

ALLOANTIBODY PRODUCTION MEASURED BY PLAQUE ASSAY
IN RELATION TO STRONG AND WEAK
HISTOINCOMPATIBILITY*

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Despite intensive investigation, the essential mechanisms of allograft rejection or tolerance have remained obscure. Although there is now no doubt that juvenile as well as adult individuals respond to foreign tissue grafts with production of serum antibodies against the alloantigens (isoantigens) of the donor, the possible roles of such antibodies in graft rejection continue to be controversial. Circulating antibodies against graft antigens are generally cytotoxic only for dissociated and especially lymphoid cells. Solid tissue transplants such as skin, kidney, or tumors are rarely vulnerable to serum antibodies alone. Indeed, such grafts often show prolonged survival or enhancement as a consequence of passive transfer of specific antiserum to the graft recipient. Much evidence suggests that specifically immune lymphocytes are the essential agents in the destruction of most types of allografts (1). This conclusion follows in particular from the often repeated finding that lymphocytes from immunized animals will adoptively transfer immunity to normal animals, whereas even hyperimmune serum from comparable donors fails to do so. Allograft rejection then has many of the characteristics of cellular hypersensitivity of the delayed- or tuberculin-type. The details of how immune lymphocytes or their transformed descendants kill foreign target cells are still unknown. Perhaps a special class of cell-bound antibodies or even induced enzymes may be involved. The recent discovery of four or more major classes of circulating immunoglobulins, each with distinctive physico-chemical and biological properties as well as intraclass differences (2, 3), adds new dimensions to the possibility that certain typical antibodies may be associated with transplantation immunity. Thus the failure of whole hyperimmune serum to mediate allograft rejection passively could be attributable to opposing effects of different molecular species of antibodies.

Recently we have found that only macroglobulin (i.e. 19S or IgM) alloantibodies are inducible in infant or juvenile mice, whereas older mice regularly produce 7S IgG alloantibodies in addition to macroglobulins (4). Yet maximal transplantation immunity of the adult type is inducible in neonates before even macroglobulin allohemagglutinins are readily detectable under diverse tactics of immunization. How-

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ever, plaque assay studies currently in progress reveal allohemolysin production by neonatal mice during the earliest period that transplantation immunity is detectible. An important question then is whether these early appearing macroglobulins are temporally and functionally associated with allograft rejection.

After much effort, a sensitive agar plaque technique has been developed that allows detection of alloantibodies against both strong and weak histocompatibility antigens in mice as well as Syrian hamsters. There appears to be no limitation in the "weakness" of the histocompatibility antigens demonstrated to evoke serum antibodies detectable by the plaque assay employed in the present study. The development of a sensitive and reproducible quantitative plaque technique to measure alloantibody production has been fraught with technical pitfalls requiring extensive empirical testing.

Materials and Methods

Animals.—Mice of the following inbred strains were used: A/J, C57BL/6, the congenic lines C57BL/10 ScSn (H-2^b) and B10.A (H-2^a), and the congenic lines C3H/DiSn (H-1^a) and C3H.K (H-1^b). The A/J (H-2^a) and C57BL/6 (H-2^b) lines also differ with respect to many non-H-2 histocompatibility loci. The immunogenetic properties of H-2 and non-H-2 histocompatibility genes and antigens in various congenic lines of mice have recently been summarized elsewhere (5). (A/J ♀ × C57BL/6 ♂) F1 hybrids were produced for tests of antibody responses to an X-linked antigen. Three inbred strains of Syrian hamsters, designated LSH, MHA, and BRC, and showing intermediate to weak histocompatibility differences, have also been under study.

Immunization.—All adult animals were immunized intravenously via the retroorbital sinus or occasionally through a tail vein. Approximately 6×10^8 allogeneic erythrocytes plus 1×10^6 buffy coat leukocytes were routinely administered to each lightly anesthetized recipient in a single inoculation of 0.1 or 0.2 ml. In pilot experiments, the intravenous route was found to be preferable to other routes for sensitization of spleen cells. Because plaque production by control spleen cells from normal, old adult animals was often high, young adults of 8 to 12 wk of age were regularly used for immunization.

Agar Plaque Technique.—The technique employed is basically similar to that devised by Jerne et al. (6) for measuring the responses of individual antibody-producing cells of mice to sheep red cells in vitro. Our method as evolved over some 2 yr of empirical testing of alloantibody (isoantibody) responses will be described, because reproducible results require more careful attention to details than is the case with the sheep cell system.

The bottom agar layer, which subsequently serves as a base for the agar overlay containing the test cells, was prepared as follows: 1% (w/v) Ionagar No. 2 is prepared in Hanks' balanced salt solution and dissolved in an Arnold sterilizer or by boiling water for 30 to 45 min. It is then cooled to about 70°C and DEAE dextran (50 mg/100 ml) is added. Sodium bicarbonate (7.5%) is then added to adjust the pH to 7. This agar is filtered under sterile conditions while still hot through a funnel containing "lint-free" cotton wrapped in fine gauze to remove coarse debris. The agar is then poured into sterile, glass Petri dishes (100 × 15 mm) with flat bottoms, about 20 ml agar per dish. Excess moisture may be removed from the plates by drying at room temperature or in a 37°C incubator for 25 to 30 min. The plates can then be stored in a refrigerator for later use up to 5 days at about 5°C.

The agar to which spleen cells and their target red cells are subsequently added was pre-

pared as follows: a 0.7% solution of Ionagar No. 2 (Oxoid Division, Consolidated Laboratories, Chicago Heights, Illinois) or Agarose (Bausch & Lomb Inc., Rochester, New York) is prepared in Hanks balanced salt solution without DEAE dextran and NaHCO_3 . The hot agar must then be ultracentrifuged at 27,000 *g* (15,000 rpm in the Sorvall RC-2B, Ivan Sorvall, Inc., Norwalk, Connecticut), for 50-ml tubes in the SS-34 rotor) for 5 min to sediment the fine particulate debris invariably present. The bottom 1 cm of solidified agar gel from each 50-ml tube containing debris is cut off and discarded. The remainder of the agar is melted, cooled to about 70°C, and DEAE dextran (50 mg/100 ml) is added with mixing. Streptomycin sulfate (0.3 mg/ml) and penicillin G sodium (300 units/ml) is then added and the pH adjusted to 7.0–7.2 with 7.5% NaHCO_3 . The agar is then distributed in 2.5-ml volumes into 2 × 15 cm tubes fitted with "Bacti-Capall" tops at 47°C until ready for use. Use of complete Eagle's medium (including amino acids and fetal calf serum) in lieu of Hanks' solution has not improved the final results.

The separate tubes of target blood cells and spleen cells are slowly warmed to room temperature or above (~25°C). After mixing well, 0.1 ml red cell suspension is pipetted into 2.5 ml agar solution at 47°C, followed by 0.2 ml spleen cell suspension. After further mixing by rotation to avoid bubbling, the test cells are immediately poured onto the center of a previously prepared Ionagar plate and quickly dispersed over the entire surface. This mixing procedure is repeated with pouring of successive tubes and the plates are left undisturbed until the overlays have completely solidified (~10 min). The cell-agar overlay suspension at the time of pouring has a pH of 7.0 or 7.1.

