Neisseria - tipificación - látex

INTRODUCTION

The course of Introduction to Immunology in our Department is directed to under-graduate students of Chemistry, Biochemistry, Biology and Pharmacy. The average number of students per year is 150 - 200. They are divided into laboratory groups (about 15 students in each) to carry out the experimental work, which consists of a protocol designed to investigate a small problem that is feasible to be solved in a period of 6 weeks of laboratory work. If it is necessary, within each group the students may focus on different approaches to the proposed problem. At the beginning of the course, they have an initial class introducing the major theoretical and experimental issues they will need in order to solve the proposed problems. Practical project subjects presented to students for them to work on must challenge and motivate them while teaching them a problem solving method. We carried out a Diagnostic Evaluation that comprised assessment of the initial state of the students' knowledge. The teacher provides them with the necessary bibliography which includes chapters of books and original papers as well as review papers prepared by the teacher concerning the different topics. It follows a stage of presentations by students and discussion with the teacher about the experiments that should be carried out to solve the proposed problem. The Diagnostic survey allowed us to adjust the teaching activity to the students' characteristics and their specific situation and facilitated significant and relevant learning for students as teaching is then based on their pre-existing knowledge (Míguez & Cáceres, 2001).

Once the experimental work is concluded, and after discussing the results extensively with the teacher, they present their results to the rest of the students and teachers in a discussion-oriented seminar. Finally, the group elaborates a report of their work that should have the structure of a scientific paper. A global evaluation comprising laboratory and seminar performances as well as the quality of the report, is used to grade the course.

The experimental protocol presented in this paper has been proposed for three consecutive years in our course. It is aimed to illustrate basic issues of the antibody response through preparation of reagents for *N. gonorroheae* typifying. With this kind of experiments, based on real problems, we want to promote significant learning and motivation in our students (Míguez et al, 1998).

The aim of this work is to illustrate basic concepts of the antibody response through the preparation of latex reagents for Ng typifying. For this purpose, we immunized two rabbits with sonicated Ng PorIA and PorIB. (NOTE: The laws regarding animal experimentation vary in different countries, including the extent to which animals may be used for class experiments. Those interested in carrying out this laboratory practical class should investigate the laws in their own country before proceeding). The students analyzed the antibody response of the rabbits along the protocol by ELISA and latex agglutination. With these data, they selected the bleeding providing the serum with maximum specific antibody titer to prepare the latex reagents. Animals (rabbits) recognized both types of Ng, so those unwanted Ab directed against shared epitopes were removed by absorption with latex sensitized with the other N. gonorroheae type. The concept of cross-reactivity and the relevance it has for the present work should be illustrated.

These latex reagents were tested with fragments of isolated Ng from clinical urethritis (disruption by freeze/thawed cycles) identified and classified by auxotype and serovar.

BACKGROUND

The genus *Neisseria* belongs to the family *Neisseriaceae* along with *Moraxella,*

Acinetobacter and Kingella. They are gram-negative cocci, nonmotile and non-spore-forming which characteristically grow in pairs with adjacent sides flattened. *Neisseria gonorrhoeae* (gonococci) and *Neisseria meningitidis* (meningococci) are pathogenic for man and are related to polymorphonuclear leukocyte or inside them. *N. gonorrhoeae* is the etiologic agent of gonorrhea, a sexually transmitted and highly contagious disease and its related clinical syndromes. The genus *Neisseria* includes a variety of other relatively or completely nonpathogenic organisms, which are important mainly because of their occasional diagnostic confusion with gonococci and meningococci. Gonococci and meningococci are closely related. They both have a polysaccharide capsule that in the case of gonnococci is a pseudocapsule.

Neisseria gonorrhoeae is heterogenous from the antigenic point of view and able to change surface structures *in vitro* and *in vivo* in order to avoid host defences.

Their most important surface structures are pili, PI (protein I), Opa (protein II), Rmp (protein III) and lipooligosacharides. Pili are arranged in individual fibrils or fibrillar aggregates and cover virtually the entire outer cell surface of the organism. They are known to increase adhesion to host tissues and resistance to phagocitosis. Protein I is the major outer membrane and porin of *N. gonorrhoea* and is used to serotype this organism. It exists in two major chemically and immunologically distinct classes designated PorIA and PorIB. A given strain possesses either PorIA or PorIB but never both. Serological typing of PI has distinguished 18 serovar of type PorIA and 28 serovar of type PorIB. Comparison of the sequence of PorIA and PorIB proteins reveals certain regions that are common to both proteins and others where there is considerable variation representing areas of antigenic diversity. Several observations suggest that PI may play a role in invasion. Incubation of radiolabeled gonococcal membranes or whole bacteria with red blood cells results in transfer of the gonococcal porin protein (PI) from the bacterial cell surface into the red cell membrane. Gonococci containing PorIA transfer PI into membranes more readily than gonococci containing PorIB. This is consistent with the fact that PorIA is more likely to cause disseminated disease than gonococci with PorIB.

