MICRODETERMINATION OF ANTIBODIES TO NERVE TISSUE GALACTOCEREBROSIDES IN THE COMPLEMENT FIXATION TEST

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Injury to and destruction of myelinated structures lie at the basis of the development of a large group of autoimmune demyelinating diseases of the nervous system, and also at the basis of degeneration of nerves after trauma and division. It has been shown that galactocerebrosides (GC), which are glycolipids not found in other tissues [11, 12], are a specific antigen for myelin and for myelin-producing cells. To study diseases accompanied by myelin destruction, both in experimental model and, in particular, in experimental allergic encephalomyelitis, and under clinical conditions, autoantibodies to the myelin components of nerve tissue (GC) must be investigated.

Since GC are hydrophobic haptens, they can take part in an immunologic reaction only in the presence of accessory lipids, cholesterol and lecithin, which induce the formation of stable lipid micelles in an aqueous medium, carrying antigenic determinants of GC on their surface. This makes the choice of optimal conditions for complete detection of antibodies to GC more difficult, but nevertheless several methods have now been developed for GC estimation in blood serum and cerebrospinal fluid: based on agglutination of a myelin suspension [10], agglutination or immunoprecipitation of labeled liposomes [4, 8], by the immunoprecipitation in agar gel test [9], by ELISA [5], and by the complement fixation test (CTF) [9]. These methods require large quantities of test material, special equipment, and highly trained staff, so that their range of usefulness and their value in experimental and diagnostic investigations are limited.

The aim of this investigation was to develop an accessible, easily performed, and highly sensitive method (a micromodification of the CFT) of determination of antibodies to GC, requiring only a small quantity of test material.

EXPERIMENTAL METHOD

Sera from 12 rabbits immunized with GC [7] and also from five control rabbits were used to develop the method. Blood for investigation was taken on the 25th-30th day after immunization.

A lipid suspension containing GC and lecithin, isolated from the brain by thin-layer chromatography on silica-gel (Woelm, West Germany) [3] and cholesterol (Serva, West Germany), was used. To analyze the 30 sera 16 ml of a suspension of antigen was prepared: chloroform-methanol (2:1) solutions containing 0.18 mg of GC and 0.60 mg of lecithin were mixed, and the solvent was removed by evaporation on a waterbath at 80°C. The residue of lipids was then dissolved by addition of 0.2 ml of hot ethanol containing 0.62 mg cholesterol, and 0.8 ml of Veronal buffer (pH 7.35) was added [9]. As a result 1 ml of an opalescent suspension was obtained and this was diluted 16-fold with the same buffer, to bring the GC concentration to 11.2 μg/ml, and this was used as the antigen for detecting antibodies to GC.

The CFT was carried out in standard polystyrene panels (12 × 22.5 cm, volume of well 2 ml), washed out with chromate mixture. Considering the anticomplementary properties of the antigen, the dose of complement was first chosen by incubating a mixture of 0.02 ml of dilutions of complement made up beforehand [1] with 0.02 ml of antigen suspension and 0.02 ml

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TABLE 1. Titers of Antibodies to GC Obtained in Rabbit Serum by Macro- and Micromethods of CFT

No. of serum	Suggested micromethod of CFT	Familiar macromethod of CFT
Serum of rabbits immunized with CFT		
1	1:512	1:512
2	1:128	1:64
3	1:32	1:32
4	1:256	1:256
5	1:128	1:128
6	1:512	1:512
7	1:32	1:32
8	1:64	1:64
9	1:128	1:128
10	1:64	1:64
Serum of control rabbits		
1	1:2	1:2
2	1:2	1:2
3	0	0
4	1:1*	1:1
5	0	0

Legend. *) Whole serum.

