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Distribution of Antibody Plaque Forming Cells in Various Tissues of Several Strains of Mice Injected with Sheep Erythrocytes.* (29628)

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The localized hemolysin ("antibody plaque") technique in agar gel is a rapid and relatively simple procedure for enumerating the number of antibody forming cells in a mixed population of cells(1). This procedure has been utilized by Jerne(1,2) to study hemolysin formation *in vitro* by spleen and lymph node cell suspensions from rabbits and mice injected with red cell antigens. Ingraham and Bussard have independently described a relatively similar localized hemolysin procedure whereby immunologically competent cells from spleens of immunized rabbits are suspended in tissue culture medium containing erythrocytes and guinea pig complement and "thickened" with carboxymethoxycellulose(3). These methods lend themselves well to the study of various aspects of antibody formation, including induction period(2,3,4), effect of immunosuppressive drugs(2,3,5) and X-irradiation(5), and role of subcellular fractions, including RNA, and ribosomes(5,6) in antibody synthesis. During several studies on antibody formation using the plaque technique(5), it was noted that various lymphoid tissues from mice injected by several routes with mammalian or avian erythrocytes were not equally efficient in plaque formation. Similarly, markedly different results were observed using different strains of inbred mice. This report is concerned with the estimation of the number of antibody plaque forming cells in various tissue suspensions from several strains of mice following primary immunization with sheep erythrocytes, using the agar plaque method of Jerne. The results indicate that there may be a marked variation in the number of plaque forming cells per given cell suspension according to strain of mouse used and whether or not lymphoid or non-lymphoid tissue is tested.

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Methods and materials. Animals. The following strains of mice were used: C_3HBL, C_{57}BL, AKR, and NIH. The mice were obtained either from Jackson Laboratory, Bar Harbor, Me., or on occasion, from a local dealer. The animals were housed in groups of 6 in plastic cages containing wood shavings. They were fed Purina mouse food and water *ad libitum.*

Immunization. Groups of 6 to 10 mice

from each strain were injected intravenously at 8 to 10 weeks of age with 5×10^9 freshly washed sheep erythrocytes (S-RBC) obtained from a single animal at a single bleeding.

Cell suspension preparation. At various times following immunization, 2 or more animals from each group were bled from the retro-orbital plexus to obtain peripheral leukocytes (buffy coat) and plasma for antibody titrations. The animals were sacrificed and various tissues, including spleen, lymph nodes, bone marrow, thymus, lung, liver, brain, thyroid and skeletal muscle, were obtained aseptically and placed into sterile, cold Hanks' solution, pH 7.2, containing 20% sterile calf serum. Excess fat was trimmed off the organs as necessary and the tissues cut into small pieces in sterile Hanks' solution. In the case of lymphoid tissue, the cells were obtained by "teasing" with fine dissecting needles in sterile Hanks' solution(7). Bone marrow cells were obtained by flushing Hanks' solution through the long limb bones. Cellular debris was removed by passage through sterile gauze. Lung, liver, and other solid tissues were first minced into small pieces and the cells forced through fine mesh, stainless steel screening. Cell suspensions from each of the tissues were kept separate and washed several times by serial centrifugation in cold Hanks' solution. The various cell preparations were resuspended as 10% suspensions (v/v) with sterile Hanks' solution. Viability of nucleated cells was determined by trypan blue dye exclusion tests. Total cell counts were estimated with a hemocytometer.

Antibody plaque method. The procedure developed by Jerne was used essentially as described(1,2). One-tenth ml cell suspension was rapidly mixed with 2.0 ml of 0.7% melted Noble agar, maintained at 48-50°C, to which had been added 1 mg DEAE-dextran (2.6×10^6 M.W.) and 0.1 ml 10% suspension freshly washed sheep erythrocytes. The warm cell-agar suspension was mixed thoroughly and then poured rapidly into a 3-mm thick base layer of solidified 1.4% agar previously prepared with sterile Hanks' solution in 100 mm round plastic petri dishes. Dilutions of each cell suspension were plated in duplicate. Plates were incubated for one hour at 37°C, then flooded with 5 ml guinea pig complement, diluted 1:10, and reincubated for 30 minutes at 37°C. Antibody plaques could be clearly visualized as zones of hemolysis against the light pink turbidity of unlysed erythrocytes. The plaques were made more distinct and the plates preserved for extended periods by flooding with benzidine-H_2O_2-acetic acid solution(2), which stained the intact erythrocytes a deep blue, leaving the plaques colorless. The number of plaques per plate was enumerated using a Quebec bacteria colony counter. The number of antibody secreting cells per number of cells plated was recorded.

Antibody titration. Plasma samples obtained from each mouse prior to sacrifice were heated at 56°C for 30 minutes and then diluted in saline in 0.025 ml volumes in micro titration plates using calibrated spiral loops. A 0.025 ml suspension of 0.25% washed sheep erythrocytes plus 2 units guinea pig complement was added to each dilution, followed by incubation for 45 minutes at 37°C. Titers were recorded as the reciprocal of the highest dilution which resulted in complete hemolysis of the added erythrocytes.