A variety of tests has been developed to detect gonococcal antigenin genital secretions. These include enzyme immunoassays with polyclonal sera against gonococcal antigens and monoclonal antibodies against gonococcal antigens. The most useful and widely available technique used for serotyping at present is based on monoclonal antibodies specific for various epitopes on outer membrane protein PorIA and PorIB. For epidemiological purposes, typing is an approach to establish patterns of transmission and virulence factors including the relationship between strain type and resistance to antimicrobial.

The aim of the present work is to obtain policlonal specifics Ab that discriminate Ng types PorIA and PorIB. Those antibodies would allow to confirm the identity and a primary classification between strains belonging to group A or B. This experimental laboratory practice is a type of problem based learning which promotes significant learning and motivation in students.

OBJECTIVES

General objective

- Prepare latex reagents to detect and type *Neisseria gonorrhoeae* type PorIA and PorIB.

Specific objectives

1. Production of polyclonal antibodies (Ab) from rabbits immunized with *Neisseria gonorrhoea* type PorIA and PorIB.

2. Determination of specific Ab titres (IgG) during the immunization protocol.

3. Evaluation of specific Ab titres (IgG) directed against share epitopes to both types of *Neisseria gonorrhoeae*.

4. Purification of specific Ab to discriminate between *Neisseria gonorrhoeae* type PorIA and PorIB.

5. Development of a latex agglutination test to confirm and type strains of *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains

This step must be done by the teacher to avoid biological risks. Two *Neisseria gonorrhoeae* strains isolated from male urethritis, identified and classified by auxotype and serovar described previosuly (Catlin, 1973; Knapp et al, 1984; Morello et al, 1985), were used. Strains 2852 (Ng PorIA) and 157 (Ng PorIB) belong to class - IA-08/ OP and IB-04 / O - respectively.

Preparation of extracts (lysates)

This step must be done by the teacher to avoid biological risks. The strains were cultured aerobically on GC agar plates containing 1% Kellog supplement at 35°C in 5% CO_2 for 18 hs. The cells were harvested and suspended in saline and lysed by freezing at -20°C for 48 - 72 hs and then sonicated in Branson B-42 sonicator in water-ice bath for three 15 seconds cycles spaced 5 min. Sonicated was centrifuged at 15000 rpm, 5 min. at 4°C and protein concentration of the supernatant was determined by the bicynchoninic acid method (BCA Protein Assay Reagent, PIERCE, Illinois, U.S.A.) using bovine serum albumin (BSA) as standard, according to the manufacturer's instructions. The samples were stored at -20°C.

Immunization protocol

The immunization may be done by the teacher or by students, depending on time availability. Two New Zealand rabbits (2 - 2,5 Kg each) were immunized according to the schedule shown in table 1. Animals were bled on days 0, 7, 14, 21 and 30.

TABLE 1. Immunization protocol

Day	Immunization
0	100 µg Ng PorIA or Ng PorIB in Freund's complete adjuvant (1:1) intradermal
7	100 µg Ng PorIA or Ng PorIB in PBS intravenously, in the marginal ear vein
14	100 µg Ng PorIA or Ng PorIB in Freund's incomplete adjuvant (1:1) intramuscular

Enzyme - linked Immunosorbent assay (ELISA)

Coat polystyrene ELISA plates (NUNC, Maxisorp, Denmark) ON at room temperature with 100 µl/well of 15 µg/ml Ng PorIA or Ng PorIB in PBS and block the non-coated part of the plastic surface by incubation for 1 h at room temperature with 200 µl/well of 1% (w/v) BSA in PBS. Wash 3 times with PBS with 0,05 % Tween 20 (PBS-T). Add rabbit serum samples (100 µl/well) diluted in PBS-T with added BSA 1% (w/v) (PBS-T-BSA), and incubate for 2 h at 37°C. Wash as above with PBS-T and add 100 µl/well of appropriately diluted peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO, Denmark) (in PBS-T-BSA) and incubate 2 h at 37 °C. Wash and add substrate solution (200 µl/well) containing 3-methyl-2-benzothiazolinone hydrazone hydrochoride, 3-dimethyl-aminobenzoic acid and H_2O_2[5]. Incubate plates

for 20 min. at room temperature with shaking. Measure optical density (OD) at 600 nm in an ELISA plate reader.

