Bull. Univ. Osaka Pref., Ser. B, Vol. 31,
大阪府立大学紀要，農学・生物学，1979

A Simple Method for Detecting Cells Producing Antibodies of Specific Immunoglobulin Classes

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(Received October 31, 1978)

Abstract

A simple method for detecting cells producing antibodies of specific immunoglobulin classes was devised based upon the hemolytic plaque formation which was under the strict dependence upon immunoglobulin-class-specific antisera. IgM-specific plaque-forming cells in immunized chickens were detected using rabbit anti-chicken μ-chain antiserum and guinea-pig complement. IgG-specific plaque-forming cells were detected using rabbit antichicken γ-chain antiserum and guinea-pig complement.

Introduction

The methods for detecting antibody-producing cells, based upon hemolytic plaque formation, elaborated by Jerne and Nordin[1] and modified thereafter by many investigators[2),3),4),5] have been used very extensively in immunological researches to evaluate the magnitude of humoral immune responses. Nevertheless, it is conceivable that there may be considerable uncertainty in the current method generally used for detecting the cells based upon the development of hemolytic plaques. Plotz, Colten and Talal[6] found the existence of IgM antibodies to sheep erythrocytes of a non-complement-fixing type in the mouse antiserum. It was known[7),8),9] that a class of IgM-antibody-producing cell is incapable of causing hemolytic plaques on a agar plate without facilitation by anti-immunoglobulin (Ig) antiserum. Experiments performed by Pasanen and Mäkela[10] suggested that cells producing IgG antibodies could produce direct plaques when heavily haptencoupled erythrocytes were used as indicator cells. Second, since the indirect plaque number representing the magnitude of immune responses of the IgG type difference between direct and anti-Ig-antiserum-facilitated plaque counts, the number is accompanied with relatively large fluctuation which is derived from the sum of variance associated with the direct plaque counts and with the counts of plaques developed by the facilitation. Thus, it would be strongly desirable to devise a method capable of distinguishing between cells producing IgM antibodies and those producing IgG antibodies with sufficient exactitude and specificity. Fortunately, antibodies of the fowl do not activate the guineapig complement indicating that the use of fowl cells, rabbit anti-chicken Ig antisera and guinea-pig complement is hopeful. Thus, the authors attempted to verify this postulate employing a combination of chicken cells to be studied, guinea-pig complement and rabbit antisera directed toward heavy chains of chicken Ig. The experiments verified the validity of the postulate.

Meterians and Methods

Animals

Animale used were chickens, White Leghorn Hy-Line. The chickens were purchased from Takeuchi Hatchery Incorporated, Osaka.

Immunization

Chickens were immunized intravenously with 4×10^8 sheep red blood cells (SRBC) at 3 and 4 weeks of age.

Preparation of cell suspensions

Spleens were dissected into cold medium 199; the tissues were cut into pieces with scissors. The suspension was filtered through layers of gauze after agitation with a pipette to break the cell aggregates. The cells were washed three times by centrifugation at 600–700 rev/minute for 10 minutes. The viability of the cells was examined by the trypan blue dye exclusion.

Preparation of antiserum directed toward μ-chain of chicken IgM

IgM was isolated from chicken serum according to the method of Benedict[11]. 2.5 mg of the IgM in 1 ml of Freund's complete adjuvant (FCA) was injected into each of the subcutis and the footpad of rabbits, and the injections with the same dose in FCA into each of the subcutis and the muscle were given after two weeks. Furthermore, 1 mg of IgM was injected intravenously two weeks later. The sera were taken 10 days after the last immunization, and were inactivated by heating for 30 minutes at 56°. Anti-L-chain antibodies were removed from the anti-IgM antiserum by the following procedures; to an appropriate volume of the antiserum was added an equal volume of a pooled serum taken from newly hatched chickens which contained IgG but not IgM at all, and the mixture was allowed to stand for 60 minutes at 37° and overnight at 4°. The supernatant was taken after centrifugating the mixture for 30 minutes at 7,000 rpm The absorption procedures were repeated until the antiserum became entirely non-agglutinable with SRBC coated with the chicken IgG. Rabbit anti-chicken μ-chain antiserum thus obtained exhibited, in the 480-fold final dilution of the antiserum, the titer of 1 : 8 in gel diffusion precipitation reaction with the chicken IgM but not react with the chicken IgG at all.