The test plates are placed in a humid 5% CO_2 incubator (National Appliance Company, Portland, model 3221) at 37°C for 30 to 35 min, followed by an additional 30 min in an air incubator at 37°C. Prolonged exposure to CO_2 may result in excessive lowering of the pH. About 4 ml of *absorbed* guinea pig complement at a dilution of 1:8 in modified barbitol buffer (5,5-diethylbarbituric acid, 2.875 g; sodium 5,5-diethylbarbiturate, 1.875 g; calcium chloride, anhydrous, 0.083 g; magnesium chloride, anhydrous, 0.238 g; sodium chloride, 42.500 g; distilled water to 1 liter to give 4x stock solution) is then added to each plate with reincubation at 37°C for 30 min. Excess complement may then be poured off and the plates are held for an additional 2½ to 3 hr at 25°C prior to final counting of the plaques. Because guinea pig complement generally contains naturally occurring antibodies for mouse cells (giving agglutination titers of 1:16 to 1:32) which may promote spurious plaques, preabsorption of complement at 0°C is desirable to remove these antibodies. One volume of washed, packed erythrocytes is sufficient to completely absorb 15 volumes of guinea pig complement or preferably 30 volumes of complement at a 1:2 dilution in modified barbitol buffer in 15 to 20 min. It is important that this antibody absorption be performed at 0°–2°C to minimize complement fixation. Concurrent control plates should include replicate immune spleen cell- and/or normal spleen cell-target red cell suspensions to which are added absorbed guinea pig complement inactivated by preheating at 56°C for 30 min. Control plates to which no complement is added sometimes yield considerable numbers of plaques after 3 hr of incubation, possibly due to spleen cell lysis in the absence of serum factors.

We could list a long series of variations in the above technique that led to less satisfactory results. The most desirable innovations are prior ultracentrifugation of overlay agar to remove fine particulate debris, inclusion of streptomycin and penicillin, incubation of test cells in a CO_2 tissue-culture environment, and use of guinea pig complement from which naturally occurring mouse-specific antibodies have been absorbed.

Preparation of Test Cells for Plaque Assays.—Target red cells are harvested via the subclavian artery or retroorbital sinus into acid-citrate-dextrose solution or isotonic citrate-saline. The blood cells are washed three times in isotonic saline in the cold. The buffy coat is commonly retained, since test results indicate that buffy coat-rich target cells yield as many

(or even slightly more) plaques as compared to buffy coat-poor red cell suspensions. About 1.2 volumes phosphate-buffered saline (pH 7.4) or Hanks' solution is added to each volume of packed, washed red cells to yield approximately 600 to 700×10^6 red cells per 0.1 ml. Each batch of red cells is counted in a Neubauer chamber and placed in an ice bath until ready for use.

The spleen cells must be harvested with special care to assure high viability and minimal clumping. A whole spleen is placed in a sterile Petri dish containing cold citrate-saline (2.0% Na_3 citrate, 0.5% NaCl) as an anticoagulant; adherent fat is removed and the spleen membrane is then cut lengthwise with fine scissors and the spleen is transferred to a Kontes Duall tissue grinder containing about 3 ml ice cold Hanks' balanced salt solution. Spleen cell aggregates are disrupted in stepwise aliquots using minimal pestle pressure and free spleen cells at each step are poured into a centrifuge tube in the cold. The combined spleen cell suspension is initially centrifuged in the cold in an International Clinical Centrifuge (zero to maximum speed and immediately back to zero) to deposit any heavy debris without appreciable loss of cells. The cell suspension is then washed twice in cold Hanks' solution in the clinical centrifuge at 400 g. A final volume of about 1.6 ml spleen cell suspension is passed through a stainless steel wire mesh filter fitted inside a 2 ml syringe to remove any remaining debris or cell clumps. This procedure provides sufficient cells for seven test plates (0.2 ml each containing 5 to 20×10^6 cells per plate) plus 0.2 ml for total nucleated cell and viability counts. Spleen cells harvested in this manner routinely show 85 to 90% viability on the basis of trypan blue or eosin-Y exclusion (0.1% w/v solutions in saline).

Technique Variations and Problems in Plaque Scoring.—Among various agar preparations tested, Oxoid Ionagar No. 2 has provided the best results with the least expense. Supposedly highly purified agars contain just as much physical debris (algal stems and fibers) and have not improved the sensitivity or reproducibility of the tests. The addition of DEAE dextran to the agar improves sensitivity, presumably by neutralizing the anticomplementary properties of the agar. Inclusion of the bottom layer of agar allows for absorption of hemoglobin from plaque-lysed red cells and assures a level surface with even distribution of the top overlay; more plaques are also discernible in general than with a single layer technique. Since spleen cells killed by freezing and thawing, or heating at 56°C for 15 min, produce very few or no plaques, plaque formation is attributable to active secretion of antibody by viable cells.

It is probable that CO_2 incubation facilitates plaque formation through maintenance of physiological conditions, including a pH near neutrality. Our CO_2 incubation time is just long enough for the phenol red indicator in the medium to turn orange or yellowish-orange; the color soon returns to red with continuing incubation in air at 37°C . Fresh mouse red cells are routinely used for plating, since cells stored in the refrigerator become increasingly prone to spontaneous lysis. Blood lymphocytes from immunized mice also produce plaques under the same conditions suitable for spleen cells.

The size of plaques produced ranges from about 0.1 to 0.7 mm. Higher plaque counts associated with substantial antibody production generally yielded more plaques of larger diameter, whereas small plaques were most frequent in conjunction with weak responses. Very tiny plaques of 0.1 to 0.2 mm diameter may be visualized and counted with the unaided eye by appropriate backlighting, or with a photographic enlarger. Plaque counts were routinely checked independently by two observers and suspicious plaques were checked again under the microscope. Although questionable "spurious plaques" may pose a problem at times, under present conditions with moderate experience, most such plaques are readily identifiable. Air bubbles or gaps in the overlay are easily discerned. In early testing, before the test components and especially the agar had been cleaned up, plaques associated with coarse debris and small clumps of spleen cells were fairly common.

Even with sterile techniques, bacterial contamination may occasionally occur from Gram-

negative bacilli and/or Gram-positive cocci found in the spleens or in commercial complement. High plaque counts may then appear even after only 3 to 4 hr of incubation, but such spurious plaques are also at least as numerous in control tests in the presence of heat-inactivated complement. In more recent testing, we have routinely added streptomycin and penicillin to the overlay agar and sterilized our complement by passage through a Swinny or other bacterial filter. Penicillin G sodium (300 units/ml) plus streptomycin sulfate (0.3 mg/ml) is sufficient to prevent bacterial proliferation up to 24 hr without impairing the secretion of antibodies. Although Kanamycin sulfate (Kantrex, Bristol Labs., New York) is an even more effective antibiotic at a concentration of 0.2 to 0.4 mg/ml with respect to the Gram-negative organisms encountered, it clearly represses alloimmune plaque production. However, no such repression is evident with this antibiotic in the mouse spleen cell-sheep red cell system under the same conditions.

