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Comparison of Three Hemolytic Plaque Assays Using Sheep and Mouse Erythrocytes as Antigens in Rats (39021)

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The formation of complement-dependent hemolytic plaques observed in a suspension of erythrocytes in agar, initially described by Jerne and Nordin (1), is a widely used method of detecting specific antibody production by lymphoid cells *in vitro*. This technique has been modified extensively and also is employed with a variety of antigens that can be covalently bound to indicator erythrocytes. Thus, antibody to polysaccharides (2), synthetic polymers (3), proteins (4) including immunoglobulins (5, 6), and haptens (7) can be measured.

Modifications of the original technique include the use of carboxymethoxycellulose (CMC) on slides without agar (8), free suspension of cells and erythrocytes in a slide technique (9), and the electrostatic binding of erythrocytes to polystyrene plastic without an immobilizing agent such as agar or CMC (10). Techniques that do not employ agar are considered to be more sensitive in detecting *in vitro* antibody production by isolated lymphoid cells (9, 10).

Hubner and Gengozian (11) showed that the maximal number of plaques that can be observed depends not only on the source of complement but also on the source of the erythrocytes used as target cells even in the classical Jerne–Nordin procedure.

The purpose of the present study was to compare the sensitivity of several hemolytic assay systems, using lymphoid cells isolated from the spleens of immunized rats. These systems include those which employ a stabilizing agent such as agarose and those which do not.

Materials and methods. Animals. Male and female COBS-CD strain rats (Charles River Breeding Lab., Cambridge, Mass.) were used at 4 to 6 months of age. $C_3 1F_1$ (C_3H/Anf Cum ♀ × 101 Cum♂ mice were obtained from Cumberland View Farms, Clinton, Tenn.

Antigens. Sheep erythrocytes (Srbc) were obtained from Baltimore Biological Lab. (Cockeysville, Md.) in Alsever's solution and stored at 4°. Mouse blood was collected from the aortic arch of mice after severing the neck with a pair of curved scissors. After collection in Alsever's solution, erythrocytes (Mrbc) were washed twice in Eisen's B solution, pH 7.5 (12), by alternate centrifugation and resuspension. Finally they were suspended in Eisen's B solution for use. Mrbc were routinely used within 4 hr of collection owing to their relative instabilty with storage. Rats were immunized with one ml of a 10% suspension of rbc given ip. The animals were killed 3 to 12 days after injection and the spleens removed.

Reagents. Rabbit anti-rat IgG (Miles Lab., Kankakee, Ill.) was heated for 30 min at 56° before each experiment in which it was used. Lyophilized guinea pig serum (Colorado Serum Co., Denver, Colo.) was reconstituted and used undiluted or at a dilution of 1:10 as a source of complement. The diluent was Hank's Balanced Salt Solution (HBSS) or Eisen's B, as indicated. Human blood, type AB RhD+, was obtained fresh from individual donors. Serum was removed after centrifugation of cells and stored at −70°. This serum was used undiluted or at a dilution of 1:7 as a source of complement. Prior to each assay, 5 vol of serum was absorbed with one vol of packed erythrocytes (mouse or sheep) at 0° for 30 min. Agarose was electrophoretic grade obtained from Bio-Rad (Richmond, Calif.) or General Biochemical (Chagrin Falls, Ohio).

Spleen cell preparation. The spleen was gently teased with Stille forceps into HBSS to give a suspension that was then aspirated several times through an ⅋ 18 gauge needle fitted to a 10 ml syringe. The suspension then was filtered through several layers of cheesecloth into 20 vol of ice-cold HBSS. The cells were washed by alternate centrifugation and suspension in HBSS and finally viability was

determined by the Trypan Blue dye exclusion test.

Direct hemolytic plaque assays. A. Modified Jerne–Nordin. An adaptation of the Jerne–Nordin asay of Yamada and Yamada (13), as modified by Duke and Harshman (14) was employed. In brief, a rbc-spleen cell-agarose suspension was dropped by pipette onto the surface of a plastic petri dish. Sixteen distinct drops/ml were deposited in which the final concentration of agarose was 0.35%. After solidification and incubation for 2 hr at 37°, each drop was flooded with complement. The plates were incubated for an additional hour and then the complement was decanted.

B. Kennedy–Axelrad assay (10). Two ml of 0.025 mg/ml of poly-L-lysine (mol wt of 30,000–70,000, Miles Lab.) in phosphate-buffered saline (PBS) was added to a single Falcon 60 × 15 mm polystyrene tissue culture dish. After 15 min, the dishes were drained and gently rinsed with PBS. Two ml of a 1% erythrocyte suspension in PBS then was added to each dish and allowed to settle and fix to the surface for 30 min while the dishes were held on a leveled, horizontal glass plate. Following incubation, the dishes were rocked gently several times and rinsed by immersion in PBS. A mixture of 2ml of a spleen cell suspension and 0.2 ml of complement was overlaid on the erythrocyte layer. The dishes were incubated 45 min at 37° on the leveled glass plate, then decanted and gently rinsed by immersion in a beaker of cold PBS.

