QUANTITATIVE STUDY OF IMMUNE HORSE SERUM ANTIBODY AGAINST CLOSTRIDIUM OEDEMATIENS BY MEANS OF PRECIPITATION ON PAPER OR PAPER-FIXED ANTIGEN

A.E. Gurvich and M.V. Ispolatovskaia

Laboratory of Physiological Chemistry (Chief – Prof. S.Ia.Kaplanskii)

Institute of Biological and Medical Chemistry (Director – V.N. Orekhovich,

Active Member Acad. Med. Sci. USSR), Acad. Med. Sci. USSR

Department of Biochemistry (Chief – V.A. Blagoveshchenskii, Candidate of Medical Sciences),

Gamaleia Institute of Epidemiology and Microbiology (Director – Prof. S.N. Muromtsev),

Acad. Med. Sci. USSR, Moscow

(Received October 21, 1957. Presented by V.N. Orekhovich, Active Member Acad. Med. Sci. USSR)

Great significance is attached to the determination of antitoxin content in various immune sera. At the present time the basic method is a study of the neutralizing capacity of serum with respect to toxin, and the degree of neutralization is established in experiments with living animals. The antitoxin content of immune sera against one of the gas-gangrene agents, Clostridium oedematiens, is determined in a similar manner under industrial conditions.

The use of experiments with animals for antibody determination has a number of substantial disadvantages: it requires considerable time, a great number of livestock, and only gives an approximate answer. It is not customary to determine antitoxin content in sera by the flocculation technique, although references are sometimes encountered in the literature concerning the feasibility of flocculation methods in similar systems [4].

We have developed methods of quantitative determination of antibody content by means of precipitation on paper [2], or by using antigen fixed on paper [3]. We undertook a study of the possible application of these methods in the determination of antibody content against Cl. oedematiens for the purpose of replacing the serum titration in animals by more accurate and speedy biochemical techniques. We were especially interested in the problem of the quantitative dependence of the flocculation of Cl. oedematiens antitoxins with corresponding immune sera.

EXPERIMENTAL METHOD

For the investigation we used Cl. oedematiens antitoxins which were obtained from E.V. Vlasov in the Department of Wound Infections of the N.F. Gamaleia Institute of Experimental Microbiology. The antisera, which were recovered through the immunization of horses, contained 2000 antitoxin units and were kindly submitted to us by O.A. Komkov of the same department. In the electrophoretic study of these sera it was found that the γ-globulin content was about 40%.

The Cl. oedematiens antitoxins were concentrated and purified by A.P. Kuz'min by sedimentation with hydrochloric acid in the isoelectric zone after a preliminary application of sodium chloride. Further purification was accomplished by sedimentation of the antitoxins with acetone in the cold with a calculation of the tempera-

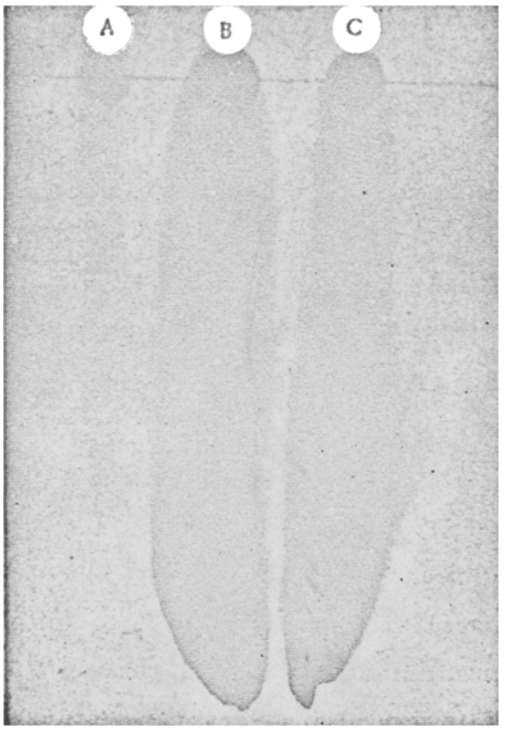

Fig. 1. Flocculation reaction on paper: A) point of application of antigen; B) point of application of antigen and immune serum; C) point of application of immune serum.

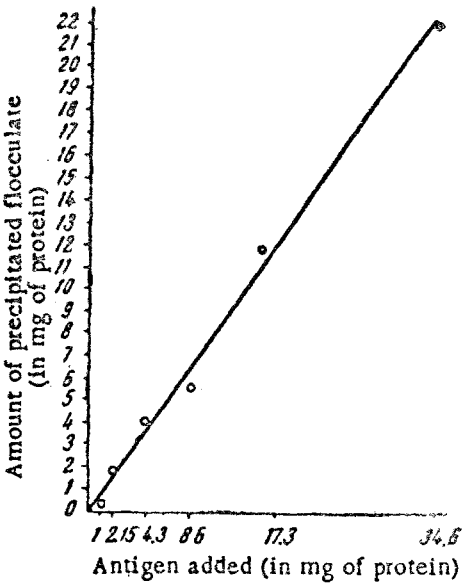

Fig. 2. Dependence between amount of antigen added and amount of precipitated flocculate.

ture, pH, and ion intensity [1]. The final result of the calculation amounted to 17 mg of protein in 1 ml.