Substantial increases in plaque counts commonly occur after prolonged incubation and the number of plaques increases with time (8 to 24 hr). Increases in plaque counts, often to the extent of two to eight times the 3 hr count, regularly occur after overnight incubation at 23°-25°C and significant increases occur even in the refrigerator. However, test plates that show few or no plaques after 3 hr of incubation usually show few additional plaques after overnight incubation. Increases in plaque counts after prolonged incubation also occur with immune spleen cells in the presence of absorbed, heat-inactivated complement. The late appearing plaques obviously deserve further study. Apart from instances of contamination with hemolytic bacteria, they may be very tentatively ascribed to (a) continuing synthesis and late release of antibodies by spleen cells, (b) cellular immunity or allogeneic inhibition that is complement-independent, (c) late lysis of red cells coated with suboptimal numbers of lytic antibodies, and (d) release of enzymes or lysins from disintegrating leukocytes. Mouse spleen cells tested against autologous red cells consistently reveal low levels of probable autoimmune plaque production. Such autoimmunity is often notably high in old animals.

Skin Grafting.—Skin grafts were made and scored by methods previously described (4, 5). Median survival times with their 95% confidence limits were determined nomographically by the method of Litchfield (7).

RESULTS

Numerous inbred and congenic strains of mice and Syrian hamsters were found to respond to intravenous inoculation of allogeneic blood cells with the appearance of plaque-forming spleen cells over a period of 3 to 13 days after primary immunization. The magnitude of the alloantibody responses in diverse strain combinations of mice was found in general to correspond to the strength of the histocompatibility disparity as measured by the median survival times (MST) of skin allografts. These results are summarized in Text-fig. 1 and Table I. The strongest histoincompatibility, as detected in the A/J → C57BL/6 combination reflecting H-2 plus non-H-2 differences, is revealed by a skin allograft MST of 8.2 ± 0.5 days and a peak average plaque production of 29.6 per 10×10^6 viable spleen cells. At the other extreme, the C3H → C3H.K congenic combination, showing quite weak histoincompatibility reflected in an average allograft survival time of 91 days, gave only feeble production of plaque-forming cells (PFC) with a peak response of 3.7 per 10×10^6 viable spleen cells. Although there were slight differences in the range of days (3 to 13) during which significant plaque production was detectible, peak plaque produc-

tion occurred much earlier (5 to 6 days) with H-2 differences than with the "weaker" H-Y or H-1 differences (9 to 10 days). In other words, weaker histocompatibility differences were associated with a somewhat longer latent period and slower recruitment of plaque-forming cells. However, these differences are relatively slight in comparison with the profound differences in allograft survival times observed. The early allohemolysin production revealed by plaque assay is clearly not temporally correlated with rejection times of allografts

TABLE I
Summary of Mouse Spleen Cell Plaque Production in Relation to Strong and Weak Histoincompatibilities

Donor strain	Recipient strain	Histocompatibility locus difference between donors and recipients	Skin allograft median survival times in days with range of rejection times in parentheses	Average plaque count per 10×10^6 spleen cells at peak primary response*	Peak plaque production	Range of primary plaque production	Average plaque count per 10×10^6 spleen cells from non-immunized control mice*
A/J	C57BL/6	H-2 plus Non-H-2	$8.2 \pm 0.5 \ddagger$ (7-10)	26.6 (29.6)	6	4-12	3.6 (4.0)
B10.A	C57BL/10	H-2	$10.3 \pm 1.2 \S$ (9-14)	15.6 (17.4)	5	3-12	1.2 (1.3)
C57BL/10	B10.A	H-2	$10.8 \pm 1.5 \S$ (9-14)	9.6 (10.7)	5	3-12	1.1 (1.2)
C57BL/6 ♂	C57BL/6 ♀	H-Y	$25.0 \pm 2.5 \parallel$ (14-75)	15.1 (16.8)	9	4-13	1.0 (1.1)
C3H.K	C3H	H-1	29 ¶ (16-43)	14.8 (16.4)	10	6-13	1.5 (1.7)
C3H	C3H.K	H-1	91 ¶ (35-124)	3.3 (3.7)	9	8-12	1.6 (1.8)

* No. of plaques recalculated ($\times 1.11$) in parentheses on the basis of 90% spleen cell viability as found by trypan blue or eosin-Y exclusion at time of testing.

‡ Cf. reference 4.

§ Nine adult females of each strain were reciprocally grafted with full-thickness skin.

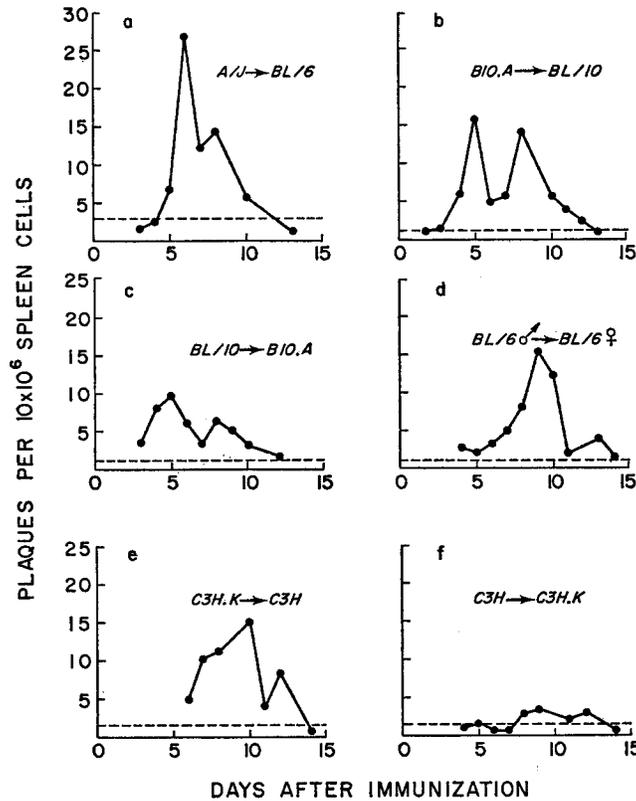
¶ Cf. reference 8.

¶ Cf. reference 9.

across non-H-2 barriers. The detailed results of these experiments are given in Table II. It should be noted that each recipient was immunized with sufficient leukocytes (1.0 to 1.2×10^6) in addition to 6 to 8×10^8 red cells to induce substantial or maximal skin transplantation immunity at the time allohemolysins were being produced (5, 10). In the combinations A/J \rightarrow BL/6, BL/10 \rightarrow B10.A, and B10.A \rightarrow BL/10, increasing the single immunizing dose two- to eightfold did not increase the plaque counts at 5, 6, or 8 days.

Immunization via orthotopic skin allograft rejection alone has led to the appearance of substantial numbers of PFC. Spleens from four B10.A females gave 1.5, 14.8, 24.2, and 26.6 plaques per 10×10^6 spleen cells on BL/10 target red cells at 2 to 3 days after complete rejection of first-set BL/10 skin allografts.

Under the same conditions in the reciprocal combination with four BL/10 female recipients, average plaque counts of 0.3, 0.7, 0.8, and 6.6 per 10×10^6 spleen cells were obtained. We have no explanation other than the late time of testing for the wide variation in numbers of PFC detected under these condi-



Text-Fig. 1. Appearance of plaque-forming spleen cells in different allogeneic and congenic mouse strain combinations following a single intravenous injection of blood cells. Each point represents the mean response of 4 to 7 mice. Dashed horizontal lines indicate average level of control plaques produced by normal spleen cells.

tions. It remains to be determined whether the time-course of appearance and recruitment of PFC induced by skin allograft rejection are commensurate to the reactions following a single intravenous injection of allogeneic cells.

