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Preparation of reagents for blood group serology: illustrating basic concepts of the antibody response

M. Miguez, M. Marco, S. Cáceres, A. Nieto

Cátedra de Inmunología, Facultad de Química, Instituto de Higiene, Universidad de la República, Montevideo, Uruguay

Abstract

The aim of the present work is to illustrate basic issues relating to the antibody response through the preparation reagents for blood group serology. Two rabbits were immunised with the stromata of A and B cells. The antibody response was analysed by ELISA and Haemagglutination. The appropriate bleeding was selected in order to prepare the reagents. They were tested by blood grouping a panel of volunteers and comparing the results with those obtained with a commercial kit.

1. Introduction

The course 'Introduction to Immunology' in our Department is aimed at undergraduate students of Chemistry, Biochemistry, Biology, Pharmacy and Veterinary Medicine. The average number of students per year is 150–200. They are divided into laboratory groups (about 15 students each) for 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 necessary, within each group the students may focus on different approaches to the problem proposed.

At the beginning of the course they have an introductory class introducing the major theoretical and experimental issues they will need to understand in order to solve the problems proposed. At the same time, 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. There is then a discussion with the teacher about the experiments that might be carried out to solve the problem.

Once the experimental work is concluded, and after discussing the results extensively with the teacher, the students reveal their results to the rest of the class and teachers in a discussion-oriented seminar. Finally, the group produces a report on their work which should be structured like a typical scientific paper. A global evaluation comprising laboratory and seminar performances as well as the quality of the report, is used to score this course.

The experimental protocol presented in this paper has been used for four consecutive years in our course. It aims to illustrate basic issues relating to the antibody response through the preparation of reagents for a well-known application of immunology, namely blood group serology.

2. Background

The surface of the human erythrocyte, in common with that of other types of cells, is coated with a large and complex mosaic of specific antigenic determinants, many of which are complex carbohydrates (oligosaccharides) [1]. To date, over 250 red blood cell antigens have been described, and the number is rapidly increasing. Included in this number are about 100 blood group determinants, belonging to 15 independent human blood group systems. Of these, the most thoroughly studied are the antigenic determinants of the ABO (or ABH) blood group system. These were discovered in 1900 by Landsteiner and are of great importance for the safe practice of blood transfusion. They are also useful for legal and even historical research purposes.

The sera of individuals of blood group A contain anti-B antibodies and those of group B contain anti-A. The blood group substances A and B are not confined to the red cells but can also be detected in body fluids (saliva, semen, etc) of secretor individuals (about 80% of the

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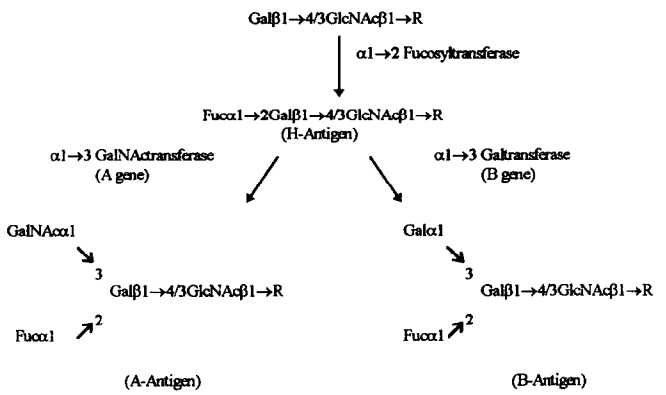

Fig. 1. Biosynthetic pathway of A, B and H antigen formation. Almost every individual express α(1→2) fucosyl-transferase, whereas the presence of A or B associated enzymes depends on the genome of each individual.

total population, which secrete ABH blood group substances according to their blood group).

