ACQUISITION OF ANTIBODY PLAQUE FORMING ACTIVITY BY NORMAL MOUSE SPLEEN CELLS TREATED IN VITRO WITH RNA EXTRACTED FROM IMMUNE DONOR SPLEENS.*

Herman Friedman
Departments of Microbiology, Albert Einstein Medical Center and Temple University School of Medicine, Philadelphia, Penna.

Received July 28, 1964

During recent years there have been several attempts to demonstrate acquisition of immunologic activity by either normal or immunologically incompetent animals following transfer of subcellular fractions prepared from antibody forming cells (Stavitsky, 1961, Cochrane and Dixon, 1962). In most cases, interpretation of results has been complicated by possible active antibody formation of recipients to antigens which may have been transferred with the fractions.

Recently, Jerne described a procedure whereby lymphoid cell suspensions from animals immunized with foreign erythrocytes form localized zones of hemolysis ("antibody plaques") when incubated in vitro in semi-solid agar containing red blood cells and complement (Jerne and Nordin, 1963). Using this procedure, Cohen and Parks reported a significant increase in antibody plaque formation by normal B_6AF_1 mouse spleen cells treated in vitro with splenic RNA extracted from donor mice previously immunized with sheep erythrocytes (Cohen and Parks, 1964). The pre-treatment "background" count of 20 to 25 plaques per 5 normal spleens was increased to an average of 90 to 100 plaques following in vitro incubation for 30 minutes at 37°C with RNA extracted from 20 immune donor mouse spleens. This report is concerned with results of experiments demonstrating acquisition of plaque forming ability by normal mouse spleen cells exposed to RNA extracts obtained at varying time intervals after a primary immunization of donor mice with sheep red blood cells. NIH mice were used in this study since they have been shown to produce high levels of anti-sheep hemolysins and form antibody plaques much more readily than more highly inbred strains of mice used by others (Friedman, 1964).

Experimental

Groups of donor adult male NIH mice were injected intraperitoneally (I.P.) with 0.5 ml 20% washed sheep erythrocytes (S-RBC). At periodic intervals after

*Supported in part by grants from the National Science Foundation (G-19581, GB-1188).

immunization (1, 2, 3, 4, 6, 10 and 14 days) three or more mice were bled from the retro-orbital plexus to obtain serum specimens for hemolysin titrations. The animals were then sacrificed and spleens obtained. A small sample from each spleen (approximately 5 mm^3) was kept separate for preparation of cell suspensions to determine the number of antibody plaque forming cells per total number of cells (Jerne and Nordin, 1963). The bulk of each spleen was quickly frozen with dry ice. RNA was prepared by phenol extraction (Gierer and Schram, 1955; Maloney, 1963). The RNA extracts were tested for hemolysins and for S-RBC antigenic determinants using standard serologic procedures. One ml extract (equivalent to RNA extracted from one donor spleen) was added to 1 ml freshly washed spleen cell suspensions (2 - 9 x 10^7 viable nucleated cells in sterile Medium 199) obtained from 6 to 8 week old normal, non-immunized adult male NIH mice. The mixtures were incubated in roller tubes at 37°C for 60 minutes. Following incubation, the RNA treated cell suspensions were washed twice in sterile Hanks solution, pH 7.2. A tenth ml of each suspension was then added to melted agar containing sheep erythrocytes and complement according to the antibody plaque procedure described by Jerne et al (Jerne, Nordin and Harvey, 1963). Resulting plaques could be visualized as small zones of hemolysis against the light pink background of unlysed erythrocytes. The number of plaques per 10^6 cells plated was recorded. As controls, samples of RNA extract were treated with RNAase (40 ug per ml) or DNAase (50 ug per ml) for 30 minutes at 28°C, followed immediately by chilling at 0°C.

Results

NIH mice injected with S-RBC responded with a rapid appearance of anti-sheep hemolysins, reaching a peak titer at 4 to 5 days post injection (Table I). At the same time, spleen cells prepared directly from groups of mice injected with S-RBC formed relatively large numbers of antibody plaques (Table I). For example, spleens obtained prior to S-RBC injection revealed only slight, if any, detectable plaque formation (averaged 2-3 plaques per 10^6 viable nucleated cells). The plaque count increased to 50 to 200 plaques per 10^6 cells one to 2 days after immunization, and reached a peak of 400-800 plaques per 10^6 cells at 4 to 5 days. The count decreased at the 7th day, reaching a low level of 10 to 80 plaques per 10^6 cells plated at 10 days. Incubation of normal spleen cells from non-immunized mice with the RNA-rich extracts prepared from donor mouse spleens at various time intervals after immunization with S-RBC, followed by plating in agar with sheep erythrocytes, resulted in varying numbers of plaques (Table I). Whereas incubation with WNA from non-immune donors did not result in significant plaque formation by normal cells, treatment of the same number of cells with RNA obtained 24 hours after primary immunization of donor mice with sheep red cells resulted in readily detectable plaque formation (8 to 17