The possibility of precipitation of a flocculate with the reacting antitoxins of Cl. oedematiens and the corresponding antisera was tested by the immunochemical method. A liberal sediment precipitated with the combination of equal volumes of antitoxin and antiserum. The relation between the amount of antigen added to the serum and the magnitude of precipitated flocculate was studied by the method of precipitation on paper [2]. These findings corresponded to the results obtained with the application of antiserum on paper discs with fixed antigen [3].

In the first series of experiments of the precipitation reaction on paper we studied the variation in the magnitude of precipitation with increasing doses of antigen added to constant volumes of antisera.

16.9 µl of immune serum was placed on several sections of chromatographic paper by means of an automatic micropipette. After the serum had dried, varying amounts of antigen were placed on the same spots. We inserted the sheet in the chromatographic chamber in such a manner that the border, where the antiserum had been applied, was immersed in a tray with veronal buffer, pH = 9.2. The buffer, flowing along the paper, carried off all the protein except the flocculate (Figure 1). The proteins from the section of paper containing the flocculate were extracted with 1 N NaOH for two hours at room temperature, the amount determined according to Lour [6], and the given quantity brought up to a volume of 1 ml.

EXPERIMENTAL RESULTS

Results of one of these experiments are presented in Figure 2. It shows that in the zone of excess antibody there existed a direct proportion between the amount of antigen introduced onto the paper and the magnitude of flocculate obtained. This proportion was maintained until all of the antibody separated out into the sediment, after which there began a diffusion of the precipitate in the excess antigen (Table 1).

In the system studied the relations between the amount of added antibody and the magnitude of sediment were entirely different from the well known system of horse serum albumin and antiserum [5]. In Figure 3 it is seen that the proportion between the magnitude of deposited sediment and the amount of added horse albumin

TABLE 1

Relation Between Amount of Antigen Added and Magnitude of Flocculate

Sample No.	Amount of antigen added in mg per ml of serum	Magnitude of flocculate	Amount of antigen*
1	8.6	6.1	–
2	17.3	10.1	–
3	34.6	20.4	18.4
4	69.2	14.9	–
5	148.4	13.6	–

*It was assumed that the precipitate contained 90% antibody [5].

was sharply changed according to the extent of increase of the amount of added antigen (Figure 3a). In contrast to this, the relation remained constant for a very broad zone of antigen concentration in the system of Cl. oedematiens antitoxin and immune serum (Figure 3b). Thus, the question arose about the relationship between the amount of antigen and antibody in the flocculate. Additional experiments were necessary to explain this.

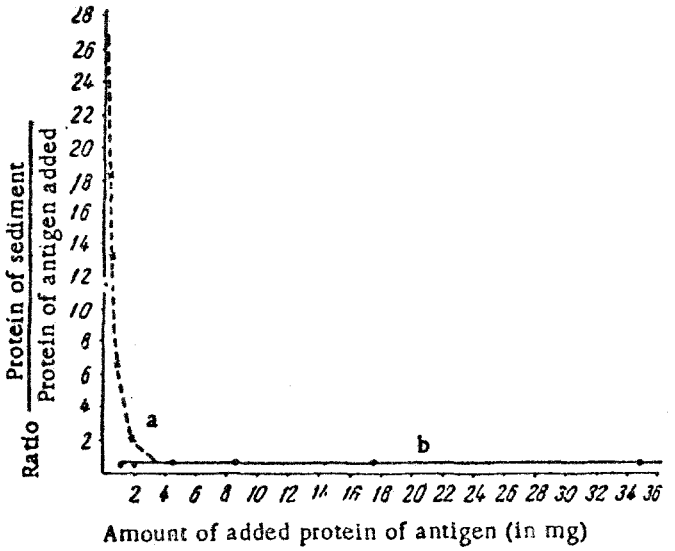

Fig. 3. Variation in the relationship between the amount of sediment and the amount of antigen added according to the degree of increased concentrations of antigen: a) system of horse albumin and immune rabbit serum; b) system of antitoxin and immune horse serum.

However, if one conditionally accepts that there is about 10% antigen contained in the flocculate, as it has been found for other systems [5], then the amount of antibody in 1 ml of the studied serum was equal to 18.4 mg.

In order to convince ourselves that we could judge the amount of antobody in the serum on the basis of our data, we tried a determination of antibody content with different dilutions of one of the immune sera at our disposal (Figure 4). It turned out that the amount of antibody was conversely proportional to the dilution of serum.