The method for studying the blood group of an individual is to test whether his/her erythrocytes are agglutinated by specific antibodies (commercial reagents generally use specific lectins), or by detecting group specific substances in saliva, if this individual is of the secretor type. The ABH glycoproteins posses a common core structure (H) and their blood group specificity resides ultimately in a relatively small structural variation [2] (Fig. 1). Everyone has H antigen present on their erythrocytes a portion of which becomes converted to A or B antigen by enzyme action depending on whether they are individuals of blood group type A or B, respectively. In group AB individuals, both the A and B-synthesizing enzymes are present and very little H activity remains. On the other hand in group O individuals, genes that encode for those enzymes are not present so that only antigen H is found on the surface of the erythrocyte [3]. The concept of cross-reactivity and its relevance for the present work should be illustrated.

The aim of this work is to illustrate basic concepts of the antibody response through the preparation of blood grouping sera for the ABO(H) human system. For this purpose we immunized two rabbits with the stromata of A and B red cells. (**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.) Students analyze the antibody response of the rabbits during the immunization protocol by ELISA and haemagglutination. With these data they select the bleeding which provides the serum with the maximum specific antibody titre for the preparation of the reagents. Animals (rabbits) recognize substances A, B, or H and other membrane proteins common to all red blood cells. The unwanted antibodies directed against red cell shared epitopes are removed by absorption with type O red cells (which carry H substance and all other common red blood cells antigens) [4]. The carbohydrate origin of the epitopes recognized is demonstrated by periodate treatment of the antigen in the ELISA assay.

The reagents (anti-A and anti-B antibodies) are tested by blood grouping a panel of volunteers and comparing the results with those obtained with a commercial kit. They are also used to determine the blood group of secretor individuals by testing their saliva by the haemagglutination inhibition test.

3. Experimental

3.1. Preparation of erythrocyte stromata (ES)

(This preparation should be done by the teacher because it requires more than the available time.) Collect type A and B blood specimens in acid–citrate–dextrose solution (7.3 g l^{-1} citric acid, 24.5 g l^{-1} glucose, 24.5 g l^{-1} trisodium citrate). **CAUTION** Blood from volunteer donors must be tested for HIV, Hepatitis B, etc. and only negative samples should be used.

Wash erythrocytes four times with PBS (0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl), centrifuging at 200**g** for 15 min at room temperature, and then haemolyze them by incubation for 30 min in 0.005 M phosphate buffer, pH 8 (5% v/v of erythrocytes in lysis buffer). Centrifuge the suspension of stromata at 20000**g** for 1 h at 10°C and wash the pellet with the same buffer until membranes are clear and free from haemoglobin. Resuspend the pellet in PBS and sonicate (3 cycles of 15 s at maximum power keeping the tube at 4°C). Centrifuge at 15000**g** for 5 min at 4°C and determine the protein concentration of the supernatant by the bicinchoninic acid method (BCA Protein Assay Reagent, Pierce, Illinois, USA) using bovine serum albumin(BSA) as standard, according to the manufacturer's instructions. The samples are finally stored at −20°C.

3.2. Immunization protocol

The immunization may be done by the teacher or by students, depending on time availability (see above Note). Two New Zealand rabbits (2–2.5 kg each) are inmunized by intravenous injections of the corresponding antigen solution into the marginal ear vein, according to the schedule shown in Table 1. The antigen solution used is a solution of sonicated stromata from human erythrocyte type A (EAS) or type B (EBS), 6.5 mg protein per ml in sterile PBS, and the suggested volumes to administer are: 0.1 ml on day 0, 1 ml on days 1–7 and 2 ml on days 8–14. Animals were bled on days 0, 3, 7, 10, 14, 17 and 24.