Results. Hemolysin response. Readily detectable hemolysins appeared within several days after immunization in the serum of all mice injected with sheep erythrocytes (Table I, II). However, NIH mice responded with the highest mean titers and reached a peak at 4 to 5 days following injection. C_3H, C_{57}, and AKR mice responded with lower mean titers. However, the peak titers with these strains were also at 4 to 5 days post injection.

Plaque formation with lymphoid tissue. Lymphoid cell suspensions from all mice injected in this study with S-RBCs revealed antibody plaque forming ability when tested in agar plates containing sheep red cells. However, there was a marked difference in the number of plaques formed per million nucleated cells plated with various lymphoid tissues from the various strains tested (Table I, II). Spleen and lymph node suspensions

TABLE I. Antibody Plaque Formation by Various Tissue Suspensions Obtained from Mice Injected I.V. Four Days Previously with 10^9 Sheep Erythrocytes.

Strain	No. of mice tested	Hemolysin titer: Mean peak	Hemolysin titer: Range	Hemolysin titer: Peak day	Antibody plaque formation per 10^6 cells with various tissue suspensions*: Spleen	Pooled lymph nodes	Peripheral leukocytes	Thymus	Bone marrow	Lung	Liver	Brain	Kidney	Skeletal muscle
NIH	68	1:825	1:240–1:2560	5	786	451	38	5	3	16	14	2	3	1
C_3H	64	1:461	1:64 –1:2560	5	314	270	21	2	5	18	10	1	1	2
C_{57}	51	1:385	1:16 –1:1280	6	270	184	14	3	6	7	14	1	1	3
AKR	55	1:135	1:8 –1:768	6	94	48	7	3	3	8	2	2	1	2

* Tenth ml of dilutions of 10% cell suspension containing approximately 10^7 viable nucleated cells per ml used for plating.

from S-RBC injected NIH mice formed the largest number of plaques. At 4 days after immunization, NIH spleen cell suspensions formed nearly twice as many antibody plaques as cell suspensions from popliteal, axillary, and mesenteric nodes. NIH thymus cell suspensions, on the other hand, revealed only a low level of plaque formation (about 4 to 5 plaques per 10^6 cell). Circulating leukocytes yielded about 20 to 40 plaque forming cells per 10^6 nucleated cells plated. Bone marrow cells were essentially negative, with less than 2 plaques per million nucleated cells. Cell suspensions from C_3H and C_{57} mice yielded about one-half the number of plaques per million nucleated lymphoid cells as compared to NIH cells (Table I, II). AKR mice yielded lymphoid cell suspensions which were markedly deficient in antibody plaque formation. Most plaques formed with AKR cells were quite small in comparison to plaques formed by cells from the other 3 strains.

Plaque formation with other tissue. All non-lymphoid tissues tested were relatively poor in antibody plaque forming ability. Preparation of cell suspensions from solid tissue was relatively difficult. The number of nucleated and viable cells (trypan blue assay stain) could be only roughly estimated using fresh, unfixed suspensions. All suspensions were tested as 10% v/v preparation, as well as in dilutions based on cell count estimation using a hemocytometer. Lung and liver cell suspensions prepared from NIH mice 4 days after immunization yielded about 10 to 15 plaques per 10^6 total cells plated, while suspensions prepared from homogenates of muscle, brain and kidney resulted in about 2 plaque forming cells per 10^6 cells plated (viability ranged between 70 to 80% for freshly prepared samples). Suspensions from the other strains tested were proportionally lower in plaque forming capacity (Table I, II).

Time of appearance of plaque forming cells. Study of the distribution of antibody plaque forming cells in relationship to time following I.V. injection of sheep erythrocytes revealed that the spleen was the first organ to show a marked rise in number of plaque

TABLE II. Typical Anti-Sheep RBC Hemolysin Titers and Antibody Plaque Formation by Spleen and Lymph Node Cell Suspensions from NIH and C_{57} Mice Injected I.V. with 10^8 Sheep Erythrocytes.

	NIH mice				C_{57} mice			
Day after injection	No. of mice tested	Mean peak hemolysin titer	No. of plaques/10^6 cells: Spleen	Lymph node	No. of mice tested	Mean peak hemolysin titer	No. of plaques/10^6 cells: Spleen	Lymph node
0*	10	<1:10	2	1	17	<1:10	0†	0†
1	17	<1:10	42	16	11	<1:10	18	12
2	16	1:83	134	38	15	1:17	38	18
3	15	1:640	395	119	15	1:88	212	40
4	16	1:936	480	240	14	1:364	242	68
5	10	1:840	472	160	18	1:360	216	51
6	19	1:320	210	148	12	1:290	105	42
10	12	1:300	140	130	11	1:278	78	30
15	10	1:72	33	17	10	1:64	17	20

* Pre-injection titer and plaque count. † Less than 1 per 10^6 viable nucleated cells plated.