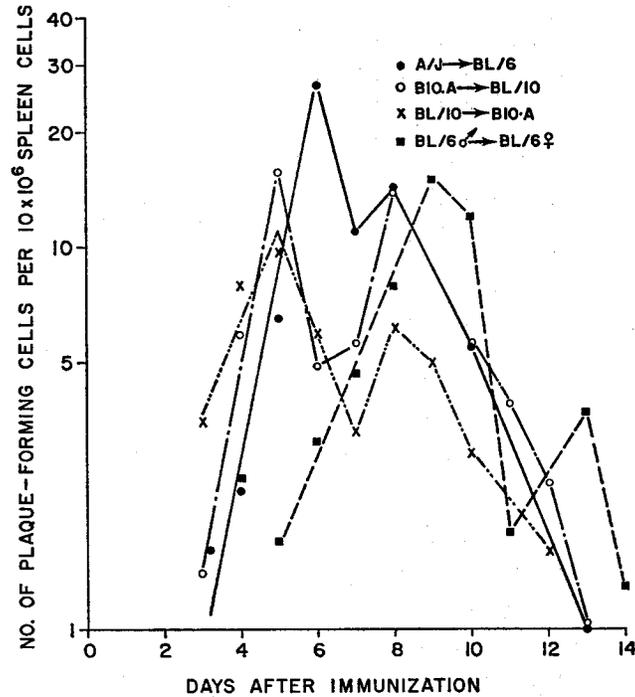
The quantitative courses of the immune reactions in the four "stronger" responses as shown in Text-figs. 1 and 2 are in accord with the assumption of an exponential increase in plaque-forming cells for 2 to 4 days until peak levels were reached. The time for doubling the number of PFC may be as short as

TABLE II
Alloimmune Hemolytic Plaque Production by Spleen Cells in Different Strain Combinations of Mice

Donor Strain	Recipient Strain*	Average No. and range in parenthesis of plaque-forming cells per 10×10^6 spleen cells at the following days after primary immunization. † Total No. nucleated spleen cells on which average count based in brackets.												Controls	
		2	3	4	5	6	7	8	9	10	11	12	13	14	
A/J	BL/6		1.6 (0.6-2.6) [98 × 10 ⁶]	2.3 (1.2-4.6) [225 × 10 ⁶]	6.5 (1.7-11.4) [296 × 10 ⁶]	26.6 (18.1-42.1) [125 × 10 ⁶]	12.0 (5.8-18.3) [166 × 10 ⁶]	14.1 (6.9-23.3) [112 × 10 ⁶]		5.5 (3.3-7.7) [72 × 10 ⁶]			1.0 (0-2.0) [55 × 10 ⁶]		3.6 (0-4.8) [290 × 10 ⁶]
B10.A	BL/10	1.2 (1.1-1.3) [132 × 10 ⁶]	1.4 (1.2-1.5) [144 × 10 ⁶]	5.9 (0.2-19.0) [292 × 10 ⁶]	15.6 (5.9-37.1) [240 × 10 ⁶]	4.9 (3.2-7.7) [275 × 10 ⁶]	5.6 (2.7-9.2) [158 × 10 ⁶]	14.0 (12.5-15.8) [80 × 10 ⁶]		5.6 (1.6-9.5) [147 × 10 ⁶]	3.9 (2.1-6.5) [202 × 10 ⁶]	2.4 (2.3-2.5) [75 × 10 ⁶]	1.0 (0.9-1.2) [91 × 10 ⁶]		1.2 (0.9-1.3) [220 × 10 ⁶]
BL/10	B10.A		3.5 (2.2-4.7) [90 × 10 ⁶]	7.9 (1.1-16.2) [308 × 10 ⁶]	9.6 (2.5-20.8) [250 × 10 ⁶]	5.9 (3.4-9.8) [280 × 10 ⁶]	3.3 (0.6-5.5) [242 × 10 ⁶]	6.2 (3.4-12.0) [278 × 10 ⁶]	5.0 (2.9-7.0) [124 × 10 ⁶]	2.9 (0.7-5.7) [228 × 10 ⁶]		1.6 (0.6-2.9) [209 × 10 ⁶]			1.1 (0-2.2) [156 × 10 ⁶]
BL/6♂†	BL/6♀			2.5 (1.6-3.2) [114 × 10 ⁶]	1.7 (1.6-1.8) [118 × 10 ⁶]	3.1 (1.0-6.5) [209 × 10 ⁶]	4.7 (2.0-7.1) [138 × 10 ⁶]	7.9 (1.8-21.2) [271 × 10 ⁶]	15.1 (8.0-24.3) [100 × 10 ⁶]	12.0 (1.0-19.4) [226 × 10 ⁶]	1.8 (1.3-2.3) [111 × 10 ⁶]		3.7 (2.6-4.8) [149 × 10 ⁶]	1.3 (0.5-2.0) [91 × 10 ⁶]	1.0 (0.8-1.1) [106 × 10 ⁶]
C3H.K	C3H					4.9 (0-8.5) [136 × 10 ⁶]	10.1 (3.5-16.5) [141 × 10 ⁶]	11.2 (26 × 10 ⁶)		14.8 (3.8-25.7) [107 × 10 ⁶]	3.9 (0.9-10.3) [327 × 10 ⁶]	8.3 (3.5-16.3) [161 × 10 ⁶]		0.2 (0-0.6) [276 × 10 ⁶]	1.5 (0.3-2.7) [115 × 10 ⁶]
C3H	C3H.K		1.0 (0.6-1.3) [106 × 10 ⁶]		1.5 (0.4-3.4) [90 × 10 ⁶]	0.6 (0-1.0) [202 × 10 ⁶]	0.5 (0-1.0) [115 × 10 ⁶]	2.8 (1.3-3.7) [157 × 10 ⁶]	3.3 (3.1-3.5) [156 × 10 ⁶]		2.1 (0.2-4.0) [63 × 10 ⁶]	2.7 (2.3-3.1) [106 × 10 ⁶]		0.3 (1.0-2.3) [339 × 10 ⁶]	1.6 (1.0-2.3) [339 × 10 ⁶]

* Each recipient given single i.v. injection of 6 to 8×10^8 donor red cells plus 1.0 to 1.2×10^8 buffy coat leukocytes on day 0.
 † Based with few exceptions on 4 to 7 adults (8 to 12 wk old) tested individually on each day indicated with counts of 4 to 6 replicate plates of each spleen cell suspension. Control values determined on spleen cells from nonimmunized animals of same age range.

7 to 8 hr during the phase of exponential increase in the most responsive A/J \rightarrow BL/6 and B10.A \rightarrow BL/10 combinations. However, this multiplication could well include commitment of preexisting virgin cells as well as proliferation of antibody-producing cells. The subsequent rapid decrease in plaque-forming cells was followed 2 to 4 days after the peak response by a moderate, renewed



Text-Fig. 2. Semilogarithmic representation of time-course of appearance of plaque-forming spleen cells in four allogeneic strain combinations of mice after a single immunization. Note exponential increases in numbers of plaque-forming cells until peak responses were reached.

rise in activity before plaque production diminished to low control levels. This secondary rise suggests interruption of continuing induction of 19S antibody synthesis, perhaps as a consequence of feedback inhibition with onset of 7S antibody production which is suboptimally revealed by guinea pig complement (11). The increases in plaque-forming cells in the congenic C3H \rightleftharpoons C3H.K combinations do not appear to be logarithmic in character. However, much larger numbers of these mice will have to be tested, especially at additional donor cell dosage levels, before this question is resolved.

