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Origin of Antibody-Dependent, Lymphoid-Cell-Independent Hemolytic Plaques in Rabbit Spleen Cell Cultures[1] (39095)

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(Introduced by S. G. Bradley)

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The *in vitro* plaque forming cell (PFC) response of dispersed rabbit spleen cells (SC) to sheep erythrocytes (SE) has been shown to require the presence of normal rabbit serum (NRS) (1). In view of the high frequency of background anti-SE antibody activity in NRS (2), it is conceivable that such antibody might play an important role in the initiation of the antibody response (3, 4, 5). While the data to be presented do not definitively resolve this issue, the study uncovered a phenomenon which may be a significant source of error in the interpretation of hemolytic plaque assays of lymphocyte cultures. As shown in this report, antibody from the normal serum in the culture becomes attached to the SE that serve as the antigenic stimulus and is the cause of spurious hemolytic plaques developed independent of lymphoid cells in the assay.

Materials and methods. Adult female New Zealand white rabbits, obtained from the NIH rodent and Rabbit Production Section, were used throughout. These animals were also the source of sterile normal rabbit serum (NRS). Fetal bovine serum (FBS) was obtained from either Gray Industries, Inc., Ft. Lauderdale, Fla., Reheis Chemical Co., Kankakee, Ill., or Microbiological Associates, Bethesda, Md.

Rabbits were exsanguinated by cardiac puncture, spleens were obtained aseptically and immersed immediately in Hanks Balanced Salt Solution (HBSS). The spleens were gently teased apart. Single cell suspensions were obtained from the supernatant fluids of the teased suspensions after a brief settling period. The cells were washed appropriately by centrifuging and resuspending the cell pellets in culture medium. Cell counting was done in the Autocytometer-II electronic cell counter (Fisher Scientific, Pittsburgh, Pa.). One milliliter cell cultures were performed as described (1). The cultured cells were harvested and assayed for hemolytic plaques as previously detailed (6) except that microscope slides were substituted for Petri dishes. Plaques were counted under a dark-field stereomicroscope (Olympus Optical Co., Tokyo, Japan) at 7–15X.

Antibody titers of NRS and culture fluids were determined by standard methods of microhemagglutination, using tris buffered saline (TBS) (6) containing 0.2% rabbit serum albumen as diluent.

Sheep erythrocytes (SE) were prepared in either HBSS or TBS as described (6, 7). Rabbit erythrocytes (RE), chicken erythrocytes (CE), and bovine erythrocytes (BE) were prepared similarly.

Actinomycin D (Calbiochem, La Jolla, Calif.) in HBSS was used as an inhibitor of RNA synthesis. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) in HBSS was used as an inhibitor of protein synthesis.

Results. Relationships between rabbit sera used as a culture supplement and hemolytic antibody plaques obtained from rabbit spleen cell cultures. Large numbers of plaques were consistently obtained when SE-inoculated cultures of cells from normal rabbits were supplemented with certain normal rabbit sera, and assayed after 3–6 days in culture (Table I). Plaque numbers were in proportion to the amount of culture suspension

[1] Part of this investigation was presented at the annual meeting of the *Federation of American Societies for Experimental Biology*, April 15–20, 1973, Atlantic City, N. J.

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TABLE I. ENUMERATION OF PLAQUES PRODUCED IN RABBIT SPLEEN CELL CULTURES SUPPLEMENTED WITH SELECTED NORMAL RABBIT SERA

Serum number	Absorbed with SE	Culture inoculated with SE	Amount of culture plated (ml)	Plaques developed/ml	Plaques per culture[a]
A30	No	No	0.1	3	42
A30	No	Yes	0.05	4083	114,324
			0.01	962	134,680
			0.005	471	131,880
A30	Yes	Yes	0.1	4	56
A32	No	Yes	0.1	2	28

[a] Equal to plaques/1.4 ml. Cultures were seeded at 1.0 ml/culture and were increased to 1.4 ml by the daily addition of nutrients.

plated. Uninoculated cultures produced very few plaques. This result was obtained with some sera but not with other sera (Table I; see also A50, Table II). Furthermore, some sera were inhibitory and had the capacity to suppress the plaque promoting ability of other sera (e.g., A43 with A30, Table II). Active, noninhibitory sera were capable of conferring apparent PFC initiation upon cells from any rabbit tested. The plaque producing capacities as well as the inhibitory capacities of respective sera could be removed by preabsorption with SE prior to the addition of such sera to the media (e.g., A30 with absorbed A43, Table II). Neither capacity was removed by preadsorption with bovine erythrocytes (BE) or with chicken erythrocytes (CE). There was no obvious relationship between the amount of antibody contained in a serum (titer) and ability to support the appearance of plaques in large numbers. It was clear, nevertheless, that removal of anti-SE agglutinins by absorption resulted in concurrent removal of the ability of a serum to support the appearance of plaques in large numbers (Tables I and II).

