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11. Supported by: American Cancer Society grant IN-53-D, NSF grant GB-747, PHS graduate training grant 5T1 GM 818-03, and PHS grant HD 00104-04. We are indebted to Mary Bens for her assistance.

24 August 1964

Antibody Plaque Formation by Normal Mouse Spleen Cell Cultures Exposed in vitro to RNA from Immune Mice

Abstract. *Suspensions of normal spleen cells from nonimmune mice were treated in vitro with RNA extracted from spleen cells from donor mice immunized 4 days previously with sheep erythrocytes. Subsequent incubation of the RNA-treated cells in tissue culture medium at 37°C for several days resulted in a marked increase in the number of localized zones of hemolysis ("antibody plaques") in relation to the number of viable cells plated in agar containing sheep erythrocytes and complement. Nonimmune cells maintained in tissue culture medium did not form plaques after incubation with either RNA from immune mice or ribonuclease-treated RNA from immune mice, or with RNA from nonimmune donor mice, or from donors immunized with chicken erythrocytes or bovine serum albumin.*

The localization of hemolysis in semisolid medium is the basis of a method for rapid estimation of the number of antibody-secreting cells present in a suspension of lymphoid cells (*1*). This procedure has been used successfully to study the kinetics of hemolysin formation by lymph node and spleen cells from experimental animals after primary or secondary immunization with sheep erythrocytes (*1*, *2*).

Cohen and Parks recently reported that spleen cells from normal B_6AF_1 mice acquired hemolysin-forming activity after incubation in vitro with RNA extracted from spleens of isologous donor mice immunized with sheep erythrocytes (*3*). They observed a three- to fivefold increase in the number of antibody plaques formed by nonimmune cells plated in agar immediately after 30 minutes incubation with RNA from immune donors. Before incubation, there were on the average 20 to 25 plaques formed with the total number of spleen cells plated from five nonimmune mice. After treatment with splenic RNA from 20 immunized donor mice, the number of plaques increased to an average of 90. Prior treatment of RNA from immune donors with ribonuclease inhibited this effect.

Spleen cells from nonimmune NIH albino mice also acquire hemolysin-forming activity after brief incubation with RNA extracted from spleen cells from donor NIH mice immunized with sheep erythrocytes (*4*). The number of specific antibody plaques formed by normal spleen cells increased 10- to 20-fold after treatment with RNA from immune donors. Maximum plaque formation occurred after treatment with RNA obtained 4 days after donor immunization. RNA obtained from donors immunized with bovine serum albumin or treated with actinomycin D prior to and after injection with sheep red blood cells did not induce plaque formation in normal cell suspensions (*4*).

This report confirms and extends the foregoing observations and describes results obtained with tissue cultures of normal mouse spleen cells after treatment in vitro with RNA from donor mice immunized with sheep erythrocytes. For these experiments, spleen cell suspensions from normal NIH mice were incubated in vitro with: (i) RNA extracted from spleen cells from mice immunized with sheep red blood cells; (ii) ribonuclease-treated or deoxyribonuclease-treated RNA from immune donors; and (iii) RNA from nonimmune donors or from donors injected with an unrelated antigen such as bovine serum albumin or chicken erythrocytes. The immune donor mice were 6- to 10-week-old NIH males injected intraperitoneally with 0.1 ml of a 20 percent suspension of fresh, washed, sheep red blood cells in buffered saline. Four days later, at the peak of antibody plaque formation, serum samples were obtained by retro-orbital puncture. Hemagglutinins and hemolysins to sheep erythrocytes were determined by standard serial dilution procedures with a microloop technique with 0.025 ml volumes of both serum dilutions and 0.5 percent sheep red blood cells (*5*).

