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LACK OF CORRELATION OF ANTIBODY TITERS AS DETERMINED BY THE PRECIPITIN AND HEMAGGLUTINATION TECHNIQUE¹

By

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Many different techniques are available today to aid in the detection of an antibody-antigen interaction. These generally fall into the categories of the complement fixation, precipitation hemagglutination and agar gel techniques. Furthermore, it is a common practice to utilize, for any particular system, the technique which has been demonstrated as being most sensitive to ascertain the presence of the antigen-antibody interaction.

It has been demonstrated that the BDB (antigen coupled to red blood cells through bis-diazotized benzidine) hemagglutination reaction is far more sensitive than the conventional precipitin method in detecting small quantities of antibody (1). Furthermore, it has also been shown that only bivalent or precipitating-type antibodies are capable of agglutinating antigen-sensitized red blood cells (2). One would therefore expect that a correlation should always exist between antibody titers as determined by the precipitin and by the hemagglutination techniques. During the past few years, however, various discrepancies between the results obtained with these two procedures

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have been noted in this laboratory. These findings are presented in this communication.

MATERIALS AND METHODS

Adult albino New Zealand rabbits were immunized with the following antigens: human serum albumin¹ (HSA), Bovine serum albumin¹ (BSA), ovalbumin¹ (OA), human gamma globulin¹ (HGG) pig serum gamma globulin¹ (PGG), whole rat serum (RS) and the soluble extract of a saline homogenate of rat liver (RL), prepared by means of a Potter Homogenizer. All of the serum protein antigens were administered intravenously, (one ml of a 2-5 per cent solution in saline) twice per week, for 1 to 6 injections. The rat liver antigen was administered intramuscularly, emulsified in Freund's adjuvant (1:1). Three injections were given, 10 days apart. All the rabbits were bled 8-15 days (early, precipitating antisera) and 75-125 days (late, non-precipitating antisera) after the last administration of the antigen. The antisera were stored at -10 °C till used.

The interfacial ring test was used for the detection of precipitating antibodies. Two-fold dilutions of the antisera were made in normal rabbit serum in order to maintain a constant density gradient between the antiserum and the antigen in the precipitin tube. Culture tubes, 40 mm × 3 mm were used.

The BDB hemagglutination technique (3), with slight modification (4) and the tanned cell hemagglutination technique (5) were used to determine the antibody titers of the antisera.

RESULTS

As can be seen in Table I, there was an almost absolute correlation between the agglutination titers and precipitin titers of the early precipitating antisera in that the antisera with the highest precipitin titers also displayed the highest hemagglutination titers. It should be noted that the BDB titers were not

¹ Pentex, Inc., Kankakee, Illinois, U.S.A.

TABLE I
Correlation Between The Precipitin and Hemagglutination Titers.

Rabbit Antiserum to BSA	Titer* of Antiserum determined by		
	Interfacial ring test	BDB** hemagglutination	Tanned***cell hemagglutination
1. Early antiserum	2	5,000	2,000
2. Early antiserum	5	10,000	4,000
3. Early antiserum	10	20,000	8,000
4. Early antiserum	10	20,000	8,000
5. Early antiserum	20	50,000	16,000
1' Late antiserum	0	10,000	2,000
2' Late antiserum	0	100,000	40,000
3' Late antiserum	0	40,000	8,000
4' Late antiserum	0	40,000	8,000
5' Late antiserum	0	25,000	5,000

* The maximum dilution of the antiserum capable of giving a reaction by the method used is referred to as the titer of the antiserum.

** Agglutination of red blood cells sensitized with antigen through the intermediary of bis-diazotized benzidine.

*** Agglutination of red blood cells sensitized with antigen after prior incubation of red cells with tannic acid (1:20,000).

TABLE II
Lack of Correlation Between Precipitin and Hemagglutination Titers.

Rabbit Antiserum to BSA	Titer* of Antiserum determined by		
	Interfacial ring test	BDB** hemagglutination	Tanned***cell hemagglutination
6.	5	10,000	2,000
7.	5	30,000	6,000
8.	5	5,000	1,000
9.	10	160	50
10.	10	320	200

* Legend same as for Table I.

** Legend same as for Table I.

*** Legend same as for Table I.

TABLE III
Relation of Hemagglutination Titer with the Type of Antigen Used.