plaques). The number of plaques per 10^6 normal spleen cells increased after treatment with RNA obtained at 48 and 72 hours post injection. Incubation with RNA obtained 96 hours after immunization resulted in maximum numbers of plaques.

Table I.

Antibody plaque formation by spleen cells from mice immunized with S-RBC and by normal mouse spleen cells following incubation in vitro with RNA extracted from donor mouse spleens at varying times after immunization.

Donor Mice*	Donor Hemolysin Titers (Means)	Direct plaque count per 10^6 spleen cells from immune mice.		Plaque count per 10^6 normal spleen cells after incubation with RNA**	
		Mean Count	Range	Mean Count	Range
None Immune	< 1:10	2	1-7	1	0-3
Immune + 24 hours	1:13	38	21-96	11	8-17
Immune + 48 hours	1:46	138	101-260	19	10-31
Immune + 72 hours	1:230	342	180-460	33	19-39
Immune + 96 hours	1:423	612	380-790	40	23-58
Immune + 144 hours	1:450	401	29-640	38	19-52
Immune + 240 Hours	1:122	17	11-80	2	0-11

* Immune mice injected I.P. with 5×10^9 S-RBC at indicated time interval prior to sacrifice, at least 3 to 4 donor mice per group tested.

** Immune RNA obtained for incubation with normal spleen cells at time interval indicated after immunization of donor mice with S-RBC.

Specificity of antibody plaque formation was demonstrated by incubating normal spleen cells in vitro with RNA obtained from mice immunized either with chicken erythrocytes (0.2 ml 20% C-RBC I.P.) four days previously, or with bovine serum albumin (BSA) 10 days previously (0.2 ml 10 mg BSA per ml complete Freund's adjuvant). Spleen cells from C-RBC or BSA immunized mice did not form plaques in agar containing sheep erythrocytes only (Table II). Normal spleen cells incubated with RNA obtained from mice immunized with chicken red cells were found to form plaques in agar containing chicken erythrocytes, but not sheep RBCs. No plaques were formed in either chicken or sheep agar plates with normal spleen cells treated with RNA prepared from mice immunized with BSA (Table II).

Table II

Antibody plaque formation in agar containing eithersheep or chicken RBCs by immune spleen cells from mice injected with sheep or chicken RBCs or BSA, and by non-immune normal spleen cells following incubation in vitro with RNA from the immunized donors.

Donor Mice*	Hemolysin titer		No. of Antibody Plaques per 10^6 spleen cells plated			
			Agar with Sheep Erythrocytes		Agar with Chicken Erythrocytes	
	Anti-Sheep	Anti-Chicken	Donor Spleen Cells	Normal Spleen Cells Treated with RNA**	Donor Spleen Cells	Normal Spleen Cells Treated with RNA**
Non-immune	<1:10	<1:10	3	2	1	0
S-RBC Immune	1:462	<1:10	264	36	4	0
C-RBC Immune	<1:10	1:264	3	0	114	12
BSA Immune	<1:10	<1:10	2	1	1	0

* Immune donors injected 4 days prior to sacrifice with either sheep (S-RBC) or chicken (C-RBC) erythrocytes, or 10 days previously with bovine serum albumin (BSA in Freund's adjuvant).

** Immune RNA from mice injected either with S-RBC, C-RBC or BSA.

Table III

Effect of various treatments of immune RNA on subsequent induction of plaque formation in normal spleen cells.

Treatment of Immune RNA*	No. of Plaques per 10^6 normal cells following incubation with indicated RNA	
	Mean Count	Range
None	40	17-53
RNAase (50 ug/ml)	2	1-12
DNAase (50 ug/ml)	43	16-59
Actinomycin-D (50 ug/ml)	39	21-52
"None" (Donors pre-injected with actinomycin-D**)	1	0-3

* Obtained from donor mouse spleens 4 days after immunization with sheep RBCs.

** Donors injected with antinomycin 1, 2 and 4 days prior to immunization with S-RBC; RNA obtained 4 days after immunization.