C. Plotz et al. assay (15). This procedure is an adaptation of the technique used by Urso and Gengozian (16). Briefly, 0.05 ml of spleen cells suspension in HBSS were added to 0.05 ml of a 10% erythrocyte suspension and 0.5 ml 0.5% agarose in 0.15 *M* NaCl solution or Eisen's B solution. The mixture was poured over the surface of a 75 × 25 mm microscopic slide. Before use, each slide was immersed in 0.5% agarose and wiped with tissue paper to leave a thin film of agarose which facilitates the fixation of the mixture. After solidification, each slide was inverted and supported on each end such that the agarose layer was suspended above a chamber approximately one mm thick. Complement was added to the chamber by capillary action. The slides were incubated for 2 hr at 37° in a humidified box.

All plaques were counted under a stereoscope at 15X.

Indirect hemolytic plaque assay. The technique of Kennedy–Axelrad was used with the following modifications. After enumeration of direct plaques, 2 ml of a dilution of anti-rat IgG was added together with 0.2 ml undiluted guinea pig or human serum. The plates were incubated 30 min at 37° and examined for further plaque development.

Results. The first parameter examined was the source of complement in the modified Jerne–Nordin assay. As shown in Table I, guinea pig serum is slightly more effective in plaque development than is human serum when Srbc are the target cells. On the other hand, human complement appears to be more effective for plaque development when Mrbc are used as target cells. As shown in Table I, more than a two-fold increase in numbers of plaques was observed with human complement over that seen with guinea pig complement when Mrbc were used as target cells. This increase was found consistently with several donors.

The modified Jerne–Nordin assay provides detection of small plaques that may not be observed in the poured plate technique since the modification is made by dropping the cell and erythrocyte mixture onto the surface of a Petri dish. The drop spreads on the surface before solidification giving a film of a few mm at the thickest point. All plaques are easily discernible with a stereoscope, which is not true with the poured plate procedure. Agarose as the immobilizing agent in this technique does stabilize Mrbc and suspensions are readily dispersed in the mixture.

Plaque development was also examined with the Kennedy–Axelrad procedure. This technique is based on the principle of electrostatic absorption of erythrocytes to poly-L-lysine fixed to a plastic surface. It was found that tissue culture polystyrene plastic dishes were necessary for optimal absorption. Prior to treatment with poly-L-lyine, glass wool wiped over the surface, or glass beads rolled in the dish may provide a more negative charge for electrostatic interaction with poly-L-lysine. Erythrocytes under these con-

TABLE I. EFFECT OF DIFFERENT COMPLEMENT SOURCES ON THE FORMATION OF HEMOLYTIC PLAQUES BY THE MODIFIED JERNE-NORDIN METHOD.

Spleen cells from rats immunized[a] with:	Target rbc	Complement	PFC/10^6	Range
Srbc	Sheep	Guinea pig	192	153–224 (17)[b]
	Sheep	Human (KBB)	162	126–205 (24)
	Mouse	Human (KBB)	0.73	0–1 (19)
Mrbc	Mouse	Guinea pig	91	57–107 (28)
	Mouse	Human (KBB)	193	173–216 (12)
Nonimmunized	Sheep	Guinea pig	2.9	0–7 (22)
	Mouse	Human (KBB)	0.75	0–2 (31)

[a] Spleen cells taken from rats immunized with 1 ml of a 10% suspension of either mouse or sheep erythrocytes 5 days before use.

[b] Numbers in parentheses indicate repetitions.

ditions formed a complete monolayer over the surface of the dish.

This assay was 15- to 25-fold more sensitive than the Jerne–Nordin technique when Srbc were used as target cells. Typical results are presented in Table II. In contrast to the findings noted above, in this technique human complement (donor RM) was more effective than guinea pig complement. However, not every source of human complement was equally effective. Complement from donor JS gave numbers of plaques comparable to guinea pig complement (318 versus 339) and less than was found with human donor RM (561).

The Kennedy-Axelrad assay is also more sensitive for detection of plaques when Mrbc are the target cells (Table II). The increased sensitivity was only about two-fold and human complement was consistently better than guinea pig complement. Although the sensitivity of this assay for Mrbc was evident, some clumping of the erythrocytes occurred when the monolayer was prepared. This clumping did not interfere with plaque development although it was more difficult to count them. A plaque could be distinguished under the stereoscope and some variation in size was noted.