Rabbit Antiserum to		Titer* of Antiserum determined by		
		Interfacial ring test	BDB** hemagglutination	Tanned***cell hemagglutination
BSA	11.	2	5,000	2,000
	12.	5	10,000	4,000
	13.	10	20,000	8,000
OA	14.	2	20,000	5,000
	15.	5	40,000	10,000
	16.	10	80,000	20,000
HGG	17.	2	300	150
	18.	5	600	300
	19.	10	1,200	600

* Legend same as for Table I.

** Legend same as for Table I.

*** Legend same as for Table I.

TABLE IV
Lack of Correlation Between BDB and Tanned Cell Hemagglutination Titers.

Rabbit Antiserum to	BDB** hemagglutination	Tanned*** cell hemagglutination
BSA	10,000	100,000
OA	10,000	30,000
HGG	20,000	1,000
PGG	5,000	160
Rat liver homogenate	0	50,000
Whole rat serum	0	10,000

* Legend same as for Table I.

** Legend same as for Table I.

*** Legend same as for Table I.

identical to the tanned cell titers but they were generally higher throughout the series. The late, non-precipitating antisera possessed higher hemagglutination titers, generally, than the early precipitating antisera (Table I). Furthermore, as can be seen from Table II, several antisera with relatively high precipitin titers displayed very low hemagglutination titers.

It was also determined that a different scale of hemagglutination titers exists for different antigen-antibody systems (Table III), even though the antisera may display similar precipitin titers when tested with their respective antigens. A most unexpected sequence of results is presented in Table IV, where it can be seen that, in two cases, one of the two hemagglutination procedures, the BDB technique, completely failed to detect antibodies in the antisera, which were identified in high titer by the tanned cell technique.

DISCUSSION

Although the hemagglutination procedures have been accepted as valid methods for the detection of antibody, the question as to whether they detect the same antibody(s) as does the precipitin test has not been adequately answered. It has been partially answered in that Gyenes and Schon (2) have recently demonstrated that only the bivalent antibody fragments possessed the capacity to agglutinate sensitized red blood cells. The papain hydrolysate of the antiserum, which contained only univalent antibody fragments, could no longer precipitate with the antigen, nor could it agglutinate sensitized red cells. These antibody fragments could, however, specifically inhibit the agglutination between the sensitized red blood cells and unadulterated antiserum, by prior incubation of the sensitized red cells with the fragmented antibodies. Furthermore, *Roberts* and *Haurowitz* (1) have demonstrated, using radioactively labelled antigen and antisera, that as little as $9 \times 10^{-3} \mu\text{g}$ of antibody could be detected by the BDB method, which is hundreds of times less than could be detected by the precipitin technique. Therefore, one should always obtain agglutination with antisera which give good precipitin tests. However, one would not expect antisera which give good precipitation tests to exhibit negative hemagglutination.

The data presented in this communication suggest that, in general, with a series of antisera directed toward the same antigen, a correlation will usually exist between the precipitin and

agglutination titers. However, with a minority of antisera, these two techniques may indeed detect different types of antibodies or antibodies directed toward antigens different from those with which the antibodies react by the precipitin test. This conclusion is based partly on the demonstration that the precipitin titers of a series of early precipitating antisera did not correlate with their hemagglutinating titers. Furthermore, although the late, non precipitating antiserum specimens did not give positive interfacial ring tests, they invariably possessed hemagglutinating antibody titers as high as, and often higher than, their early precipitating counterparts (Table I) (6,7). A similar observation concerning the lack of correlation between precipitating and agglutinating antibody titers has been reported by *Fiset* (8). Precipitating antisera were absorbed with the antigen in a step-wise fashion to the point that precipitating antibody could no longer be detected. However, these "absorbed" antisera were found to co-precipitate upon the addition of the specific antigen and homologous precipitating antisera although they were negative by the tanned cell method. This findings was interpreted by the author as evidence for the existence of "univalent" antibody, which would be unable to agglutinate sensitized cells (2) but which would be capable of co-precipitating with homologous antigen-antibody complexes. However, the author made no attempt to explain why, during the absorption procedure, the antigen should selectively react with only the precipitating antibodies and not the "univalent", non-precipitating antibodies. It is highly probable that what the author was actually detecting were not "univalent" non-precipitating antibodies, but rather, soluble antigen-antibody complexes. These latter would, of course, co-precipitate with homologous antigen and precipitating antibody but would not agglutinate sensitized red cells, since they were prepared in antigen excess.