Sufficient tests have been done with other allogeneic combinations of mice

and Syrian hamsters to demonstrate that our plaque assay technique is suitable for measurement of allohemolysin responses in general. High levels of PFC were found in A/J females at 5 to 7 days after immunization with H-2 incompatible C57BL/6 female blood cells. The H-Y antigen as expressed in the A/J male \rightarrow A/J female combination has in a limited number of tests led to the appearance of fewer PFC than found in the C57BL/6 male \rightarrow C57BL/6 female combination. A moderately strong X-linked histocompatibility antigen was found to distinguish the A/J and C57BL/6 strains such that single C57BL/6 male skin grafts were rejected by 9 (A/J \varnothing \times BL/6 σ^7)F1 adult males with a median survival time of 28.0 days with 95% confidence limits of 23.8 to 33.1 days. A single intravenous immunization of (A/J \varnothing \times BL/6 σ^7)F1 males with 6×10^8 F1 sibling female red cells, involving the same X-linked antigen, gave a peak plaque production of about 15 per 10×10^6 spleen cells at 10 days. From these results, it may be concluded that the various non-H-2 histocompatibility antigens thus far investigated are also blood group antigens capable of eliciting the production of hemolysins. Although we presume on the basis of earlier work that these antigens are more abundantly present in leukocytes than erythrocytes, quantitative evaluation of this question in terms of relative immunogenicities for stimulation of PFC has not been attempted. However, leukocyte-enriched and leukocyte-depleted preparations of target red cells appear in general to be about equally susceptible to plaque production.

Preliminary results of plaque formation by spleen cells following a single primary immunization with 0.5 to 1.0×10^9 red cells plus buffy coat leukocytes have been obtained with the following strain combinations of hamsters: BRC \rightleftharpoons MHA, LSH \rightleftharpoons MHA, and BRC \rightarrow LSH. Peak responses ranging from 14 to 24 PFC per 10×10^6 spleen cells were detected in all combinations at about 9 to 11 days after immunization. This consistent, early alloantibody response was surprising on two counts: (a) the immunity manifested toward first-set skin allografts ranges from very weak in LSH \rightleftharpoons MHA (12) to intermediate in the BRC interstrain combinations with MST's of about 4 wk (13); (b) even after hyperimmunization, many previous attempts to detect serum alloantibodies by diverse serological techniques have given only irregular low titers or negative results.

After rest periods of 2 to 4 wk, booster immunizations of hamsters have yielded heightened and earlier secondary responses reflected in two- to four-fold increases in PFC over the maximal plaque counts observed in primary responses. Antiserums taken from hyperimmunized hamsters at times when PFC activity was high have exhibited sufficient titers of hemolysins in standard tests with guinea pig complement to facilitate absorption analyses of strain-specific differences. Such analyses are now in progress. Several-fold increases over peak primary PFC counts have also been observed in pre-

liminary tests of mice given booster inoculations of allogeneic blood cells. In these situations, optimal schedules and tactics of immunization remain to be determined and may well depend on the "strength" of the antigenic disparity involved.

Five adult A/J female mice immunized with 5×10^8 sheep red cells by the intravenous route and tested for PFC after 4 days by the same method applied in the allogeneic combinations have given an average of 1310 plaques per 10×10^6 spleen cells with mouse cell-absorbed complement. Slightly but not significantly higher plaque counts were obtained in concurrent tests with unabsorbed complement, indicating that the naturally occurring mouse-specific antibodies in guinea pig serum do not block the release of antibodies by mouse spleen cells. Otherwise identical tests employing unabsorbed or absorbed, heat-inactivated complement gave no plaques or very few plaques.

The various types of hemolytic plaques produced by immune spleen cells in the major test systems studied are illustrated comparatively in Figs. 1 to 6. In general, alloimmune plaques are smaller and develop less rapidly than those produced against sheep erythrocytes by either mouse or hamster spleen cells.

DISCUSSION

Although the agar plaque technique as employed in the present studies is essentially simple, close attention to technical details has proved important in the attainment of reproducible results. Moreover, prior ultracentrifugation of overlay agar to remove particulate debris, addition of streptomycin and penicillin to inhibit spleen-derived bacteria, incubation of test cells in a 5% CO₂ environment, and use of guinea pig complement from which natural mouse-specific antibodies have been absorbed appear to be important. Such refinements appear to be less critical in the sheep red cell-mouse spleen cell system where ten- to fiftyfold higher plaque counts are readily obtainable (14, 15) under comparable conditions. This heightened responsiveness to sheep cells in comparison with allogeneic cells is not surprising in view of the relative antigenic disparities involved. Reasonably reproducible plaque counts in our hands have required testing whole spleens rather than portions thereof, a finding consistent with the evidence of Nakano and Braun (16) that PFC occur in spleens in a nonrandom distribution. In this connection, we are still concerned about relative plating efficiency; i.e., the number of plaques obtained as a function of the number of potential PFC present in a given spleen. Spleen cell suspensions from immunized animals often appear to be more "sticky" than those from normal animals. Cells which tend to remain in clumps are finally removed by filtration and are therefore not tested; on the other hand, excessive manipulation to disperse all clumped cells has resulted in unacceptably low (50 to 70%) viability counts.

Calculations of numbers of PFC by other investigators have not taken spleen cell viability determinations into account. For comparative purposes, PFC counts given in the present paper unless otherwise noted are also calculated on the basis of 100% viability. However, with our actual viability counts regularly around 90%, it should be noted that the numbers of PFC per 10×10^6 *viable* spleen cells are increased by a factor of 1.11 (see Table I). The control level of 1 to 2 PFC per 10×10^6 spleen cells found in young adults of most mouse and hamster strains is 4 to 5 times less than the number of control plaques found with respect to sheep red cells (14, 15, 17). In our intraspecific experiments, the same low levels of PFC are found regardless whether the normal spleen cells are tested against autologous or allogeneic red cells. We presume that these PFC are largely autoimmune since they often increase substantially with advanced age (18). In the sheep cell-mouse system, the higher levels of normal PFC may be attributable partly to naturally occurring immunization with heterophile antigens having determinants similar to some found on sheep cells and partly to spurious plaques. Alternatively, Jerne (15) favors the idea that these PFC differentiate spontaneously and may be present in the absence of an antigenic stimulus.

Since nucleated cell counts of whole spleens even from animals of the same strain and age may vary from about 80 to 150 million cells, it is important to express plaque counts per 10^6 spleen cells or multiples thereof rather than per spleen. The number of plaques obtained under the conditions described was not significantly affected by the sex of the donors or recipients in the BL/10 \rightarrow B10.A and C3H \rightarrow C3H.K combinations. In other combinations not involving sex-associated antigens only female donors and recipients were tested. Although we have not systematically investigated the dosage of donor cells as an experimental variable in all strain combinations studied, over the range of 1×10^8 to 4×10^9 red cells, closely similar responses have been obtained. This dosage range which includes 160,000 to 6.4 million buffy coat leukocytes may be suboptimal in the weaker histoincompatible combinations on a single injection basis, even though similar dosages yield maximal responses toward sheep cells (15). It is entirely possible that the dosage of allogeneic donor leukocytes is more decisive than the red cell dosage, since the former are known to be a much richer source of most alloantigens (cf. references 1, 11).