TABLE II. EFFECT OF DIFFERENT COMPLEMENT SOURCES ON THE FORMATION OF HEMOLYTIC PLAQUES BY THE KENNEDY–AXELRAD METHOD.

Spleen cells from rats immunized with:	Target rbc	Complement	PFC/10^5	Range
Srbc	Sheep	Human (RM)	561	515–674
		Human (JS)	318	259–365
		Guinea pig	339	329–357
		Inactivated human (RM)	3.3	3–4
		None	1.8	0.3
Mrbc	Mouse	Human (P.N.)	42	41–43
		Guinea pig	26	18–33

This technique presents several problems which require added attention when plaques are evaluated. These include: (1) mouse erythrocytes tend to clump; (2) if the plates are not leveled, there tends to be unequal distribution of erythrocytes, or unequal distribution of plaques; (3) the plates must be left undisturbed since the cells are in free suspension; (4) following incubation, the cells and fluids are decanted so that cells are not present within plaques when observed; and (5) plaque size varies probably owing to the varying proximity of the secreting cell to the monolayer.

The third technique evaluated was that of Plotz *et al.* Preliminary results indicated that Eisen's B solution was superior to HBSS as diluent; thus, it was used throughout this technique. Since it was also noted that guinea pig complement was consistently good for plaque development with Srbc, this was the only complement source used with Srbc (Table III). However, human complement appeared to be superior to guinea pig complement in the Mrbc system and it was therefore used exclusively for this system in the Plotz technique. These results are presented in Table III.

TABLE III. EFFECT OF DIFFERENT COMPLEMENT SOURCES ON THE FORMATION OF HEMOLYTIC PLAQUES BY THE PLOTZ *et al.* METHOD.

Spleen cells from rats immunized with:	Target rbc	Complement	PFC/10^5	Range
Srbc	Sheep[a]	Guinea pig	221	198–245
	Sheep[b]	Guinea pig	160	143–177
Mrbc	Mouse[a]	Human (P.N.)	120	116–124
		Guinea pig	32	22–38

[a] Target rbc suspended in Eisen's B solution.
[b] Target rbc suspended in HBSS.

TABLE IV. INDIRECT KENNEDY–AXELRAD ASSAY WITH SPLEEN CELLS TAKEN FROM RATS 5 DAYS AFTER INJECTION WITH Srbc.

Anti-IgG added dilution	Direct plaques PFC/10^5	Indirect PFC/10^5	% Total plaques developed by added serum
1:30	164	8	6
1:60	153	7	7
1:100	148	0	0
11 days after injection with Srbc			
1:60	20	49	67
1:100	16	41	70
Normal serum added 1:60	21	0	0

The increased sensitivity and size variation of plaques in the Kennedy–Axelrad assay for detection of antibody producing cells to Srbc, suggested that cells producing IgG might be responsible for the increased number of plaques observed. IgG producing cells are normally detected by adding anti-IgG serum to augment complement activation needed for erythrocyte lysis with IgG. This suggestion was evaluated by adding anti-IgG globulin in a second incubation. These results are presented in Table IV. It is evident that when spleen cells were prepared at 5 days after injection of antigen, very few plaques were of the indirect (IgG) variety.

TABLE V. KENNEDY–AXELRAD ASSAY WITH SPLEEN CELLS TAKEN FROM RATS 5 DAYS AFTER INJECTION WITH Srbc: ANTI-IgG ADDED PRIOR TO DIRECT PLAQUE PRODUCTION.

Anti-IgG added	Direct PFC/10^5	Range	Reduction (%)
None	749	600–857	
1:30	293	256–320	60
1:60	748	690–793	0

TABLE VI. SUMMARY OF PREFERRED CONDITIONS FOR HEMOLYTIC PLAQUE DEVELOPMENT.

Method	Immunizing cell type	Target cell	Complement	PFC/10^6
Jerne–Nordin	Srbc	Sheep	Guinea pig	192
	Mrbc	Mouse	Human	193
Kennedy–Axelrad	Srbc	Sheep	Guinea pig	3390
	Mrbc	Mouse	Human	420
Plotz *et al.*	Srbc	Sheep	Guinea pig	2210
		Mouse		
	Mrbc		Human	1200

When cells were prepared at 11 days after injection of antigen, the majority of plaques were detected only after addition of anti-IgG. It may be tentatively concluded that the increased sensitivity of the assay was not due to indirect plaques being detected as direct plaques.

Anti-IgG was added in the initial incubation period of the Kennedy–Axelrad technique (Table V) and it was found that the reagent actually reduced the number of direct plaques (60% reduction with a 1:30 dilution of anti-IgG globulin).