The antitoxin of Cl. oedematiens studied by us presented an intricate complex of six antigens [7]. In the study of the antigen composition of this antitoxin by the method of precipitation on agar, we observed the forma-

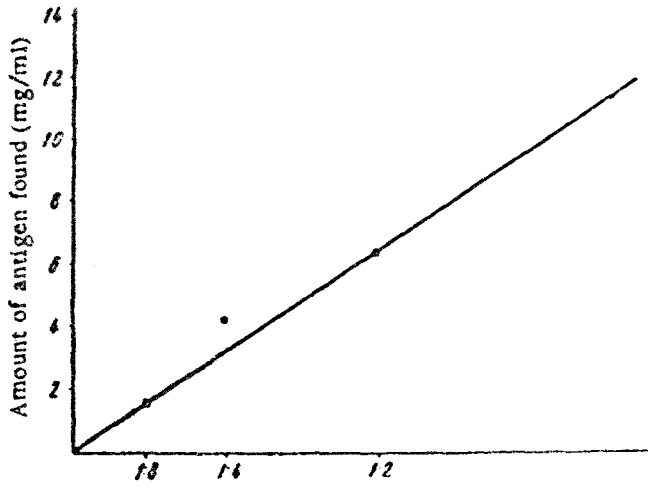

Fig. 4. Relation between amount of antibody found by described method and dilution of antiserum studied.

tion of five to seven zones of precipitation. It is possible that not all of the antibody, which was formed in the immunization of this complex mixture, precipitated in the reaction with the corresponding antigens. We applied a method of antibody determination using antigens fixed on paper [3], inasmuch as it allowed us to determine not only the amount of precipitating antibody but also all of the remaining antibody not forming a precipitate.

For this series of experiments paper discs were prepared on which Cl. oedematiens antigen was fixed chemically by an excess of alkyl halide [3]. The antigen did not lose its immunological activity with fixation in this manner. Hence, if immune serum was applied on a disc with fixed antigen, and all the unreacted protein was removed by a stream of veronal buffer, then only fixed antigen and antibody attached to it remained on the disc. Having determined the protein increment on the disc, we were able to determine the amount of antibody in the experimental serum.

It was established by this method that the amount of antibody in the serum against Cl. oedematiens consisted of 17.53 mg/ml (an average of 9 determinations).

Thus, the same amount of antibody was found by means of fixed antigens as in the determination by the method of precipitation on paper. Some discrepancies in values can be partially explained by different methods. In addition, it is possible to determine only the amount of antibody protein by the method of fixed antigen, but by the method of precipitation on paper the entire amount of protein can be determined in the sediment, where an accurate antigen-antibody relationship is unknown in the system studied. The amount of antigen in the sediment can be somewhat higher than the 10% which we obtained.

Both methods conform in their results in respect to an absence of an appreciable amount of nonreacting antibody in the studied serum. Thus, serum antibody against Cl. oedematiens by both techniques represents the actual amount of antibody. Application of chemical methods is considerably more accurate than serum titration in animals and can be accomplished in a briefer period of time.

However, one must take into account that because of the complexity of the antigen formation of Cl. oedematiens antitoxin and the presence in the serum of a number of antibodies, the over-all determination of their general amount did not allow us always to obtain a detailed characterization of the immune serum. For future investigations of a similar nature individual fractions separated from the antitoxin will be desirable to use as antigen.

SUMMARY

Reaction of flocculation between the antitoxin of Cl. oedematiens and the corresponding immune horse serum was studied with the aid of precipitation on paper. When toxin and immune serum were placed on the same

place on the paper, a flocculate appears, which unlike all the rest of the proteins is not washed off the paper by the veronal buffer. The flocculate is precipitated at the zone of considerable surplus of antitoxin. The quantity of precipitate is increased in proportion to the quantity of the added antigen up to the time when the whole quantity of the antigen is precipitated. Then the precipitate begins to dissolve in the surplus of the antigen. Data which were obtained by the method of precipitation on paper correspond to the results of determination of the quantity of the antibodies in the same serum with the aid of the antigen fixed on paper through diazoconnection and the residual haloidalkylate. This points to the possibility of replacement of the biological method of determination of the antigens of Cl. oedematiens by the immunochemical method.

LITERATURE CITED

[1] V.A. Blagoveshchenskii, I.V. Bulanova, N.V. Zakharova, M.V. Ispolatovskaia, I.P. Malorov and L.Ia. Marmalevskaia, Reports of Proceedings of Inter-Institutional Scientific Conference on the Problem of "Anaerobiasis" [in Russian] (Moscow, 1956).

[2] A.E. Gurvich, Biokhimiia 20, 5, 550 (1955).

[3] A.E. Gurvich, Biokhimiia 22, 6, 1028-1034 (1957).*

[4] V.A. Krestovnikova, O.A. Komkova, P.M. Dreitser and S.P. Zaeva, Zhur. Mikrobiol., Epidemiol., i Immunobiol. 11, 3 (1933).

[5] E. Kabat and M. Heidelberger, J. Exper. Med. 66, 2, 229 (1937).

[6] C.L. Oakley, G.H. Warrack and P.H. Clarke, J. Gen. Microbiol. 1, 1, 91 (1941).

*Original Russian pagination. See C.B. translation.