The results reveal an overall lack of correspondence between the times or rates of skin allograft rejection and the time of appearance of plaque-forming cells. In other words, prolonged allograft survival was not correlated with a commensurate delay in the initial occurrence of plaque-forming cells. Nevertheless, peak plaque production occurred earlier and reached higher levels across the stronger histocompatibility barriers in general accord with the rapidity of allograft rejection. Weak transplantation barriers were not associated with proportionate delays in the induction of antibodies, but were reflected in the quantitative antibody response thereafter.

Much evidence strongly suggests that transplantation immunity and humoral alloantibody production are elicited by antigens with the same or similar determinant groups (1). The present evidence supports various earlier indications that these two manifestations of alloimmune responsiveness depend upon distinctive immunological processes (4, 19). The times of inception of transplantation immunity and hemolysin production were disparate in conjunction with weak histocompatibility differences. The question arises whether additional subpopulations of antibodies are being produced, but remain undetected by the present plaque assay. It is generally assumed that only 19S antibodies are produced in significant amounts in an early primary response. However, adult C57BL/6 or A/J mice may produce readily detectable titers of both 19S and 7S alloantibodies as revealed by analytical ultracentrifugation (4) within 9 days after reciprocal primary immunization. The extensive studies of Winn (11) indicate that guinea pig complement is efficiently activated only by 19S mouse antibodies, whereas rabbit complement works efficiently with 7S mouse antibodies. Thus it is possible that numerous PFC elaborating 7S hemolysins, especially late in the primary response, were missed in the present analysis. Replicate assays employing both guinea pig and rabbit complements should clarify this point. In a strict sense then, the relationships discerned in the present study may be relevant only to 19S hemolysins. Other molecular species of antibodies may also be produced relatively early and require other means of detection.

The results show that H-1, H-Y, and H-X antigens in the mouse and other weak H antigens in Syrian hamsters are also erythrocyte antigens. For as yet unknown reasons, detection of these weak histocompatibility antigens by standard serological techniques has not been achieved on a reproducible basis in the past. Further testing in relation to known times of peak plaque production should prove fruitful, especially following booster injections.

SUMMARY

A plaque technique which provides a sensitive and quantitative determination of allohemolysin (isohemolysin) production by lymphoid cells of mice and Syrian hamsters is described. Much ongoing attention has been given to minimizing sources of spurious plaques while improving the reproducibility of the technique. Primary intravenous immunization with allogeneic blood cells led to the early appearance of plaque-forming spleen cells responding to both strong and weak histocompatibility antigens in inbred strains of mice. The strongest histoincompatibility (A/J → BL/6), characterized by H-2 plus non-H-2 differences and a median skin allograft survival time of 8.2 ± 0.5 days, gave a peak average plaque production of $29.6 \text{ per } 10 \times 10^6$ viable spleen cells after 6 days. At the other extreme with an average allograft survival time of 91 days, C3H(H-1^a) → C3H.K(H-1^b) showed a feeble production of plaque-forming cells with a peak response of $3.7 \text{ per } 10 \times 10^6$ viable spleen

cells at 9 days. Histocompatibility antigens determined by genes at the H-1, H-Y, and H-X loci were demonstrated to be erythrocyte antigens as well. Peak plaque production occurred earlier and reached higher levels across the stronger histocompatibility barriers (H-2 plus non-H-2 or H-2 alone) in temporal accord with the rapidity of skin allograft rejection. Weak H-Y or H-1 differences leading to prolonged allograft survival were associated with moderate, but not commensurate delays in the initial occurrence and recruitment of plaque-forming cells. With one exception (C57BL/10 → B10.A), the average plaque counts found at the peak of the primary responses were inversely proportional to the median survival times of skin allografts in the same strain combinations. Nevertheless, in light of the disparity between the times of allograft rejection and the time course of appearance of plaque-forming cells with the "weaker" congenic combinations, the early 19S antibodies do not appear to be directly involved in allograft rejection.

There appears to be no limitation in the weakness of histocompatibility antigens capable of evoking antibodies detectible by plaque assay. The extent to which molecular species of mouse antibodies other than 19S hemolysins are induced and detected under the conditions employed remains to be determined. The responses in strain combinations involving stronger histocompatibility barriers were characterized by exponential increases in plaque-forming cells, for 2 to 4 days until maximal levels were reached. Repeated immunization in several trials led to substantial increases over the maximum primary response in the number of demonstrable plaque-forming cells. Further preliminary results are reported concerning alloimmune plaque production in three strains of Syrian hamsters exhibiting intermediate to weak histocompatibility differences.