Discussion. This study was undertaken to compare three hemolytic plaque assay systems in current use and to recommend the appropriate procedure for specific conditions. Erythrocytes from sheep and mice were used as antigens. Although the original plaque assay employed Srbc as antigen, erythrocytes from a variety of species including goat, mouse, and chicken have been used in adaptations of the technique. On the basis of these studies, summarized in Table VI, it would appear that the Kennedy–Axelrad technique is the most sensitive when using Srbc.

However, in contrast, the Plotz technique appears to be the most sensitive method for evaluating the response to Mrbc. In addition, the use of human complement is preferred with Mrbc whereas, guinea pig or human complement give comparable results when studying the immune response to Srbc. Thus, results in which hemolytic plaques have been developed solely by the addition of guinea pig complement may be questioned. Hubner and Gengozian (11) also showed that plaque size and number are contingent on the source of complement and further, concluded that one species does not respond as well to erythrocyte antigens as another.

Our results show that depending upon the *in vitro* techniques to evaluate the immune response of an animal, the qualitative and quantitative data may not actually reflect the *in vivo* response. Data collected with spleen cells of rats with Mrbc as antigen and guinea pig complement suggest a low response when compared to that with Srbc as antigen although the titrations of antisera are equivalent (data not presented). This lowered *in vitro* response is misleading since the total number of plaques that can be demonstrated with human complement is similar to that seen with Srbc and guinea pig complement. Rats than do respond equally well to Mrbc and Srbc in the plaque assay when the appropriate complement is used.

Although the Kennedy–Axelrad and Plotz techniques appear to be more sensitive for the sheep and mouse systems respectively, it should be noted that the Jerne–Nordin technique gave comparable results with both erythrocyte systems and thus may be preferred when making comparative studies.

Although agar was used in the hemolytic plaque assay developed by Jerne and Nordin (1), agar does have anticomplementary activity (6, 11). Agarose, a purified agar, has had the anticomplementary polysaccharide removed during preparation and is preferred as the stabilizing agent.

In both methods that employed agarose, human complement is more effective than guinea pig complement. The reason for this is not clear although guinea pig complement is more dependent upon the concentration of Mg^{2+} and Ca^{2+} than human complement (6) and optimal levels of these cations for fixation of human complement to Mrbc may be present.

The variation in plaque size in the Kennedy–Axelrad assay is of interest. The absence of agarose in the method would allow antibody to diffuse a greater distance from the surface of a secreting lymphoid cell. Diffusion would result in larger plaques if the lymphoid cell is in direct contact with the erythrocyte monolayer. Since the cells are in free suspension above the monolayer, however, the zone of antibody enveloping a cell would be in varying depth in relation to the position of the cell to the monolayer. Thus, the cells closest to the erythrocytes at the beginning of incubation should produce larger plaques than cells further from the monolayer. Overall, this would result in a diversity of plaque sizes.

A more plausible explanation may be suggested for the results found in the Kennedy-Axelrad assay. The increased numbers of plaques may be the smaller variety that are not observable in assay systems that utilize agarose. Such plaques could be the result of lymphoid cells that produce either decreased amounts of antibody or antibody of low affinity.

The increased number of plaques observed with the Kennedy–Axelrad procedure does not appear to be due to cells producing IgG, usually detected by adding anti-IgG serum. In fact, the addition of such antiserum inhibited plaque formation (direct plaques). This observation is similar to reports by others who showed a reduction in numbers of plaques by addition of anti-immunoglobulin (17, 18) to spleen cells obtained 4–6 days after injection of antigen although increases are noted when the addition of anti-IgG is made to cells obtained later during immunization (18).

Summary. Several hemolytic plaque assays using spleen cells from rats immunized with sheep or mouse erythrocytes were compared. Modifications of the assays were made to define optimal conditions. Mrbc in assays with guinea pig complement yield a much reduced number of plaques than is observed when human complement is used. In con-

trast, no difference in numbers is noted with Srbc regardless of complement source.

The use of Srbc in an assay technique that does not employ agarose is more sensitive than techniques that require agarose; the result is a 50–100% increase in the number of plaques. This increase in plaque number was observed with either guinea pig or human complement. In contrast, there was considerable clumping of Mrbc in the absence of agarose which tended to obscure distinct plaque formation. Increased sensitivity of plaque development for Srbc in the absence of agarose was due to increased numbers of direct (IgM) plaque detection and not to concomitant detection of cells producing IgG (indirect plaques).

Maximal detection of plaques with Mrbc was observed when human complement was used in assays containing agarose. These assays gave a threefold increase in plaque numbers when compared with assays without agarose.

It is evident from the above that the most sensitive and reproducible hemolytic plaque assay to be used by an investigator will depend primarily upon the antigen, and on reagents selected for the test.

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