Influence of Metabolic Inhibitors on the Capacity of Spleen Cells to Form Hemolytic Plaques and Rosettes* (33989)

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(Introduced by C. A. Evans)

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The formation of hemolytic plaques by cells from animals injected with foreign erythrocytes has been shown to require an intact respiratory pathway (1–3). The necessity of active protein synthesis as a prerequisite of plaque formation has been studied and contradictory results were reported (1, 4, 5). Despite these contradictions, plaque formation has been generally accepted as a measure of the capacity of such cells to synthesize antibody (Ab). On the other hand, the formation of red cell rosettes by Ab-carrying cells has been shown to occur with actively as well as passively "immunized" cells. Indirect evidence suggests, however, that the only cells which become immunized passively in the Ab-producing animal are macrophages and that in the primary immune response Ab synthesis is the basis of rosette formation by lymphatic cells other than macrophages (6, 7). In the present work, puromycin (PM) and sodium fluoride (NaF) were used to study the significance of the hemolytic plaque test and the rosette test for measuring actively-produced and passively-adsorbed Ab.

Methods. For induction of a primary antibody response 0.2 ml of a 30% suspension of washed sheep red blood cells (SRBC) in phosphate buffered saline (PBS) was injected ip into adult C57BL/Ks mice. Five to 6 days thereafter spleens obtained from 5 to 8 mice were pooled, single cell suspensions were prepared (8), and washed three times in Hanks' balanced salt solution (HBSS).

Drug treatment was done with spleen cells suspended at 1×10^7 cells/ml in complete Hanks' medium [10% calf serum, 0.1% proteose peptone (Difco), 0.1% yeast extract (Difco), 50 units of penicillin/ml, and 50 μg of streptomycin/ml in HBSS]. Puromycin and NaF were dissolved in sterile distilled H_2O and 1 vol of the solution was added to 19 vol of the cell suspension. The final drug concentrations were 1.2×10^{-4} or $2.9 \times 10^{-4}M$ PM; or 2.0×10^{-3} or 10^{-2} M NaF. Control samples received the same quantity of distilled H_2O. Parallel samples of drug-containing and control cell suspensions received 2 μCi/ml of L-leucine-^{3}H. The mixtures were incubated at 37° in 16 × 125-mm screwcapped tubes in a rotating drum. Unless otherwise stated, samples were removed at 1, 2, and 3 hr. The cells were washed twice in the cold with HBSS, and subjected to the rosette test (8) and to the modified Jerne plaque test (3, 9).

Cells labeled with leucine-^{3}H were washed twice, resuspended in 1 ml of PBS, and ruptured by alternate freezing and thawing five times. After centrifugation at 1500 g, the supernatant was precipitated in the cold with 1 ml of 10% trichloroacetic acid (TCA). After centrifugation, the precipitate was redissolved in 2 ml of distilled H_2O. Half of this sample was used to determine protein concentration with the Lowry method (10). The other half was used to measure TCA-precipitable radioactivity in a Packard liquid-scintillation counter.

Hyperimmune sera to SRBC, used for passive sensitization of cells, were produced as described previously (7),. The hemagglutinin titers of the sera were >1:40,000.

The peritoneal exudate cells used for pas-

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TABLE I. Relative Numbers of Plaque-Forming Cells (PFC) and Rosette-Forming Cells (RFC) in spleen cells from Sheep Red Blood Cell (SRBC)-Injected Animals and in Normal Peritoneal Exudate Cells Treated *in Vitro* with Anti-SRBC Serum.[a]

		Antibody positive cells			
			Av (%) of nonincubated cells;[b] after incubation at 37° for:		
		Before incubation (no.) (hr):	1	2	3
A. Spleen cells					
RFC ($p \cdot 10^6$)		5 and 6 days after injection of SRBC, 22,000–100,000	99 (±38)	54 (±27)	31 (±10)
PFC ($p \cdot 10^6$)		5-day av, 933 6-day av, 384	52 (±18)	44 (±17)	20 (±10)
B. Peritoneal exudate cells					
RFC ($p \cdot 10^6$)	Untreated	3000	nd[c]	nd	nd
	Ab treated	158,000–230,000	20 (±9.7)	18 (±23)	8 (±1)
PFC ($p \cdot 10^6$)	Untreated	0	nd	nd	nd
	Ab treated	1200–18,200	44 (±66)	1.6 (±1.8)	0.6 (±0.9)