Origin of hemolytic antibody plaques in spleen cell cultures supplemented with normal rabbit serum. Summarized in Table III are the results of experiments which individually and collectively demonstrate the fact that NRS that appears to support a vigorous primary-type PFC response to SE in spleen cell cultures actually has a different role. The data show that metabolic inhibitors used in excess

TABLE II. EFFECT OF MIXING[a] EFFECTIVE AND INEFFECTIVE NORMAL RABBIT SERA FOR CULTURE SUPPLEMENTATION ON THE NUMBER OF PLAQUES PRODUCED IN RABBIT SPLEEN CELL CULTURES

Serum	Serum HA[b] titer^{-1}	Absorbed with SE	Plaques per culture
A30	16	No	$> 10^5$
A43	16	No	$< 10^2$
A50	16	No	$< 10^2$
A30	0	Yes	$< 10^2$
A30		No	$< 10^2$
+A43		No	
A30		No	$> 10^5$
+A43		Yes	
A30		No	$> 10^5$
+A50		No	
A30		Yes	$< 10^2$
+A50		No	

[a] At a 50:50 ratio.
[b] Hemagglutination.

in order to inhibit effectively synthesis of RNA or proteins failed to affect the number of plaques formed. Kidney cells (not known to produce antibody in mammals) cultured in the presence of SE and an effective NRS produced high numbers of plaques. Moreover, cell-free supernatant fluids obtained from uninoculated SC cultures which had been supplemented with an effective NRS had the property to produce similar large numbers of plaques when mock-cultured with SE. More or less visible aggregates were later observed to form progressively in the culture dishes of either SE-inoculated SC cultures or mock cultures of the supernatant fluids of uninoculated cultures which had been supplemented with an effective NRS. These antibody-sensitized aggregates of SE survived the usual cell harvest procedure at the end of the culture period, including vortex mixing of cell suspensions. The harvested SE-aggregates were then mixed inadvertently with the nonsensitized target SE contained in the agarose gelling medium and plated as usual in the plaque assay. The subsequent addition of complement resulted in development of two types of plaques, those formed by authentic PFC and those caused by lysis of the more numerous sensitized aggregates of SE. Further identification of the origin of the predominant number of such plaques was

TABLE III. CHARACTERISTICS OF PLAQUES FROM SE-INOCULATED RABBIT SPLEEN CELL CULTURES SUPPLEMENTED WITH AN EFFECTIVE RABBIT SERUM[a]

Culture	Amount of culture plated (ml)	Plaques developed/ml	Plaques per culture[b]
Control, rabbit spleen cells	0.01	1097	153,580
	0.005	546	152,880
+ actinomycin (25 μg/ml)	0.01	1222	171,080
+ cycloheximide (25 μg/ml)	0.005	923	258,440
Kidney cells + SE	0.01	1097	153,580
SE only	0.005	555	155,400
SE only, plated in RE[c]	0.005	501	140,280
Uninoculated culture supernatant fluids (SF) mock-cultured[d] with SE			
Vortex mixed (1 sec)	0.005	591	165,480
Not vortex mixed			
Mock-culture SF[e]	0.005	155	43,400
Pellet[f]	0.005	> 1000	> 280,000
SF from mock-culture, preabsorbed with SE, mock-cultured again with SE			
Vortex mixed	0.1	0	0
Not Vortex mixed	0.1	0	0

[a] From rabbit # *A30*.
[b] Equal to plaques/1.4 ml. See footnote[a], Table I.
[c] Rabbit erythrocyte target cell lawn.
[d] SF was inoculated with SE and incubated to simulate an SC culture.
[e] Not centrifuged, but siphoned from culture dish.
[f] From the sediment after (e) above.

provided by observing them in similar high numbers in plaque assays using irrelevant, homologous, nonsensitized rabbit erythrocytes as target cells in the agarose gel. The majority of the plaques that were formed in these cultures did not involve lymphoid cells; they have been designated lymphoid-cell-independent plaques (LCIP).

Characteristics of rabbit spleen cell PFC responses to sheep erythrocytes in cultures supplemented with fetal bovine serum. Results obtained in 15 experiments in which primary immunizations with SE of rabbit spleen PFC cultures were performed are shown in Table IV. These cultures were supplemented with one of three fetal bovine sera, none of which contained LCIP predisposing anti-SE binding antibody, but which had been found to support mouse primary-type PFC responses in culture. Less than half of the individual rabbit spleen cells tested responded. Those that did respond, responded in cultures supported by either FBS A, B, or C (e.g., rabbits A45, A102, A104 and A116). Those that did not respond, failed to respond in the presence of either serum (e.g., A57 and A105). The positive responses shown were, in our experience, low as compared with a mouse culture counterpart. This is in general agreement with most of the early literature concerning rabbit cell responses *in vitro* (Table IV).