Rabbit serum of the corresponding optimum extraction was used as reference in every plate. Specific antibody titers were expressed as arbitrary units (AU) referred to this serum. Units were defined so that their values were the same for dilutions of the reference serum exhibiting similar low OD (approx. 0,1) in each test. AU and OD corresponding to dilutions of the reference serum were correlated by linear regression. OD corresponding to each sample was converted to AU of this reference pool.

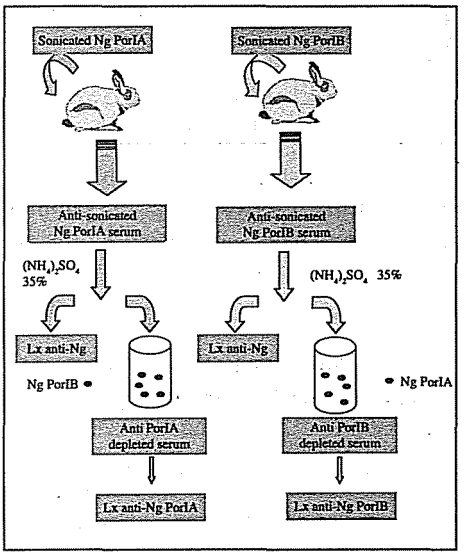

FIGURE 1. Experimental design.

Neisseria gonorrhoeae latex

Latex reagents reactive to rabbit anti-bacterial antibodies were prepared by passive sensitization of sonicated Ng PorIA or Ng PorIB to latéx microspheres.

To prepare 5 ml of final reactive, mix 2mg of sonicated Ng PorIA or Ng PorIB and add PBS to obtain a final volume of 4,6 mL. Under magnetic stirring, add quickly 0,4 ml of a solution of 10% solids-latex polystyrene microspheres (Estapor® Ref k30, Prolabo France, 0,3 µm) to a final latex solid concentration of 8 mg/ml. After gentle mixing for 2 hs at room temperature, block non-reactive sites by the addition of 30 ml of a solution 100 mg/ml of BSA. Mix well and store at 4°C ON. On the second day, centrifuge at 15000 rpm for 10 min. at 4°C and resuspend by magnetic stirring in PBS-BSA 0,1%. Filter by 8 µm polycarbonate membranes and store at 4°C.

Isolation of IgG fractions

The IgG fractions of each antisera were isolated by precipitation with 37% saturated ammonium sulfate, pH 7.0 for 2 hs. The precipitates were centrifuged, washed twice with 37% saturated ammonium sulfate solution, pH 7.0, and resuspended in PBS. After dialysis in PBS, the protein concentration was assayed by the bicynchoninic acid method (BCA Protein Assay Reagent, PIERCE, Illinois, U.S.A.) using BSA as standard, according to the manufacturer's instructions. The samples were stored at -20°C until use.

Anti *Neisseria gonorrhoeae* latex

Latex reagents reactive to bacteria were prepared by passive sensitization of rabbit anti-bacterial antibodies to latéx microspheres. Dialize antibacterial IgG antibodies versus glycine 0,02 M pH 9.5 buffer containing 0.1% sodium azide.

To prepare 5 ml of final reactive, mix 2mg of dialized antibodies with the same buffer to obtain a final volume of 2,1 ml. Under magnetic stirring, add quickly 0,4 ml of a solution of 10% solids-latex polystyrene microspheres (Estapor® Ref K30, Prolabo France, 0,3 µm) to a final latex solid concentration of 8 mg/ml. After gentle mixing

for 2 hs at room temperature, block non-reactive sites by the addition of 50 µl of a solution 100 mg/mL of BSA. Mix gently for 10 min. at room temperature Add 2,5 ml of glycine 0,2 M pH 8 buffer containing 0.2% sodium azide, 17 g/L NaCl, 120 g/L urea and 120 ml/L nn-dimetil formamide. Mix well and filter by 8 mm polycarbonate membranes. Store at 4°C.

Depletion of cross-reacting antibodies from rabbit sera

Incubate in a tube 15 mg of the isolated IgG fraction anti-Ng PorIA or anti-Ng PorIB with 4 ml of *Neissera gonorrhoeae* PorIB and PorIA latex respectively to eliminate cross reacting Ab from rabbit sera. Mix gently for at least 90 min. at 37°C and centrifugue at 15000 rpm for 10 min.

Completeness of depletion was confirmed by látex. If it is necessary, repeat the above operation until no more agglutination of *Neissera gonorrhoeae* PorIB and PorIA latex is observed.