[a] Effect of incubation of the cells at 37° on PFC and RFC.
[b] In parentheses, 1 SD.
[c] nd = not done.

sive immunization were obtained from mice 3 days after the last two doses of 1 ml of 0.5% (w/v) oyster glycogen in PBS given at an interval of 3 days. The cells were washed once in HBSS and resuspended in HBSS to a concentration of 50×10^6 cells/ml. One half vol of heat-inactivated hyperimmune serum was added and the mixture was incubated at 37° for 1 hr. During incubation the cells were gently mixed with a capillary pipette every 5–10 min. After two washes with 10 vol of HBSS, the cells were resuspended at a concentration of 3.5×10^6 cells/ml. In the case of passively immunized cells treated with drugs for 1 hr, the suspending medium used was HBSS without added nutrients. Cells treated with inhibitors for 3 hr were suspended in the complete Hanks' medium described above. Puromycin, NaF, and/or L-leucine-^{3}H were added to passively immunized cells in the same concentrations as were used for the immune spleen cells. In as much as roller tubes were unsatisfactory for macrophages because cells adhered to the walls of the tubes, peritoneal cells were incubated in conical tubes without rolling and gently mixed with a capillary pipette every 10 min. After drug treatment, passively immunized peritoneal cells were washed and tested as described above for the spleen cells.

Materials. Puromycin-2HCl, Sigma Chemical Company; oyster glycogen, Eastman Organic Chemicals; L-leucine-4,5-^{3}H, 5 Ci/mmole, New England Nuclear.

Results. Table IA shows the relative numbers of rosette-forming cells (RFC) and plaque-forming cells (PFC) which were found in immune spleens. Incubation at 37° without addition of drugs decreased the RFC and PFC titers, probably due to an overall loss of cell activity under the *in vitro* conditions. The RFC counts dropped in 1 hr to 99%, in 2 hr to 54% and in 3 hr to 31% of the values obtained without incubation (Table IA). Plaque formation was even more strongly diminished by mere incubation at 37°. In 1 hr the PFC counts were reduced to 52%, in 2 hr to 44% and in 3 hr to 20% of control values without incubation (Table IA). The reduction in antibody activity must have been due to more subtle changes than are detected by the trypan blue exclusion test; viability of the cells averaegd 78% in the nonincubated cells and was only slightly re-

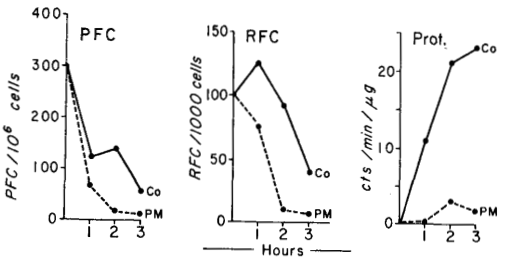

FIG. 1. Effect of puromycin (PM) on plaque-forming cells (PFC), rosette forming cells (RFC) and protein synthesis (Expt. 6): abscissa, hours of incubation with puromycin (PM), or without (Co). Leucine-^{3}H incorporation into protein of about 10^7 cells at 3 hr: control = 690 cpm; PM = 45 cpm.

duced by incubation at 37° in 1 hr to 77%, in 2 hr to 74%, and in 3 hr to 72% viable cells.

An example of the effect of puromycin on immune spleen cells is shown in Fig. 1. Protein synthesis was almost completely depressed in the first hour of incubation with PM. Puromycin decreased plaque and rosette formation during the first hour of incubation and the effect became maximal during the second hour. It is not evident why the number of RFC in the control sample increased during the first hour of incubation. Possibly this effect was due to uptake by spleen macrophages of cytophilic antibodies released during the incubation. The course of the decrease of PFC and RFC during incubation with the drugs varied from experiment to experiment. The strongest suppression was evident either at 1, 2, or 3 hr. For this reason the results obtained at various time intervals were averaged in the tables. The data are expressed as percentage of the control since the absolute numbers of RFC and PFC observed without incubation and after treatment with inhibitors varied greatly.