Preperation of antiserum directed toward γ-chain of chicken IgG

IgG was isolated from chicken serum according to the method[11] of Benedict. Unabsorbed rabbit antiserum to chicken IgG was prepared by the same method with that to prepare the anti-chicken IgM antiserum. Anti-L-chain antibodies were removed from the unabsorbed anti-IgG antiserum by the following procedures; 2% solution of $F(ab')_2$ obtained by pepsin digestion of the chicken IgG at pH 4.5 followed by gel filtration on a Sephadex G-200 column was added to an equal volume of the antiserum, and the mixture was allowed to stand for 60 minutes at 37° and overnight at 4°. The supernatant was taken after centrifugating the mixture for 30 minutes at 7,000 rpm. The absorption procedures were repeated until the antiserum became completely non-agglutinable with SRBC coated with the chicken IgM. The rabbit anti-chicken γ-chain antiserum thus

obtained reacted, in the 480-fold final dilution, with the chicken IgG in gel diffusion precipitation reaction exhibiting the titer of 1 : 16 but not with the chicken IgM at all.

Complement

Fresh pooled serum taken from several guinea-pigs was used as the complement. The complement was added to the assay mixture in a 8-fold final dilution.

Procedures for detecting hemolytic plaques

Eour pieces (25 x 5 mm) of usual vinyl tape were laid across a clean microscopic slide (25 x 75 mm) dividing it into three equal areas. The preparation of a reaction mixture for the plaque assay was carried out using a dropper of microtitration which was controlled so that one drop may contain a 250-μl volume. The assay mixture was prepared by mixing 3 drops of a splenic cell suspension of 1×10^7 cells per milliliter, 3 drops of a 15% SRBC suspension in phosphate buffer saline (PBS), pH 7.2, 3 drops of rabbit antiserum against heavy chains of chicken Ig diluted with PBS in appropriate concentrations and 9 drops of complement. One drop of the mixture was dropped on each section of the slide, and immediately a coverslip (18 x 24 mm) was laid onto the two tape pieces as shown in Fig. 1. When plaque-forming cells of IgM type were detected, the

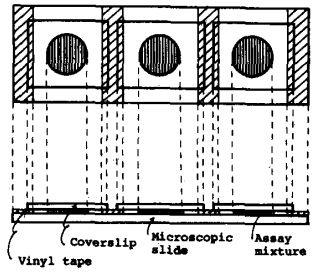

Fig. 1. A plate for plaque assay.

assay mixture containing anti-μ-chain antiserum was used, and that containing anti-γ-chain antiserum was used when IgG plaque-forming cells were detected. The assay mixture was naturally spread to form a thin layer between the coverslip and the slide. The slide was allowed to stand horizontally for 20 minutes in a incubator controlled at 37°. Plaques of hemolysis were detected on an immunoviewer, a box equipped with a light. When the plaques were doubtful in observation with the naked eye, they were detected using a microscope.

Results

Development of hemolytic plaques under various conditions

A splenic cell suspension was prepared from 5-week-old chickens immunized with SRBC at 3 and 4 weeks of age. The assay mixtures made from the splenic cells, SRBC, various dilutions of antisera against heavy chains and complement were dropped on the slide, and a coverslip was laid onto that. That slide was incubated at 37°. Hemolytic plaques began to appear after about 10 minutes. The appearance of the plaques reached

the maximum after incubation for about 20 minutes. The plaques appearing with the anit-Ig antisera of higher concentrations than 300-fold final dilution were relatively obscure in visibility. The size of plaques enlarged with time during incubation, and reached the maximum after about 20 minutes. The plaques disappeared after incubation for about 60 minutes, because of the fusion of many localized plaques into large hemolytic areas. When lymphoid cells to be tested were contained in excess in the assay mixture, the plaques became obscure after incubation for about 30 minutes. The plaque number varied depending upon the concentration of of anti-μ-chain autiserum showing the maximum in 480-fold final dilution (Table 1). The plaque formation was inhibited