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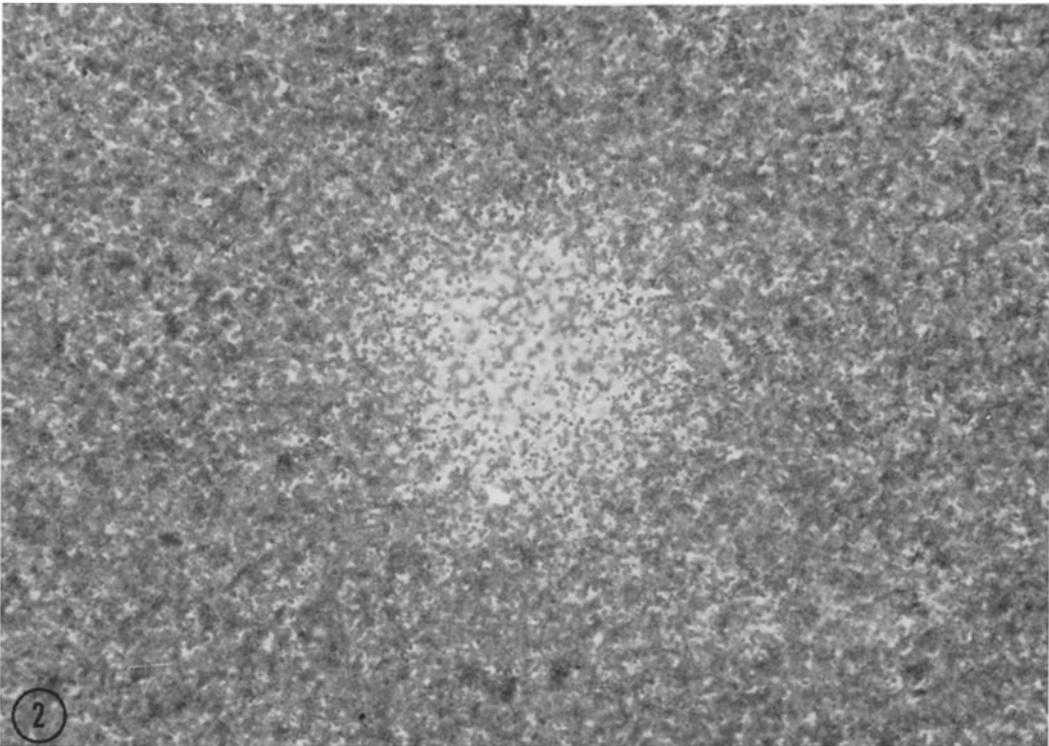
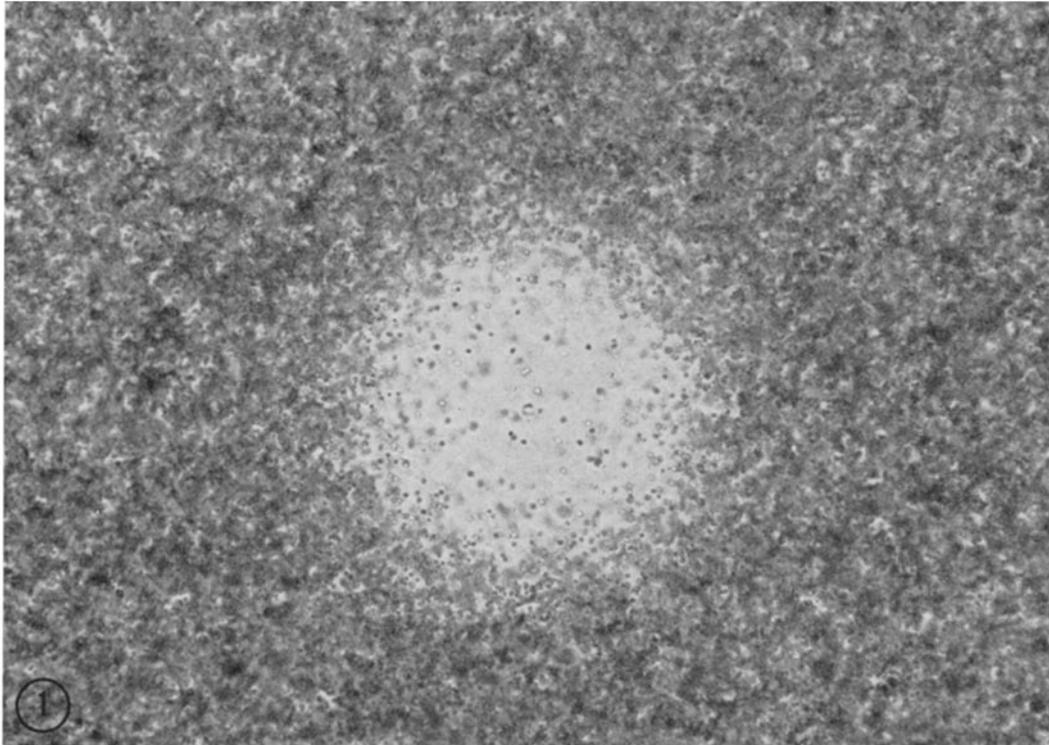
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EXPLANATION OF PLATES

PLATE 88

FIG. 1. Typical individual plaque produced by immune A/J mouse spleen cells against sheep erythrocytes in presence of absorbed guinea pig complement. Notable variation in size and clarity of individual plaques is the rule. Approximately $\times 100$.

FIG. 2. Larger alloimmune plaque produced by C57BL/6 mouse spleen cells against A/J erythrocytes in presence of absorbed guinea pig complement at 6 days after immunization. These larger plaques, though uncommon, are often associated with small clumps of spleen cells. Approximately $\times 100$.

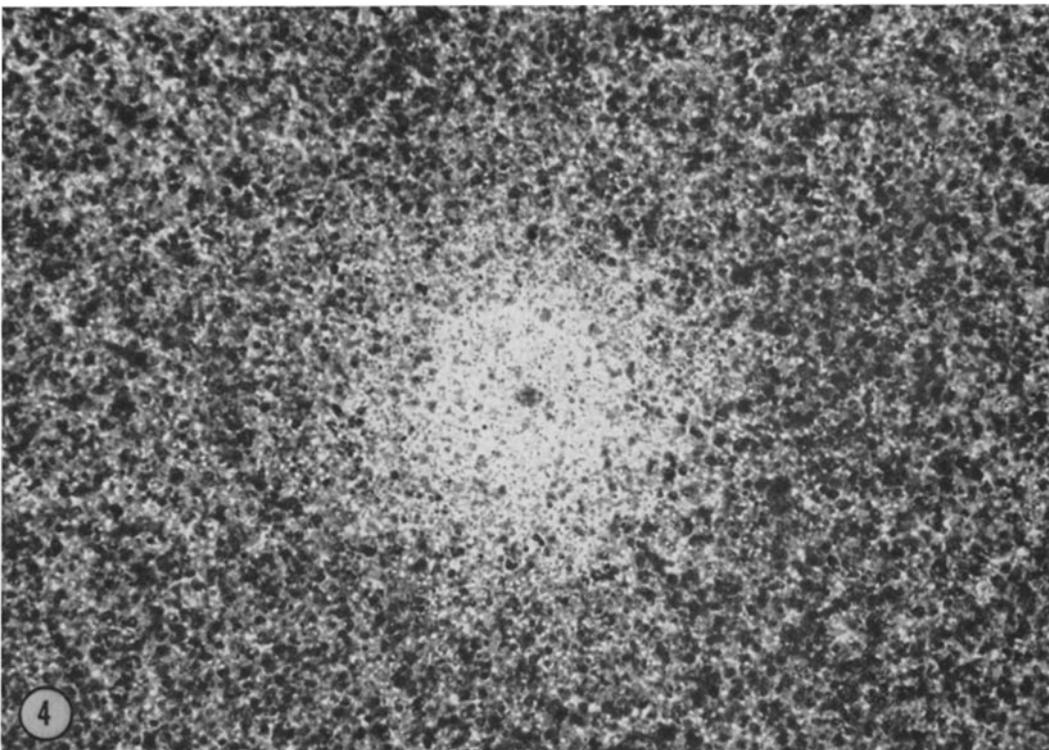
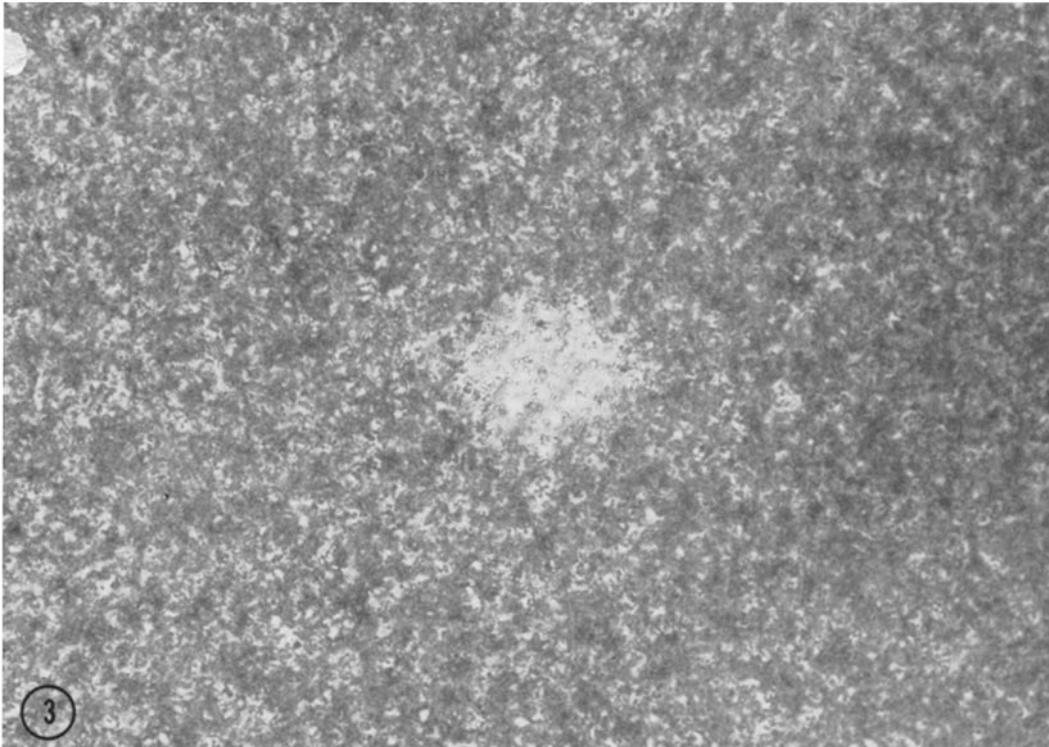