The effect of PM and NaF on immune spleen cells is shown in Table IIA. Puromycin suppressed protein synthesis to between 7 and 24% of control activity. Plaque-forming capacity was decreased to 13–50% of the control titers. The rosette-forming capacity was clearly decreased by PM in 2 of 5 experiments (5 and 6 Table IIA); in three experiments only a slight decrease took place.

Sodium fluoride in the dosages used caused some depression of protein synthesis (Table IIA). In experiments 3–5 where protein synthesis was clearly depressed, PFC were significantly lowered. The maximum suppression of RFC counts by NaF was 58% (Expt. 4); in general the effect of NaF on RFC was variable and not correlated with the action on protein synthesis.

Treatment with the inhibitors did not significantly reduce viability of the cells as judged by the trypan blue exclusion test. Cell viability average 74 ± 6.4% in the control samples, 73 ± 7.6% in the NaF treated, and 69 ± 9% in the PM-treated samples. Selective death of antibody-active cells can not be excluded with certainty, however.

In an attempt to determine whether the effects of PM and NaF were an indication that active Ab synthesis was necessary to the activity of RFC and PFC of immune spleens, the same drugs were tested on glycogen-induced peritoneal exudate cells coated with cytophilic Ab. The behavior of PFC and RFC in untreated peritoneal exudate cells and in cells treated with hyperimmune serum and incubated at 37° without the drugs is shown in Table IB. In peritoneal cell preparations not incubated in cytophilic Ab, a maximum of 3000 RFC/10^6 cells developed but no PFC were found. Cells incubated in hyperimmune serum and washed twice contained between 158,000 and 230,000 RFC/10^6 cells and between 1200 and 18,200 PFC/10^6 cells. "Passively immunized" PFC were specific and complement-dependent as shown in Fig. 2. Incubation at 37° of cells coated with cytophilic Ab decreased RFC in 1 hr to 20%, in 2 hr to 18% and in 3 hr to 8% of the values before incubation (Table IB); the PFC were decreased to 44%, 1.6 and 0.6%, respectively (Table IB). As shown in Table IIB, incubation with PM depressed the protein synthesis of normal peritoneal cells coated with cytophilic Ab as much as that of immune spleen cells (Table IIA). Plaque formation, however, was not reduced by PM and rosette formation, was, if anything,

TABLE II. Effect of Puromycin and NaF on Plaque-Forming Cells (PFC), Rosette-Forming Cells (RFC) and Protein Synthesis.

Expt. no.	After SRBC (days)	Dosage (M)	Times of incubation (hr)	(%) PFC[a]	(%) RFC[a]	(%) Protein synthesis[a]
A. Effect on actively immune spleen cells.						
		PM				
1	5	1.2×10^{-4}	0.5, 2, 5	50	86	24
2	5	2.9×10^{-4}	4.5	13	88	7
5	6	2.9×10^{-4}	1, 2, 3	13	58	8
6	6	2.9×10^{-4}	1, 2, 3	29	33	13
7	6	2.9×10^{-4}	1, 2, 3	41	85	9
Mean (5 expts.)				29.2	70	12.2
		NaF				
3	5	2.0×10^{-3}	0.5, 1, 2.5, 4	62	60	52
3	5	1.0×10^{-2}	0.5, 1, 2.5, 4	56	72	nd
4	5	1.0×10^{-2}	1, 2, 3	66	42	20
5	6	2.0×10^{-3}	1, 2, 3	46	130	30
6	6	2.0×10^{-3}	1, 2, 3	84	100	71
7	6	2.0×10^{-3}	1, 2, 3	122	95	141
Mean (6 expts.)				72.7	82.5	63
B. Effect on passively sensitized peritoneal exudate cells						
		PM				
1	—	2.9×10^{-4}	1	nd	133	nd
2	—	2.9×10^{-4}	0.5, 1	104	115	4.6
3	—	2.9×10^{-4}	0.5, 1	94	97	2.3
4	—	2.9×10^{-4}	1, 2, 3	100	152	36
5	—	2.9×10^{-4}	1, 2, 3	100	152	nd
Mean (5 expts.)				99.5	129.8	14.3
		NaF				
1	—	0.6×10^{-3}	1	nd	99	nd
1	—	1.0×10^{-2}	1	nd	96	nd
2	—	2.0×10^{-3}	0.5, 1	96	85	77
3	—	2.0×10^{-3}	0.5, 1	101	88	90
4	—	2.0×10^{-3}	1, 2, 3	110	239	73
Mean (5 expts.)				102	121	80