Those mice with serum titers of 1:512 or higher were killed, and their spleens were immediately frozen at −60°C with dry ice. Prior to freezing, spleen samples, approximately 5 mm^3, were chosen at random and used for preparation of cell suspensions to determine the number of antibody-forming cells per million viable nucleated donor spleen cells (*1*). RNA was extracted from the frozen spleens as follows (*6*). The spleens were weighed and suspended in three volumes of cold 88 percent phenol containing 4.3 mg of bentonite powder per milliliter. To this mixture was added an equal volume of cold 0.02*M* phosphate buffer, *p*H 7.2, containing 0.01*M* sodium EDTA (ethylenediaminetetraacetate). The mixture of spleen cells, phenol, and buffer was homogenized at 0°C for 10 minutes with a high-speed VirTis tissue homogenizer. The aqueous phase was separated by centrifugation at 2400*g* for 15 minutes in the cold and was freed of phenol by five extractions with equal volumes of cold ethyl ether. Two volumes of absolute ethanol were added to the concentrated extract. The precipitate was recovered by centrifugation at 1200*g* for 10 minutes in the cold. The resulting pellet was suspended in 5.0 ml of cold sterile phosphate buffer. The RNA concentration was estimated by ultraviolet spectrophotometry and by the orcinol reaction (*7*).

As controls, 40 μg of ribonuclease or deoxyribonuclease was added to 1.0 ml samples of extract, and the mixtures were incubated at 26°C for 30 minutes and then chilled in an ice bath. A separate 1.0-ml sample was heated at 100°C for 10 minutes. As additional control preparations, RNA-rich material was extracted from spleens from nonimmunized normal mice and from mice injected 4 to 6 days previously either with chicken erythrocytes (0.1 ml of a 20 percent suspension injected intraperitoneally) or with bovine serum albumin (BSA) (0.2 ml of an emulsion of 10.0 mg of

BSA per milliliter of complete Freund's adjuvant injected subcutaneously).

Normal "recipient" spleen cell suspensions for incubation with RNA were obtained from nonimmunized adult NIH mice, 6 to 12 weeks of age, whose serum had no demonstrable hemolysins or agglutinins to sheep erythrocytes. Suspensions containing 20×10^6 to 5×10^5 viable nucleated cells per milliliter of sterile Hanks solution (*p*H 7.2) were prepared, trypan blue dye exclusion tests being used for estimation of viability and a hemocytometer for cell counts. In several experiments, portions of normal cell suspensions were heated at 60°C for 30 minutes prior to treatment with RNA.

For incubation, 1.0 ml of RNA extract was added to 1.0 ml of suspensions of the nonimmune spleen cells in sterile Medium 199 (Hanks base), *p*H 7.2, containing penicillin (100 μg per milliliter). The RNA-cell suspensions were incubated in slowly rotating roller tubes at 37°C for 30 minutes. At the end of this time, the tubes were centrifuged at 2000*g* in the cold. The sedimented cells were washed twice with sterile medium, suspended in 5 ml of medium and incubated at 37°C. Samples were obtained prior to incubation, at the end of the first 30 minutes of incubation, and at the end of 2, 6, 24, 48, 72, 96, and 144 hours of incubation. Each sampling was used for preparation of a smear for microscopic examination and for determination of total cell count and viability. A small sample was stored in the cold for determination of hemolysins to sheep cells by the microloop technique.

One tenth of a milliliter of each cell suspension remaining from that obtained during the sampling periods was carefully added to 2.0 ml of melted Noble agar (0.7 percent), maintained at 48° to 52°C, containing 1 mg of diethylaminoethyldextran and 0.1 ml of a 10 percent suspension of freshly washed sheep erythrocytes. After gentle mixing, the warm agar suspension was carefully layered over a base (3 mm thick) of 1.4 percent Noble agar in a plastic petri dish (100 mm diameter). The plates were incubated at 37°C for 1 hour and then further incubated for 30 minutes with 5 ml of 1:10 guinea pig complement. Antibody plaques were readily seen as small zones of hemolysis against a light pink background of unlysed red cells. Plaques were made more distinct by treatment of the plates with a cold freshly prepared solution of benzidine, hydrogen peroxide, and acetic acid (*1*). The number of antibody plaques counted in relation to the total number of viable nucleated cells plated from each culture tube was recorded.