Table 1
Direct agglutination. Anti-RBC A, B and O specific Ab titres were evaluated by direct agglutination in the selected extractions and the depleted ones for both animals

	Anti-EAS serum		Anti-EBS serum	
	Non-depleted	Depleted	Non-depleted	Depleted
RBC A	1024	128	128	0
RBC B	128	0	512	128
RBC O	128	0	64	0

3.3. Depletion of cross-reacting antibodies

All sera must be first incubated at 56°C for 30 min to inactivate complement (this would otherwise cause lysis of erythrocytes recognized by rabbit antibodies). Incubate one volume of each of the anti-EAS or anti-EBS sera with five volumes of packed washed type O red blood cells (from volunteer donors tested as described above) to eliminate cross reacting antibodies from rabbit sera. Mix gently for at least 2 h at 4°C and centrifuge at 200**g** for 10 min. Completeness of depletion should be confirmed by haemagglutination. If necessary repeat the above operation until no more agglutination of type O red cells is observed.

3.4. Haemagglutination

Add 25 μl of PBS to all the wells (except the first column) of a micro-haemagglutination tray (V-shaped bottom) using a multi-channel automatic pipette. Add to each well of the first column of the tray 50 μl of: each rabbit immune serum to be tested, as well as pre-immune rabbit serum (day 0) and PBS as a negative control (complement in all sera must first be inactivated at 56°C for 30 min.). Make serial dilutions of each serum directly into the tray. Add 50 μl of human red blood cells (RBCA or B) in PBS (2% v/v) to all the wells.

Cover the tray and mix the contents of the wells by gentle shaking. Leave at room temperature for 1 h and read the plate on a white surface. Positive agglutination is recorded when the cells form a continuous carpet on the base of the well. If no agglutination has occurred the cells roll down to form a tight button to the bottom of the well. Define titre as the reciprocal of the last dilution showing detectable agglutination.

3.5. Enzyme-linked immunosorbent assay (ELISA)

Coat polystyrene ELISA plates (Nunc, Maxisorp, Denmark) overnight at room temperature with 100 μl/well of 15 μg ml^{-1} EAS or EBS 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) bovine serum albumin(BSA) in PBS. Wash three 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 pig 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 and then measure the absorbance at 600 nm in an ELISA plate reader.

Rabbit serum of the corresponding optimum bleeding was used as reference in every plate. Specific antibody titres were expressed in 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 absorbance corresponding to dilutions of the reference serum were correlated by linear regression. Absorbancies corresponding to each sample was converted to AU of this reference pool.

3.6. Sodium metaperiodate treatment of native antigen

Treat antigen adsorbed on ELISA plates with 20 mM sodium metaperiodate solution in 50 mM sodium acetate buffer, pH 4.5 (100 μl/well) in the dark for 1 h [6]. Rinse with acetate buffer and add 100 μl/well of a solution of 50 mM $NaBH_4$ in PBS during 30 min in the dark. Wash with PBS–T and continue with ELISA as previously described.

AU were assigned as described above. The difference between the AU without (corresponding to total Ab) and with periodate treatment (corresponding to Ab recognizing peptide and other periodate resistant epitopes) was assumed to estimate the Ab titre against carbohydrate epitopes.

3.7. Direct agglutination

Add 15 μl of serial dilutions of each selected rabbit serum in PBS to an appropriate glass slide. Add 15 μl of A, B or O blood type 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.

3.8. Blood grouping

Add 15 μl of the blood to be tested to each of two separate glass slides. Add, to each sample, 15 μl of anti-A or anti-B appropriately depleted serum at the previously determined optimum working dilution and mix well.

Rock the slide and observe agglutination over 2 min. Do the same with the corresponding commercial kit.

3.9. *Inhibition test using saliva*

Dilute saliva samples with an approx equal volume of PBS and place them in a boiling water bath for 10 min to inactivate enzymes. Centrifuge at 15000**g** for 5 min at 4°C and use the clear supernatant fluids in the following test. For each saliva sample, make serial dilutions of anti-A and anti-B selected rabbit sera in PBS directly into the tray as in haemagglutination technique (25 μl per well). Choose suitable initial dilutions in order to give titres of approximately 32. Add equal volumes of saliva to each well and include one volume of PBS in place of saliva as a control for each antiserum. Mix gently and allow to stand for 30 min at room temperature. Add 50 μl of the appropriate A or B red cell suspension (2% v/v) to each well. After 1 h incubation at room temperature, observe agglutination. Those individuals whose saliva contain the appropriate ABO group specific substances normally show complete inhibition of agglutination.