[a] PFC, RFC, and protein synthesis are given as percentage of the control (untreated samples). Each value represents the mean of 2–4 samples taken at the indicated intervals in one experiment.

[b] nd = not done.

slightly enhanced by PM. Incubation with NaF (Table IIB) slightly inhibited protein synthesis, but rosette and plaque formation were not diminished.

The comparative action of PM and NaF on actively and passively immunized cells is summarized in Table III. No significant difference between the two cell populations is seen with respect to the effect of PM and NaF on protein synthesis. However, the difference between the action of PM on actively and passively immunized PFC and RFC is significant. Sodium fluoride discriminated to a significant degree between actively and passively immunized PFC, but not RFC.

Discussion. It is presumed that antibodies synthesized by a cell will lead to plaque formation if the antibodies are secreted. It

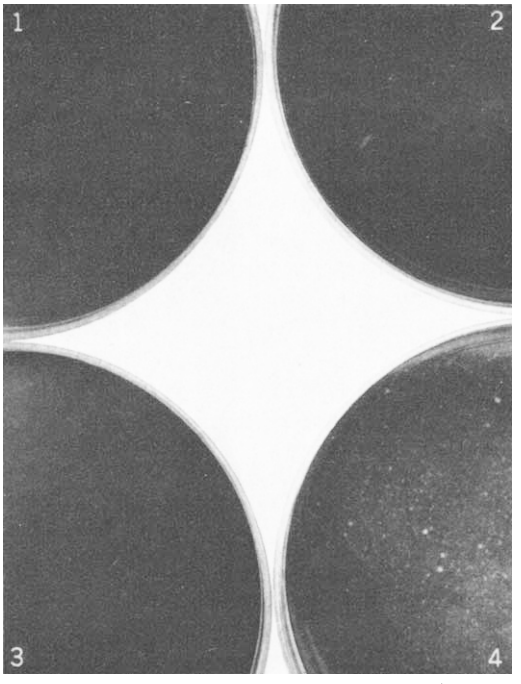

FIG. 2. Plaque formation by peritoneal exudate cells: Plate 1, nontreated cells plus SRBC plus C′; Plates 2, 3, 4, cells treated with anti-SRBC antiserum; Plate 2, plus mouse RBC plus C′; Plate 3, plus SRBC plus heat inactivated C′ (56°, 30 plus SRBC plus heat inactivated C′ (56°, 30 min); and Plate 4, plus SRBC plus C′.

has been proposed that continuous secretion of Ab is also the basis of rosette formation by Ab-synthesizing cells, rather than adherence of previously secreted Ab (11).

The present comparison of the effects of PM on actively and passively immunized cells suggests that both plaque formation and rosette formation in a population of actively immune spleen cells are mainly due to active Ab synthesis and not to passive Ab adsorption. Puromycin in the dosages used here has been shown by Helmreich *et al.* (12), to completely inhibit protein synthesis in lymph node cells; however, secretion of preformed gamma globulin proceeded for about 2–3 hr at a decreased rate. The partial independence of secretion from protein synthesis may be the basis for the remaining PFC and RFC counts after PM treatment, i.e., some Ab produced before treatment would still be secreted. Some Ab production might also have continued during treatment with PM, since protein synthesis was usually not completely blocked (Table II). In addition, some of the remaining positive cells might have been macrophages bearing cytophilic Ab which was bound independently of protein synthesis. The greater decrease of PFC than RFC by mere incubation at 37° as well as by drug treatment probably reflects the relative insensitivity of the Jerne test (13). A few Ab molecules remaining attached to an immune cell after suppression of protein synthesis or after some non specific damage due to incubation *in vitro* would not result in a visible plaque, but would probably still cause SRBC adherence. This sensitivity of the rosette test strongly influences the interpretation whether in a given situation the majority of the cells have actively synthesized the rosette forming Abs. It appears advisable to use the Jerne test in combination with puromycin rather than the rosette test in experi-