The findings presented in Table I could, theoretically, be explained on the basis of the following suppositions:

1. The antibodies in the early, precipitating antisera are different from those in the late, non-precipitating antisera, not

only on the basis of their physico-chemical properties, but also on the basis of a different immunological specificity.

2. None of the antigens used are pure, homogeneous "single antigen" systems. They are all heterogenous, antigenically, if not physico-chemically. It is therefore possible that some rabbits respond with antibody formation directed toward antigenic constituents of the antigen system which do not adhere or couple to the red blood cell, or do so minimally. Under these circumstances, the antiserum will give a positive interfacial ring test but may not agglutinate the sensitized red blood cells. This may be the explanation for the findings in Table II. The reasons why the antigen(s) may not adhere to the cell surface may be as follows:

- (a) It may be present in low concentration relative to the other proteins in the antigen system and therefore cannot optimally sensitize the red blood cells, using either the BDB or the tanned cell method, due to competition of the antigens.
- (b) The antigen(s) in question may be deficient in tyrosine groups, and therefore would not be capable of sensitizing red blood cells using the BDB technique, which requires the presence of tyrosine groups.

However, it should be possible to sensitize red cells by the tanned cell method with such an antigen since this method does not require the presence of tyrosine groups as a prerequisite to its utilization.

- (c) The antigen in question may be a carbohydrate or lipid constituent of the antigen system, easily dissociable from its linkage to protein molecules and as such could not sensitize red blood cells by the BDB technique. However, here again, it should be possible to sensitize tanned red blood cells with such an antigen.

Insofar as supposition No. 1 is concerned, it has been demonstrated, by means of the reversed BDB technique (9,10), that both the precipitating and non-precipitating antibodies,

observed in the same rabbit bled at different intervals of time, are directed toward identical antigenic sites of the antigen system (11). They therefore possess the same immunologic specificity.

Insofar as supposition No. 2 concerned, it would appear that explanation (a) is the most logical to explain these findings, since it has been verified experimentally in our laboratory using two non-cross-reacting systems. It was demonstrated that rabbit anti-BSA will agglutinate red cells sensitized with BSA but not if the BSA had been mixed with a heterologous protein prior to the sensitization, even though the absolute concentration of the BSA was kept constant (12). This explanation is supported by the results presented in Table IV. Although the antisera to the various protein antigens possessed similar precipitin titers, their hemagglutination titers varied markedly. If one accepts the thesis that all of the antigens used were heterogeneous, antigenically, and that optimal sensitization of the red cells necessitates a minimal concentration of that particular antigen in the antigen complex, one could explain the results obtained on the basis that the component in the antigen system present in highest concentration need not be the best antigen in that system. Therefore, antibody titers using BSA sensitized cells (anti-BSA antiserum) will not be identical to those using HGG sensitized cells (anti-HGG antiserum), even though both preparations of red blood cells are optimally sensitized with the respective antigens and the precipitin titers of the respective antisera are identical. It would therefore appear that, insofar as any particular antiserum specimen is considered, it would be wise to deploy both the BDB and tanned cell procedures, as well as the precipitin test, before stating with any degree of assurance that antibody is not present in the specimen. The data presented in Table IV support the suggestion that both hemagglutination techniques should be attempted with each antibody-antigen system. With several of the antigens (HGG and PGG), the BDB method was by far superior to the tanned cell method for the demonstration of antibody, whereas the converse was true for two other antigen

systems (rat liver homogenate and whole rat serum). Thus, one cannot, with any degree of certainty, decide beforehand which of these two hemagglutination procedures will best serve as the detector system for the presence of antibodies.

Several other observations concerning the efficacy of the two hemagglutination techniques deserve to be mentioned. Firstly, for any particular system, the BDB technique was found to impart a higher antibody titer to the antiserum than did the tanned cell method. This finding is probably due to the fact that antigen is constantly being liberated from the sensitized tanned red cell (13) and is thus capable of reacting with and neutralizing small quantities of antibodies in solution. Such a state of affairs does not transpire with the BDB technique in which the antigen is chemically bound to the red cell stroma (1).