forming cells, regardless of strain. Prior to immunization there was a "background" of 2 to 3 plaques per 10^6 spleen cells (about 100-150 plaque forming cells per spleen) with NIH mice. The background count was much lower with lymph node cells (4 to 5 plaques per 10^9 cells). Background was even lower with the other strains. Within a day after immunization, there was a readily detectable increase of antibody plaque formation. By the fourth to fifth day after injection, when hemolysin titers were reaching a peak, there was a sharp rise in number of plaque forming cells per splenic tissue (Table II). Spleen cell suspensions from NIH mice yielded an average of 1 plaque forming cell per 2000 cells 4 days after immunization. Spleen cell suspensions from C_{57} and C_3H mice resulted in one plaque forming cell per 4 to 5000 cells 4 days after injection. Spleen cells from AKR mice resulted in the poorest plaque formation, with about one plaque per 10,000 cells at the fourth to fifth day after immunization. Cell suspensions prepared from pooled lymph nodes resulted in about one-half the number of plaques formed per number of viable nucleated cells plated, as compared to spleen cell suspensions. However, the peak number of plaques with lymph nodes was also at the fourth day (Table II). The background, pre-immunization plaque count with solid tissue suspensions was nearly undetectable (1-4 plaques/10^8 cells plated). Although the count at 4 days post injection was still low (Table I), the number of plaques signified a marked increase.

Discussion. The localized hemolysin procedures developed by Jerne(1,2) and by Ingraham and Bussard(3) permit rapid evaluation of the relative number of antibody releasing cells in a given population of mixed cells. Whereas other techniques, such as fluorescent microscopy or single cell micro drop procedures, are relatively time consuming and laborious, the antibody plaque technique can be performed without specialized equipment or material. Using this method, it has been shown in several studies(1-5) that the number of cells actively secreting hemolysins increase rapidly in spleens and lymph nodes of animals receiving an initial injection of sheep erythrocytes. Formation of these zones of hemolysis in agar containing erythrocytes and complement is related to the immune status of the donor and the number of cells plated (1-5). The number of plaques formed per number of cells plated is also related to the dosage and route of immunization, as well as to the presence or absence of agents such as protein or nucleic acid antagonists, X-irradiation, adjuvants, and the like.

The observations reported here using the antibody plaque method indicate that the total number of hemolysin secreting cells in a population of lymphoid cells varies markedly according to strain of mouse. This should not be surprising since it is widely recognized that various strains of mice may

have markedly different immunologic responses following primary and secondary immunization with soluble or particulate antigens. However, the level of circulating antibody need not be related to the total number of antibody secreting cells. Rate of antibody formation per individual cell, rate of release of newly formed antibody, gamma globulin catabolism, and binding of free antibody to tissue may vary from strain to strain. These factors can only be estimated by measuring levels of circulating antibody. The experiments reported here indicate that variations in serum hemolysin titers among the strains tested may be related to the number of hemolysin secreting cells per animals. Serum hemolysin titers in these experiments apparently are a reflection of the number of antibody secreting lymphoid cells per animal. This suggestion is strengthened by additional observations, not reported here, that spleen cell suspensions from 4 different inbred strains of hamsters result in different degrees of antibody plaque forming capacity following immunization with sheep erythrocytes.

Plating of cell suspensions obtained from various other mouse tissues besides spleen and lymph nodes indicated that hemolysin secreting cells were either absent or in low concentration. It is difficult to prepare cell suspensions from solid tissues and still maintain viability and biologic activity. This may account for the low plaque counts. The low count may also reflect the relatively low number of lymphoid cells in suspensions prepared from solid tissue. It should be noted that significant numbers of antibody plaque forming cells were detected among circulating leukocytes obtained from venous blood specimens. Very few plaques were obtained with thymus or bone marrow cells.

The results of the experiments reported here are being used as guides for further work on formation of antibody plaques in agar with subcellular particles, such as ribosomes, from immune spleen cell suspensions, and for studies of the transfer of antibody plaque forming capabilities to non-immune spleen cells *in vivo* and *in vitro* by means of subcellular extracts, including RNA fractions, from immune lymphoid cells(5).

Summary. Lymphoid cell suspensions from several strains of mice injected with a single inoculum of sheep erythrocytes were capable of forming localized zones of hemolysis ("antibody plaques") in agar containing sheep erythrocytes and complement. The ability of lymphoid and non-lymphoid tissue from immunized NIH, C_3H, C_{57}, and AKR mice to form antibody plaques in agar were compared. Optimum time for plaque formation with the strains tested was 4 to 5 days following intravenous immunization. Cells from NIH mice resulted in the most consistent and highest levels of plaque formation. C_3H and C_{57} mice yielded lymphoid cells which were less than half as efficient in plaque forming ability as compared to NIH cells. Lymphoid cell from AKR mice had the least plaque forming capability. Spleen cell suspensions formed the largest number of plaques following I.V. injection. Lymph node cell suspensions were somewhat less efficient. Suspensions of peripheral leukocytes (obtained from "buffy coats") had low, but significant antibody plaque forming ability. Thymus and bone marrow cells had no significant plaque forming ability. Cell suspensions prepared from lungs, kidney, liver, brain, and skeletal muscle exhibited either low levels of plaque formation or were negative.

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