The results obtained in a typical series of experiments are presented in Table 1. When 10 to 20×10^6 spleen cells from nonimmune donor mice were treated in tissue culture medium with 20 μg of RNA from immune donors, the number of plaques generally increased five- to tenfold within 30 to 60 minutes after initial incubation. The number of plaques increased sharply after 24 to 48 hours of incubation in tissue culture medium. Plaque formation reached a plateau and then decreased after 72 and 96 hours. Cell viability varied from 75 to 85 percent during the first 2 to 3 days, then decreased markedly, as indicated by the trypan blue stain. Few if any cells appeared viable after 4 to 5 days incubation. In no instance was there serologic evidence of hemolysins or hemagglutinins to sheep erythrocytes in any supernatant fluids from RNA-treated spleen cell cultures. Nor did serologic tests reveal any antibody activity or detectable erythrocyte antigens in the RNA extracts from the immune donors.

Table 1. Number of antibody plaques formed by 10^6 viable tissue culture cells of normal mouse spleen suspensions incubated for various time periods at 37°C after prior treatment in vitro with splenic RNA from immune or control donor mice. Each figure represents average plaque count of four to six samples. Nucleated spleen cells (20×10^6 per milliliter Medium 199) cultured at 37°C after incubation with 1.0 ml RNA extract (15 to 20 μg).

RNA donor immunization*	RNA treatment	Hours								
		0	0.5	2	6	24	48	72	96	144
Nonimmune mouse spleen (nontreated)										
Sheep RBC	None	2	16	25	38	85	98	90	47	12
Sheep RBC	Ribonuclease	1	3	7	3	4	6	2	0	1
Sheep RBC	Deoxyribonuclease	3	12	33	46	88	95	98	26	6
None	None	1	3	2	5	2	1	1	0	0
Chicken RBC	None	2	3	2	5	1	3	0	0	0
BSA	None	0	2	3	4	2	3	0	0	1
No RNA		1	0	0	3	3	1	2	0	0
Nonimmune mouse spleen (heated 56°C, 30 min)										
Sheep RBC	None	0	1	0	3	0	2	0	0	2
Sheep erythrocyte immunized mice										
No RNA		213			276	390	486	520	512	395

* RNA obtained by phenol extraction (*6*) of spleen cells from donor mice immunized either with sheep or chicken erythrocytes (RBC) or with bovine serum albumin.

Table 2. Number of antibody plaques formed by 10^6 tissue culture cells of normal spleen after treatment in vitro with graded concentrations of RNA extracted from mice immunized 4 days previously with sheep erythrocytes. Normal spleen cells (NSC) (20×10^6) in 1.0 ml of Medium 199 incubated with a 1.0 ml volume of RNA extract.

RNA concn. added to NSC (μg)	Hours			
	0	2	24	48
350.5	2	11	26	31
34.5	1	18	52	56
19.1	0	34	78	98
9.7	2	20	70	84
3.4	2	10	7	7
None	3	1	2	1

Incubation of a 1.0-ml suspension of 20 million spleen cells from the normal mouse with an equal volume of undiluted RNA extract containing 250 to 350 μg was not as effective in inducing subsequent plaque formation as incubation with a 1:10 to a 1:20 dilution of the extract. A greater dilution was less effective (Table 2). Incubation of lower numbers of cells with undiluted RNA extract was similarly less effective than incubation with more diluted extract.

Treatment of the normal spleen cell suspensions with RNA from immune donors, followed by centrifugation and washing with medium to remove excess RNA, had little advantage over uninterrupted incubation with RNA. Treatment of RNA from immune donors with ribonuclease or with heat at 100°C for 10 minutes prior to incubation with spleen cells interfered with induction of plaque formation (Table 1). Treatment with deoxyribonuclease had no inhibitory effect. Incubation of normal cells with RNA from nonimmune donors or from donors immunized previously with chicken erythrocytes or bovine serum albumin failed to result in plaque formation toward sheep erythrocytes (Table 1). Specifici-

ty of plaque formation was further demonstrated in several experiments by incubating normal spleen cells with RNA from donors immunized to sheep cells and then by plating of the cells in agar containing either sheep or chicken red blood cells. Plaques were observed only in the plates containing the sheep erythrocytes.

The increase in the number of plaque-forming cells in cultures of normal spleen cells treated in vitro with RNA extracts from immune donor mice suggests that the ability to synthesize sheep hemolysin had been actively induced. The continued increase during incubation in tissue culture medium may have occurred either by an increase in the total number of cells responding to the RNA, or by proliferation of the initial number of cells which were "activated" by RNA. Removal of RNA from the cultures by several washings with buffer had no detectable effect on plaque formation.