TABLE III. Comparison of the Effects of Puromycin (PM) and NaF on Actively versus Passively Immunized Cells.

Test	Active[a]	Passive[a]	p[b]
PM			
Protein synthesis	13.1 ± 11.5	17.4 ± 22.0	>0.4
PFC	31.9 ± 20.8	110.0 ± 29.8	<0.001
RFC	67.5 ± 26.5	131.0 ± 49.0	<0.005
NaF[c]			
Protein synthesis	61.0 ± 33.5	81.0 ± 17.3	>0.1
PFC	76.3 ± 29.2	109.0 ± 16.8	<0.01
RFC	96.2 ± 44.0	137.0 ± 83.0	>0.2

[a] Plaque-forming cells (PFC), rosette-forming cells (RFC) and protein synthesis are given as percentage of the control (untreated samples); means and 1 SD based on all the single observations; the small differences in the means in this table and the means in Table II occur because the number of samples taken in each experiment varied between 1 and 4.

[b] p of the difference of the averages obtained with actively and passively immunized cells was calculated using the Student's t test.

[c] Only the experiments using 2×10^{-3} M NaF are compared.

ments where it is crucial to differentiate between cells which have actively or passively acquired Abs.

Cohn *et al.* (14), have shown that NaF, in concentrations of 10^{-2} to 10^{-3} *M*, reduces membrane activity of mouse macrophages. In the present study on actively immunized cells, the reduction of membrane activity produced by NaF probably interfered with the secretory mechanism and therefore, secondarily influenced plaque formation and to some extent rosette formation. In addition some of the effect may have been due to the depression of protein synthesis, which possibly occurred secondary to inhibition of glycolysis.

The fact that cells passively coated with Ab gave rise to plaque formation demands precaution in interpretations dealing with PFC from immune spleens. A similar observation recently made by Roseman *et al.* (15), led these authors to conclude that part of the PFC found late in the primary Ab response were due to adsorbed Ab. The adsorbed Ab was probably bound to cytophilic sites of macrophages. The amount of cytophilic Ab bound to the macrophage cell membrane decreased during incubation at 37°; the disappearance was due to elution into the medium and to engulfment by the macrophages (16). Puromycin appears to have stabilized some of the rosette forming Ab on passively immunized cells (Table IIB). This effect might have been due to interference with the process of cell membrane renewal, which probably demands protein synthesis. Thus engulfment of the Ab might have been inhibited. That this effect was not apparent in PFC suggests that most of the adsorbed Ab was lost by elution into the medium and that stabilization of the minor proportion which would otherwise have been lost by engulfment was not detectable by the relatively insensitive plaque test. It follows that dissociation of the Ab from the cell membrane was not influenced by PM or NaF. Accordingly no difference in the amount of hemagglutinating Ab eluted into the media was detectable between control and drug-treated samples.

Summary. Treatment of normal peritoneal exudate cells with antisheep red blood cell antibodies passively immunized some of the cells (presumably macrophages), i.e., conferred on them the capacity to form hemolytic plaques and rosettes. Preincubation with puromycin inhibited plaque and rosette formation by immune spleen cells of actively immunized animals but not by passively immunized peritoneal exucate cells. Puromycin inhibited protein synthesis in both cell populations equally strongly. Sodium fluoride depressed plaque formation by immune spleen cells of actively immunized animals, but not by passively immunized cells. Sodium fluoride treatment had no effect on rosette formation by either actively or passively immunized cells.

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