Secondly, it has also been noted that, with certain antigen-antibody systems *i.e.* tetanus toxoid – anti-tetanus toxoid antiserum, a prozone of non-agglutinated sensitized cells may occur, extending over a series of 8–12 tubes before a positive agglutination pattern will be observed (14). This is not a property of the antiserum since similar results were obtained with rabbit and horse antisera. A similar observation of a prozone effect has been reported by *Kaplan & Jandl* (15) using the rheumatoid factor-gamma-globulin system.

SUMMARY

1. With most precipitating antisera (rabbit), the hemagglutination techniques are about 1000 times more sensitive than the precipitation methods for the detection of antibody.
2. With a proportion of antisera, there is no correlation between the precipitin and hemagglutination, both BDB and tanned cell, titers.
3. Antisera obtained from rabbits several months after immunization will not precipitate with the antigen but will give a high titer by either of the hemagglutination tests used.

4. Using highly heterogeneous antigen systems, one of the hemagglutination tests may give completely negative results, whereas the other may give a high antibody titer.

REFERENCES

1. *Roberts, A. N. & Haurowitz, F.*: Quantitative Studies on the Bis-Diazotized Benzidine Method of Hemagglutination. *J. Immunol.* 89, 348, 1962.
2. *Gyenes, L. & Schon, A. H.*: The Mechanism of the Hemagglutination Reaction. *J. Immunol.* 89, 483, 1962.
3. *Pressman, D., Campbell, D. H. & Pauling, L.*: The Agglutination of Intact Azo-Erythrocytes by Antisera Homologous to the Attached Groups. *J. Immunol.* 44, 101, 1942.
4. *Stavitsky, A. B. & Arquilla, E. R.*: Micromethods for the Study of Proteins and Antibodies. III. Procedures and Application of Hemagglutination-Inhibition Reactions with Bis-Diazotized Benzidine and Protein-Conjugated Red Blood Cells. *J. Immunol.* 74, 306, 1955.
5. *Boyden, S. V.*: The Absorption of Proteins on Erythrocytes Treated with Tannic Acid and Subsequent Hemagglutination by Antiprotein Sera. *J. Exp. Med.* 93, 107, 1951.
6. *Richter, M. & Haurowitz, F. H.*: Continuous Synthesis Antibody After Primary Immunization with Protein Antigens. *J. Immunol.* 84, 420, 1960.
7. *Blumer, H., Richter, M., Cua-Lim, F. & Rose, B.*: Precipitating and Non-Precipitating Antibodies in the Primary and Secondary Immune Responses, Their Rate of Decline, Anaphylaxis Sensitizing Capacity and the Effect of Cortisone. *Canad. J. Biochem. and Physiol.* 40, 105, 1962.
8. *Fiset, L. G.*: Non-Precipitating Rabbit Antibodies: A Study of their Reactivity in vitro by Means of the Quantitative Precipitin and Tannic Acid Hemagglutination Techniques. *J. Immunol.* 8, 580, 1963.
9. *Cua-Lim, F., Richter, M. & Rose, B.*: The Reversed BDB Technique. The Agglutination of Antibody-Coated Red Blood Cells by Antigen. *J. Allergy* 34, 142, 1963.
10. *Richter, M., Cua-Lim, F. & Rose, B.*: The Agglutination of Antibody-Coated Red Cells by Antigen. *Biochem. and Biophys. Res. Comm.* 7, 241, 1962.
11. *Richter, M., Cua-Lim, F. & Rose, B.*: The Identity of Immunological Specificity of Both Precipitating and Non-Precipitating Antibodies. *Int. Arch. Allergy* 23, 115, 1963.
12. *Richter, M. & Cohen, J. J.*: The Role of Antigen Concentration in the BDB Hemagglutination Reaction. - To be submitted for publication.
13. *Feinberg, R. T. & Flick, J. A.*: Elution of Pollen Antigens from Tannic Acid-Treated Erythrocytes. *Proc. Soc. Exp. Biol. and Med.* 96, 71, 1957.
14. *Cohen, J. J. & Richter, M.*: (Unpublished results).
15. *Kaplan, M. E. & Jandl, J. H.*: The Effect of Rheumatoid Factors and of Antiglobulins on Immune Hemolysis in Vivo. *J. Exp. Med.* 117, 105, 1963.

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