The results presented here do not offer a definitive explanation concerning the mechanism of the acquisition of antibody plaque-forming activity by normal spleen cells treated in vitro with RNA from immune donors. They do, however, extend previous observations on acquisition of immunologic capabilities by nonimmune individuals (*8*) and by normal lymphoid cells after treatment with RNA or subcellular fractions derived from immunized donors (*3, 4, 9*). Whether RNA extracts contain an antibody "coding" mechanism, an immunogenic antigen–nucleic acid complex, or an antibody precursor which may induce immunologic activity in nonimmune cells is not known. However, experiments with gradient centrifugation and chromatography techniques suggest that the RNA in the extracts used in this study has properties consistent with low molecular weight RNA (*10*).

These experiments, together with those reported by others (*1, 2*) demonstrate that localized antibody plaque formation in gel is a valuable procedure for investigation of antibody production by cells cultured in vitro as well as by cells obtained directly from animals immunized with foreign erythrocytes.

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17 September 1964

Enzootic Sendai Virus Infections in Mouse Breeder Colonies within the United States

Abstract. *Naturally occurring enzootic infections of Sendai virus have been detected by serologic monitoring and virus isolation in mouse breeder colonies from five states in the United States of America. Six of 21 colonies tested were found to be infected with Sendai virus, and the mean incidence of infection within infected colonies was 76 percent. Forty-one of 120 attempts to isolate the virus were successful.*

Sendai virus belongs to *Myxovirus parainfluenzae* type 1 and is identical with newborn pneumonitis virus, hemagglutinating virus of Japan (HVJ), and influenza D virus (*1*). Although the related human pathogen, hemadsorption virus 2, is worldwide in distribution, naturally occurring infections with Sendai virus have been reported only from Japan, China, and the U.S.S.R. (*2*). The infection was reported to be distributed widely among laboratory mice in Japan (*1*), and the virus has been isolated from mice, swine, and hamsters (*3*). Sendai virus is the only member of the parainfluenza virus group known to naturally infect mice. The question of human infection by Sendai virus has been obscured by technical problems in virus isolation and by serological cross reactivity with other parainfluenza viruses, although one apparently valid virus isolation has been made (*4*).

During studies to determine the cause of high mortality rates in suckling mice obtained from various breeders and held in our experimental animal-holding facility, it was discovered that serums from many of the mice inhibited hemagglutination by Sendai virus. Concurrently, during routine serologic monitoring of mouse breeder colonies (*5*), a mouse breeding colony was found to have a high incidence of hemagglutination-inhibition (HI) antibody for Sendai virus. Since naturally occurring Sendai virus infection heretofore had not been reported in the United States, studies were undertaken to identify further the infecting agent and to determine to what extent the virus was present in other mouse breeder colonies within the United States.

The Sendai/52 strain of Sendai virus was obtained from R. M. Chanock, and two stock pools of virus were prepared for use in serologic testing, the virus for one pool being cultured in kidney tissue of the Rhesus monkey, and the virus for the other, in chick allantoic fluid. Mice used in this study were obtained from various commercial and institutional mouse breeder colonies. A minimum of 25 mice, and in most cases more than 50 mice, were tested from each colony. Mice tested were of both sexes and generally were more than 5 months of age. Individual mouse serums were prepared as previously reported (*6*). All serums were heated at 56°C for 30 minutes prior to testing. For screening the mouse serums we conducted hemagglutination-inhibition and complement fixation tests, utilizing Microtiter (*7*) serological plates. Hemagglutination-inhibition tests were performed at room temperature, four hemagglutination units of antigen and human type O RBC being used, and the results were read by the pattern method (*6*).

Virus isolations were performed in duplicate in both embryonated hens' eggs and tissue culture. For each isolation, 0.05 ml of a 10-percent homogenate of mouse lung was inoculated amniotically into 8-day-old embryonated hens' eggs and the amniotic fluids were tested for hemagglutinins 4 days later. Positive hemagglutinating fluids were tested by hemagglutination-inhibition with antiserum specific for the Sendai virus prepared in germfree mice. Test tubes of primary cultures