Table 1. Development of hemolytic plaques in various conditions

Antiserum	Final dilution	Mean number of plaques and their standard deviation*
Anti- -chain antiserum	X 60	2,640 ±463
	X 300	3,768 ±228
	X 480	3,936 ±420
	X 600	3,432 ±564
Anti- -chain antiserum	X 300	4,392 ±367
	X 480	5,424 ±581
	X 600	5,232 ±682
———		0

* Mean number of hemolytic plaques and their standard deviation per 10^6 spleen cells.

by anti-μ-antiserum of higher concentrations than 300-fold final dilution, and was low in the antiserum finally diluted 600-fold. In contrast, the degree of plaque development by anti-γ-chain antiserum was roughly equal in the three tested concentrations of the antiserum. None of hemolytic plaques were developed at all by operation of guinea-pig complement alone in the absence of rabbit antisera specific for heavy chains of chicken Ig in accordance with the prior postulation; every antibody-producing cell formed a hemolytic plaque under the absolute dependence upon the rabbit antisera against heavy chains. It is evident that the plaque induced by anti-μ-chain serum is a localized hemolysis caused by complement activated with rabbit anti-μ antibody under the cooperation with 'IgM' antigen (IgM-typed hemolysin) secreted by one chicken cell, and that the plaque induced by anti-γ serum is a localized hemolysis by complement activated with rabbit anti-γ antibody under the cooperation with 'IgG' antigen (IgG-typed hemolysin) secreted by another chicken lymphoid cell.

The standard procedures for detecting plaque-forming cells specific for immunoglobulin-classes

In the light of the above experimental results the authors established, as follows, the standard conditions under which plaque-forming cells specific for Ig-classes are detected in the lymphoid organ of the chicken:n: (a) When plaque-forming cells of the IgM type are detected, fresh pooled serum isolated from guineapigs is used as complement together with an appropriate dilution of the highly specific rabbit antiserum directed toward

μ-chain determinant of chicken IgM (for example, 480-fold final dilution of the original antiserum having the titer of f 1 : 8 in gel diffusion precipitation reaction). (b) When plaque-forming cells of the IgG type are detected, the fresh serum from guinea-pigs is used as complement together with an appropriate dilution of the highly specific rabbit antiserum directed toward γ-chain determinant of chicken Ig (for example, 480-fold final dilution of the original antiserum having the titer of 1 : 16 in gel diffusion precipitation reaction). (c) A liquid plate technique described in the section of Materials and Methods is recommended because of its high sensitivity and simplicity. 25 μl of the assay mixture consisting of 75 μl of cell suspension (1×10^7 cells/ml), 75 μl of 15% SRBC suspension, 75 μl of anti-heavy chain antiserum and 225 μl of complement is plated on a microscopic slide with the aid of vinyl tape pieces and of a coverslip. After incubating the slide for 20 minutes at 37°, the plaque number is counted for less than 30 minutes.

Discussion

A method to detect cells producing antibodies of specific Ig classes was devised. The method can detect plaque-forming cells with pronounced discrimination between the plaques of IgM type and of IgG type. This assertion is verified by the following results: The plaque development was induced only be operation of the highly specific antiserum directed toward – or –chain determinant of the chicken Ig.

The present method for detecting plaque-forming cells is more simple than that devised by Cunningham and Szenberg[12] on the basis of plaque assays in liquid media. Simple procedures for the plaque assay described in the section of Materials and Methods did not disturb successful achievement of plaque detection at all mainly because of short incubation.

There exist a varity of evidences showing notable uncertainty in the current method for detecting Ig-class-specific plaque-forming cells, as shown in the section of Introduction. It was verified in the present study that such uncertainties are solved in the chicken system by using the present method. If antibodies produced by the mammalian cells will not activate chicken complement through the antigen-antibody reaction, the detection of plaque-forming cells absolutely dependent upon the addition of the chicken antimammalian heavy chain antiserum should be possible.

Acknowledgements

We should like to express our great thankfulness to Professor K. Sato, University of Nagoya, for his technical instruction of the detection of plaque-forming cells. We are also grateful to Mr. T. Masui for his generous donation of valuable F $(ab')_2$ of the chicken IgG.

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