4. Results

We illustrated, by different techniques, the antibody response of an animal against the inoculated antigen. Anti-EAS and EBS specific antibody titres were evaluated by ELISA throughout the protocol in serum samples from rabbits [Fig. 2(a)]. Both titres peaked at day 17 (bleeding No. 5), and so this was the selected bleeding for preparing the reagents. The whole profile was also evaluated by haemagglutination [Fig. 2(b)] being similar to that obtained by ELISA.

The existence of shared epitopes between the three types of human red blood cells was demonstrated by direct agglutination. Titres of the selected extraction (anti-EAS and EBS) before depletion, against human red blood cells A, B and O are presented in Table 1. Each serum showed a high titre against the corresponding red cells and a low one, but not nil, against the others.

The depletion process that eliminates antibodies directed against shared epitopes was also checked. Once depleted, both extractions (anti-EAS and EBS) were checked by direct agglutination against human red blood cells A, B and O. The results are shown in Table 1. With these results we selected the optimal dilution of each depleted serum so that when it is used as reagent, cross-reaction with the other blood group types is not observed. The results of typing 100 volunteers by direct agglutination, comparing our reagent with a commercial kit, showed 100% coincidence. An alternative blood grouping method using saliva samples was also tried. Saliva samples were obtained from ten of the 100 volunteers mentioned above. The results of the inhibition test are shown in Table 2. We also studied the percentage of antibodies generated against carbohydrate epitopes in the selected extractions. They were 70% of anti-EAS antibodies and 82% anti-EBS, respectively, before deple-

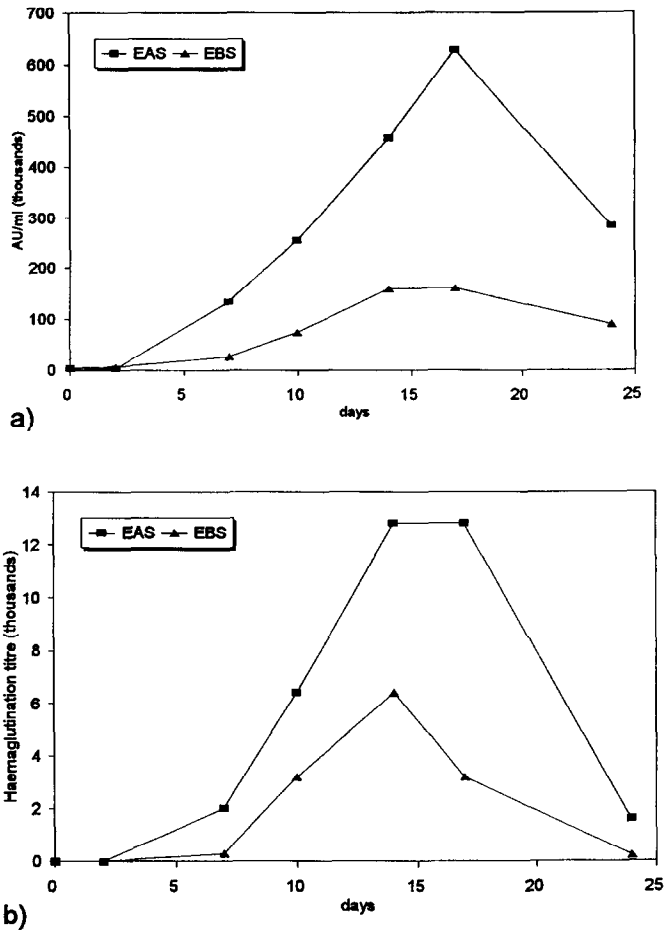

Fig. 2. Titres of specific anti-EAS and EBS antibodies. Anti-EAS and EBS specific ELISA (a) and haemagglutination (b) titres of serum samples extracted from rabbits are shown at different times during the protocol.