Discussion. The hemolytic plaque assay was first applied to suspensions of spleen cells obtained directly from immunized or control animals, where there is little chance of the proper SE anti-SE association necessary to form LCIP. When this method was applied to spleen cells from culture, precautions against artifacts were taken (8), but as the method has become more routine, these precautions have probably been relaxed in other laboratories. The risk of such omission is high when cultures are supplemented with rabbit serum or with any serum containing antibody against the erythrocytes chosen as the antigen.

Our results have shown in a rabbit SC culture system that plaques were produced by an antibody mediated, but lymphoid-cell-independent process. When intact SE were

TABLE IV. PFC RESPONSES[a] OF SE-INOCULATED FBS SUPPLEMENTED RABBIT SPLEEN CELL CULTURES

Rabbit number	Plaques per culture								
	FBS[b]								
	A		B		C		NRS[c] (SE-absorbed)		
	SE-inoculated								O-time[d] (background)
	No[e]	Yes[e]	No	Yes	No	Yes	No	Yes	
A62	—[f]	—	—	—	—	—	0	0	40
A41	0	0	—	—	—	—	—	—	120
A45	60	1380	40	320	—	—	—	—	< 1
A57	0	0	0	0	0	0	—	—	< 1
A81	—	—	0	100	—	—	—	—	< 1
A102	100	2000	100	1600	100	1520	—	—	80
A104	100	160	—	—	80	60	—	—	80
A105	0	0	0	0	—	—	0	0	40
A110	0	0	—	—	—	—	—	—	40
A112	—	—	40	1300	—	—	—	—	160
A113	0	0	—	—	—	—	—	—	40
A116	60	20	60	200	60	140	—	—	40
A118	—	—	—	—	—	—	0	0	40
B4	0	300	—	—	—	—	—	—	< 1
B15	0	0	—	—	—	—	—	—	< 1

[a] After 4 days in culture.

[b] Fetal bovine serum A, B, or C each represent a tested lot purchased from one of the three suppliers listed in Materials and Methods.

[c] Normal rabbit serum.

[d] By preculture assay of the seeding SC suspension. Plaques per 20×10^6 cells seeded per culture.

[e] refers to SE inoculation.

[f] Indicates, not tested.

added to provide the antigenic stimulus in cultures which contained rabbit serum with binding activity for SE, the SE were sensitized and formed more or less tightly bound aggregates. These were subsequently lysed focally within a lawn of nonsensitized SE by the addition of complement yielding LCIP. In these primary-type cultures of cells from noninjected rabbits, LCIP were generated by the sensitizing activity of background antibody contained in the rabbit serum of the culture medium. We have also observed that spleen cell cultures from SE immune rabbits may coproduce two kinds of plaques. Some are formed by authentic PFC and others are LCIP as a result of free antibody secreted into the medium by PFC (manuscript submitted). It should be pointed out that the central appearance of lymphoid cells, particularly B lymphocytes, within plaques may not be sufficient evidence to exclude LCIP. It has been shown that antigen–antibody complexes may bind to B-cells by either a complement receptor (9) or an Ig receptor (10). Since antibody-complement lysed erythrocyte membranes are in fact antigen–antibody complexes, B-lymphocytes might be expected within some LCIP.

Since preformed background anti-SE antibody had been observed in the sera of 99% of a large number of normal rabbits in a previous study (2), our original intention was to test the possibility that some portion, possibly a particular type of such normal anti-SE antibody might have served as a "primer." Our premise was conceptually similar to that of others (3, 4, 5) who have postulated that an "IgT" would be a primer. However, our discovery of the artifactual plaque-forming mechanism renders this rabbit cell culture system unsuitable to identify the primer.

We do not presently understand the existence of both effective and ineffective antibody containing sera, nor do we understand the finding that some ineffective sera

were actually inhibitory (e.g., A43 Table II) and were able to suppress the plaques formed by an effective serum (A30). The ability to remove both the LCIP promoting and the LCIP inhibiting activities by specific absorptions with SE but not with CE or BE suggests that both activities are antigen specific. The antibody-like factor, having either or both LCIP promoting and LCIP inhibiting properties, remains to be isolated and identified.

Summary. This report describes a phenomenon that may be a significant source of error in the data acquired from hemolytic plaque assays of lymphocyte cultures. The data show that background antibody in the normal-serum supplement of the culture medium does not actually support initiation of a vigorous primary-type plaque-forming cell (PFC) response to sheep erythrocytes (SE). Instead, the background antibody causes the development of numerous spurious lymphoid-cell-independent plaques (LCIP). In general agreement with the literature, primary-type rabbit spleen cell responses *in vitro* were low in culture media supplemented with fetal bovine serum, a serum which does not contain background antibodies to SE.

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