RNAase treatment of immune donor RNA (obtained 96 hours after immunization) prior to incubation with normal spleen cells suppressed expected plaque formation. Treatment with DNAase had no detectable effect (Table III).

Incubation of immune RNA in vitro with actinomycin-D (10 ug/ml) had no effect on antibody plaque inducing activity. However, prior treatment of prospective donor mice with 50 ug actinomycin-D (administered I.P. 1, 2 and 4 days prior to S-RBC immunization) completely suppressed direct antibody plaque formation by donor spleen cells (Table III). Actinomycin treatment also interfered with subsequent induction of plaque formation in normal cells by RNA extracted from the treated donors.

Discussion

The results reported here confirm and extend the observation of Cohen and Parks concerning acquisition of hemolysin forming capacity by normal mouse spleen cell suspensions incubated in vitro with RNA-rich extracts from immune spleen cells (Cohen and Parks, 1964). Whereas Cohen and Parks observed a 3 to 5 fold increase in plaque formation, the experiments reported here, using NIH mice, demonstrated a 20 to 40 fold increment in specific plaque forming ability following incubation of normal cells with immune RNA.

In addition, these experiments demonstrate that an increase in plaque forming ability by immune mouse spleen cells is reflected in an increased ability of extracted splenic RNA to "induce" plaque formation in non-immune normal cells. It appears unlikely that such induction is due primarily to antigenic determinants transferred with the RNA extracts. Incubation of normal spleen cells in tissue culture with RBCs does not induce antibody activity in vitro (Cohen and Parks, 1964; Friedman, 1964a). However, it has been observed in other experiments that treatment of normal spleen cells with immune RNA, followed by incubation in tissue culture medium at 37°C for a period of several days, results in a marked increase in the number of antibody plaques, as compared to the number obtained immediately after RNA treatment (Friedman, H., 1964b). Although immune RNA extracts in the study reported here were found to be free of serologically detectable antibody or antigens, the possibility exists that RNA extracts contain potent immunogenic nucleic acid-antigen complexes (Garvey and Campbell, 1957, Haurowitz, 1959, Friedman, 1963) which may rapidly convert normal cells to antibody secreters.

Treatment of the immune extracts with RNAase, but not with DNAase, completely suppressed biologic activity. Actinomycin-D treatment in vitro did not affect plaque induction activity. However, pre-treatment of prospective donor mice with actinomycin-D prevented plaque formation by donor spleen cells and also resulted in failure of subsequently extracted RNA to induce plaque formation by normal spleen cells. These observations suggest that production of new RNA is essential for antibody formation. These experiments also indicate that the antibody plaque procedure of Jerne is a valuable tool for studying subcellular mechanisms in antibody synthesis.

Acknowledgement

The excellent technical assistance of Mrs. Leony Mills during these experiments is acknowledged.

Summary

RNA rich extracts prepared from spleen cells from NIH mice immunized with sheep RBCs were incubated in vitro with suspensions of normal NIH spleen cells. Subsequent incubation in agar containing sheep erythrocytes and complement resulted in appearance of a significant number of antibody plaques. RNA obtained from donor mouse spleens 4 days after primary immunization (at the peak of hemolysin formation) was most efficient. Treatment of immune RNA extracts with RNAase, but not DNAase, inhibited this effect. Actinomycin-D treatment of donor mice in vivo, but not of RNA extracts in vitro, was capable of suppressing plaque inducing activity.

References

1. Stavitsky, A., Adv. in Immunol., 1: 211 (1961).
2. Cochrane, A., and Dixon, F., Adv. in Immunol., 2: 201 (1962).
3. Cohen, E. P., and Parks, J. J., Science, 144: 1012 (1964).
4. Garvey, J. S., and Campbell, D. H., J. Exptl. Med., 105: 361 (1957).
5. Gierer, A., and Schramm, M., Nature, 177: 702 (1956).
6. Friedman, H., Proc. Soc. Exp. Biol. Med., 113: 1040 (1963).
7. Friedman, H., Proc. Soc. Exp. Biol. Med., in press (1964).
8. Friedman, H., Science, submitted for publication, 1964.
9. Haurowitz, F., and Crampton, C. F., J. Immunol., 68: 73 (1957).
10. Jerne, N. K., and Nordin, A. A., Science, 140: 405 (1963).
11. Jerne, N. K., Nordin, A. A., and Henry, C., in Cell-bound Antibodies, 109, Wistar Institute Press, Phila., Pa. (1963).
12. Maloney, J. B., Acta Internat'l Contre. Cancre, 19: 250 (1963).