TABLE 2. Titers of Antibodies to GC in Immune Sera Inactivated by Heating on the Day of the Experiment or the Previous Day

No. of serum	Sera heated on day before experiment	Sera heated on day of experiment
1	1:4	1:64
2	1:8	1:128
3	1:4	1:64
4	1:4	1:64
5	1:2	1:64

of Veronal-Medinal buffer (pH 7.35) at 37°C, in dry-air incubator for 45 min. Next 0.4 ml of the hemolytic system was added (a mixture of equal volumes of a 0.8% suspension of washed sheep's erythrocytes with hemolytic serum in a standard dilution). The panels were incubated at 40°C in a waterbath for 5 min and the results were read on the basis of 100% hemolysis of the erythrocytees, and the working dilution of complement was obtained [2] in order to determine anti-GC-antibodies in the CFT.

Complement in the working dilution was mixed with an equal volume of antigen suspension (32 ml of mixture was obtained to analyze 30 sera) immediately before use (must not be kept on ice!). The sera for testing were incubated for 30 min at 56°C to inactivate endogenous complement, after which a series of double dilutions in physiological saline, in a volume of 0.02 ml, was prepared from them directly in the panels for the main experiment. To each well was added 0.04 ml of the mixture of antigen with complement. Controls for anticomplementary activity of antigen and serum [1] were set up in wells in the same panel, after which the panels were sandwiched between two empty panels and incubated in the dry air incubator at 37°C for 45 min. Next 0.4 ml of fresh hemolytic system was added to each well, incubation was repeated in a waterbath at 40°C until complete hemolysis appeared in the control wells (3-5 min), and immediately thereafter the results of the test were read in transmitted light on the basis of complete hemolysis. The maximal dilution of serum at which complete hemolysis of the erythrocytes was observed was taken to be the titer of anti-GC antibodies.

EXPERIMENTAL RESULTS

The use of microsystems to determine antibody titers in biological fluids has been widely adopted. It has been shown that titers of antibodies to protein antigens, determined in serologic tests performed by macro- and micromethods, with no change in the conditions of the test, are identical [13]. However, the development of a micromodification of the CFT to determine antibodies to the lipid antigen of GC required changes in the conditions of the test (temperature and duration of incubation, time of preparation and conditions of keeping the antigen-complement suspension, times of performance of the whole test, and so on). These changes are required because of the physicochemical properties of the antigen, its insolubility in water, its property of being adsorbed on polystyrene, the necessity of using accessory lipids in the reaction, the necessity of having ethanol in the antigen suspension, and also the instability of the antigen suspension, its separation into layers in the cold, modification of its anticomplementary properties and its increased ability to induce hemolysis of etythrocytes after long-term keeping. To estimate the suitability of the suggested conditions of the micromethod for determination of antibodies to GC in the CFT, we compared results obtained by this method with the results of analysis of antibodies to GC obtained by the familiar macromethod of CFT [9] (Table 1).

It will be clear from Table 1 that the titers of antibodies determined in the same serum by the two methods are identical. It can thus be concluded that the suggested conditions for conduct of the CFT are perfectly suitable for determination of antibodies to a glycolipid antigen such as GC, and they do not reduce the sensitivity of the method. To achieve reproducibility and accuracy of the results of determination of antibodies to GC in accordance with the suggested method, the conditions stipulated and all details of the experimental method must be followed strictly.

It is important that inactivation of endogenous complement in the test sera (30 min, 56°C) be carried out immediately before their use in the CFT, for otherwise, if inactivation of complement is done on the previous day (as is the case with most modifications of the CFT), the titers of antibodies to GC will be sharply depressed (Table 2).

This is evidently because the antibodies are less resistant to the lipid hapten of GC than antibodies to protein and glycoprotein antigens [6].

The suggested micromodification of the CFT thus avoids artefacts which arise when antibodies to GC are determined by long periods of incubation in the cold and by storing the inactivated sera, which are features of most methods. Because of the ability of the lipid suspension of the antigen to be adsorbed on polystyrene, special attention must be paid to the need for washing the polystyrene panels with chromate mixture, to prevent any distortion of the results. The method described above can reduce the consumption of the components of the test and shorten the time required to carry it out from 3 days to 1 day. The method developed is suitable for any laboratory which has CFT facilities.

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