(Hildemann and Pinkerton: Alloantibody production and histoincompatibility)

PLATE 89

FIG. 3. Typical small alloimmune plaque produced by C57BL/6 mouse spleen cells against A/J erythrocytes in presence of absorbed guinea pig complement at 6 days after immunization. Note single spleen cell in center of plaque. Compare with Figs. 1 and 2 at same magnification. Approximately $\times 100$.

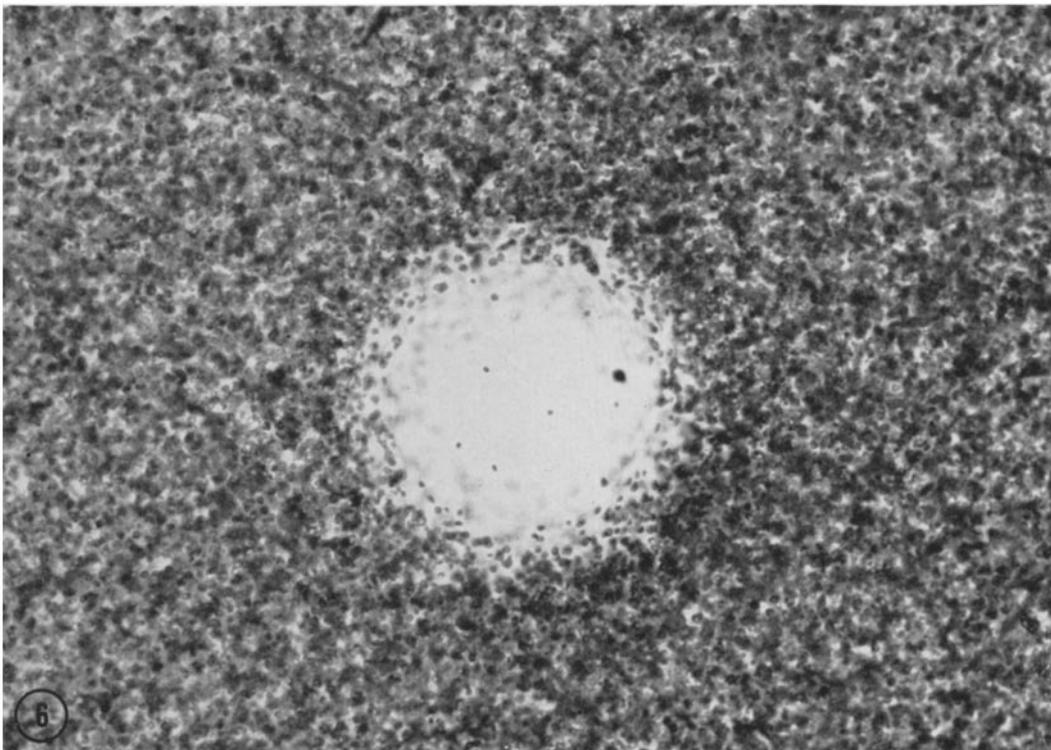
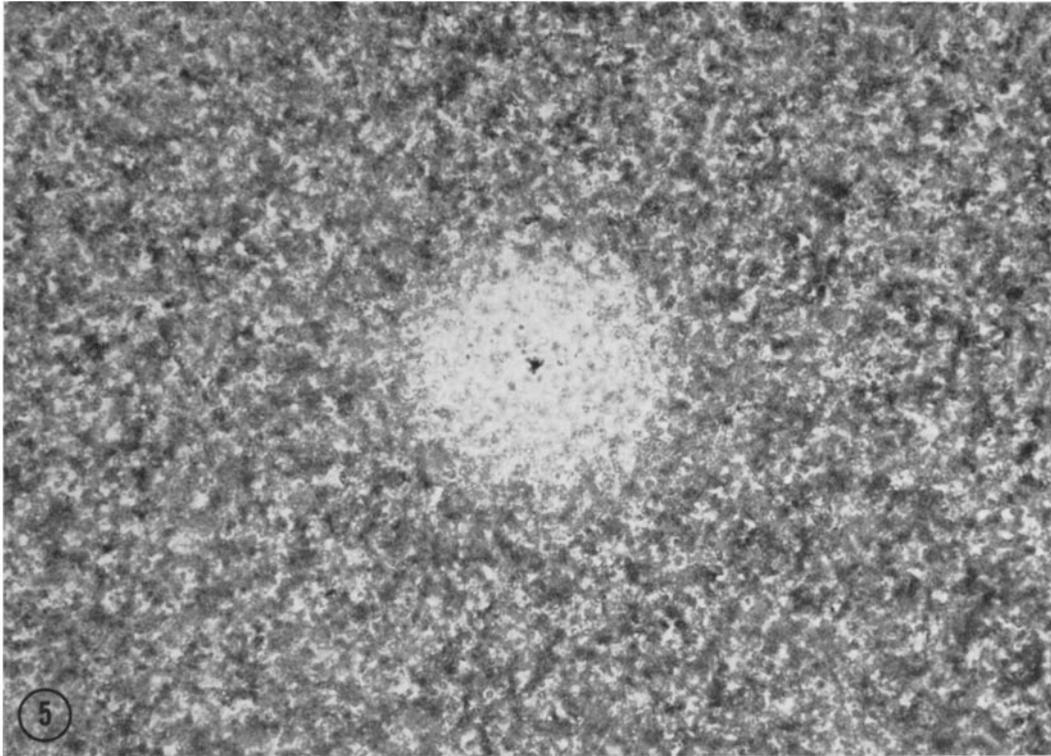
FIG. 4. An individual alloimmune plaque produced by MHA hamster spleen cells against BRC erythrocytes in presence of absorbed guinea pig complement at 13 days after immunization. Approximately $\times 100$.



(Hildemann and Pinkerton: Alloantibody production and histoincompatibility)

PLATE 90

FIGS. 5 and 6. Individual alloimmune plaques produced by MHA hamster spleen cells against BRC erythrocytes in presence of absorbed guinea pig complement at 13 days after immunization. Note substantial variation in margins and diffuseness of plaques in Figs. 4, 5, and 6 under magnification. Approximately $\times 100$.



(Hildemann and Pinkerton: Alloantibody production and histoincompatibility)