Table 2
Blood grouping by inhibition test. Saliva from 10 volunteers was tested for blood group by inhibition of haemagglutination

Sample number	Inhibition test result	Commercial kit
1	B	B
2	A	A
3	AB	AB
4	O or not secretor	O
5	AB	AB
6	O or not secretor	B
7	A	A
8	O or not secretor	A
9	O or nor secretor	A
10	O or not secretor	O

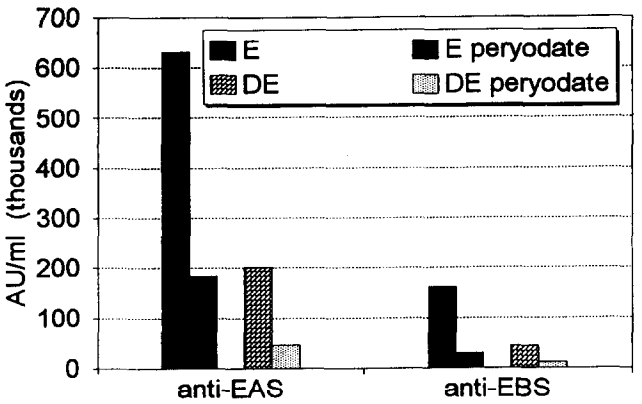

Fig. 3. Titres of specific anti-EAS and EBS antibodies with and without periodate treatment. Anti-EAS and EBS specific antibody titres of the selected extraction and corresponding depleted sera, were determined by ELISA.

tion. The depleted ones showed 77 and 73%, respectively, of antibodies to carbohydrate epitopes.

5. Discussion

This model system was used to demonstrate to the students the antibody response against different antigens with similar structures (in this case complex mixtures of glycolipoproteins). The issues that we highlighted included the nature of antigens, the different immunization routes and the production of specific Abs against the immunizing Ag. The antibody response was followed by two techniques, ELISA and haemagglutination (quantitative and semiquantitative, respectively) and the advantages and disadvantages of each were discussed.

The results obtained (see Fig. 2) allowed the students to observe the development of specific antibody titre during the immunization. In order to obtain the blood group typing reagents we selected bleeding No. 5 because it showed the maximum titre of specific antibodies. The results shown by the selected anti-EAS and EBS bleedings using direct agglutination demonstrated the existence of antibodies directed against shared epitopes between the three types of human red blood cell antigens. This was expected considering the structure of the antigen used to immunize the rabbits. The students were able to understand the need to eliminate unwanted antibodies in order to obtain an appropriate reagent for typing and were able to conclude for themselves the feasibility of doing this by affinity chromatography using human red blood cells type O as immunoadsorbent.

The titration by direct agglutination of both depleted sera allowed not only us to check the efficiency of depletion but also to determine the appropriate working dilution. It was very interesting to find similarities between the percentage of specific antibodies against substances A and B that are present in each of the extractions selected. We found, after depletion, 32% of anti-EAS Ab and 27% of anti-EBS in the selected extractions.

The students were, of course, very enthusiastic to perform typing themselves with their own reagents and compare the results with the commercial kit. Alternatively, blood grouping of saliva of secreting individuals by a haemagglutination inhibition test allowed them to verify the presence of these substances in secretions (see Table 2).

Furthermore we could use this system to show the generation of high levels of specific antibodies against the carbohydrate epitopes which determine the differences between substances A, B and H. The presence of these antibodies was shown by periodate treatment of the corresponding antigen before performing the ELISA test (see Fig. 3).

The first time this laboratory work was proposed we began immunizing animals two months before the beginning of the course and tried to bleed them exhaustively so that we could count on there being sufficient material for several courses. It is possible to perform this experiment dividing the work and assigning one part of it to groups of smaller numbers of students and having a final discussion integrating the different aspects of the experimental protocol at the end of the course.

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