Titration of specific anti-Ng antibodies by latex agglutination

Add 25 µl of serial dilutions of each selected rabbit serum in PBS within 1 cm diameter black circles on plastic or glass slides. Add 17 µl of *Neissera gonorrhoeae* PorIB or PorIA latex and mix well. Rock the slide and observe the agglutination over 2 min. Define the titre as the reciprocal of the last dilution showing detectable agglutination.

Latex agglutination

Add 25 µl of bacterial suspensions including a positive control and PBS as a negative within 1 cm diameter black circles on plastic or glass slides. Add 17 µl of anti *Neissera gonorrhoeae* PorIB or PorIA latex and mix well. Rock the slide and observe the agglutination over 2 min.

RESULTS

We evidenced, by different techniques, the antibody response of an animal against the inoculated Ag. Anti-Ng PorIA and Ng PorIB specific antibody titres were evaluated by ELISA and latex throughout the protocol in serum samples from rabbits (fig. 2 and table 2). Both profiles were similar showing the 5th extraction (day 30) the maximum titre by ELISA and latex. So this was the selected extraction to prepare the reagents.

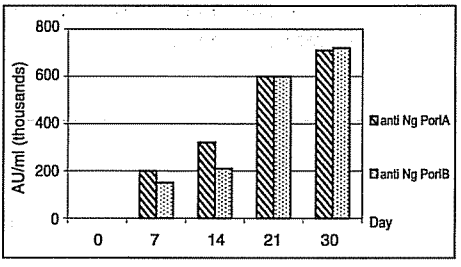

FIGURE 2. Anti-Ng PorIA and Ng PorIB specific ELISA titres of serum samples extracted from rabbits are shown at different times during the protocol.

TABLE 2. Titres of specific anti Ng PorIA and Ng PorIB antibodies by latex

Day	Titre	
	Anti Ng PorIA serum	Anti Ng PorIB serum
7	8	32
14	16	64
21	32	128
30	128	256

The existence of shared epitopes between both types of Ng was demonstrated by ELISA and latex (table 3) in the selected extractions (day 30).

Table 3. Anti-Ng PorIA and NG PorIB specific Ab titres were evaluated by latex agglutination and ELISA in the selected extractions for both animals.

Cross-reactions between strains	
Anti-PorIA serum *vs.* Ng PorIB	
Titre	AU/mL
16	460000
Anti-PorIB serum *vs.* Ng PorIA	
Titre	AU/mL
32	150000

The depletion process that eliminates antibodies directed against shared epitopes was checked by Ng latex. Each sera showed high latex titres against the corresponding type and were nule against the other.

Latex reagents anti-Ng PorIA and PorIB were tested by direct agglutination with fragments of isolated Ng from clinical urethritis (disruption by freeze/thawed cycles) identified and classified by auxotype and serovar. The results showed 100% of concordance.

DISCUSSION

The results obtained indicate that high specific Ab titres were obtained and that depleted sera discriminated between both types of *N. gonorrhoeae*. These results suggest the possibility to obtain diagnostic reactives to confirm presumptive identifications by a rapid and simple method.

This model was used to show to the students the antibody response against different Ags with similar structures. The issues that we highlighted included the nature of Ags, the different immunization routes and obtention of specific Abs against the immunizing Ag. Follow up of the antibody response was done by two techniques, ELISA and latex (quantitative and semiquantitative, respectively) advantages and disadvantages of each one were discussed. The results obtained (see fig. 2) allowed the students to observe the evolution of specific Ab titre during the whole protocol. In order to obtain the typing reagents, we selected extraction 5 because it showed the maximum titre of specific antibodies.

The results shown by the selected anti-PorIA and PorIB extractions using latex agglutination, demonstrated the existence of Abs directed against shared epitopes between them. This was expected considering the structure of the Ag used to immunize the rabbits. The students were able to realize the need to eliminate unwanted Abs in order to obtain an appropriate reagent for typing and concluded the feasibility of solving that by affinity chromatography using latex sensitized with the other Ng type as immunoabsorbent. The titration by latex agglutination of both depleted sera allowed to check the efficiency of depletion.

The students were very enthusiastic to perform typing with their own reagents. We found 100% coincidence between results obtained typing Ng with our reagents and by auxotype and serovar.

It is possible to perform this experiment dividing the work and assigning one part of it to groups of lower number of students making a final discussion integrating the different aspects of the experimental protocol at the end of the course. In our 3 years experience with this experiment students showed themselves very interested with it and the Problem Based Learning methodology.

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