IN VITRO STIMULATION OF ANTIBODY FORMATION
BY PERITONEAL CELLS

II. Cell Interactions and Effects of Immunochemical
or Metabolic Inhibitors

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In the first paper of this series (1), we have described a new and more sensitive method allowing the detection of plaques of hemolysis formed in thin monolayer cultures by mouse peritoneal cells (PC).[1] Evidence was presented which showed that the plaques were caused by the secretion of a product from a living cell, and that lysis was dependent on complement. In this paper, we consider in greater depth the question of whether the hemolytic product responsible for plaque appearance is antibody. We also discuss the role of cell interaction in its causation and the effects of various metabolic inhibitors on the phenomenon.

Materials and Methods

These were essentially the same as those described in the first paper (1).

Thoracic Duct Cannulation.—This was according to the method of Boak and Woodruff (2), as modified by Miller and Mitchell (3).

Antimouse Immunoglobulin Sera.—These were kindly provided by Dr. H. Bazin. They were obtained by immunization of rabbits with IgM or IgG fractions of mouse serum. They were repeatedly absorbed with either IgG- or IgM-containing preparations to obtain sera apparently monospecific for the class of immunoglobulins which was used as the immunizing agent. We checked this monospecificity extensively, using immunoelectrophoretic analysis and tanned cell hemagglutination. In the latter test, the anti-IgM sera, which will be most critical to our argument, had titers of <1:10 with erythrocytes sensitized with mouse IgG, while the anti-IgG sera had titers of 1:652,000 with the same erythrocytes. The reverse situation pertained, though anti-IgM titers were lower; thus the sera were effectively able to discriminate between 19S and 7S classes. No attempt was made to prepare sera able to discriminate between the different mouse gamma globulin subclasses. Further evidence for the discriminatory capacity of the sera is given under Results.

RESULTS

Behavior In Vitro of Lymphoid Cells from Different Sources.—It has already been shown by one of us (4) that mouse lymph node cells and spleen cells,

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[1] *Abbreviations used in this paper:* CMC, carboxymethylcellulose; PC, mouse peritoneal cells; PFC, plaque-forming cells.

placed in the original system described by Ingraham and Bussard (5) (carboxymethylcellulose [CMC] medium sealed between slide and coverslip—"closed system") were unable to develop hemolytic plaques during incubation periods of up to 72 hr, in contradistinction to the behavior of PC. The new technique (ultrathin layers under paraffin oil—"open system"), described in the previous article (1), was used to assay again the plaque-forming capacity of different lymphoid cells, since this technique is about twice as sensitive for the detection of plaque-forming cells than the closed technique.

TABLE I

Plaque-Forming Capacity of Lymphoid Cells from Nonimmunized Mice. Young Male Mice, Open System

Organ	Concentration of cells	No. of plaques/10^6 cells
Spleen	5 $\times$ 10^6	18
Mesenteric: Exp. 1	20 $\times$ 10^6	0
"	10 $\times$ 10^6	20
"	5 $\times$ 10^6	0
Lymph Node: Exp. 2	5 $\times$ 10^6	8
Thymus: Exp. 1	5 $\times$ 10^6	10
Exp. 2	5 $\times$ 10^6	0
Bone marrow: Exp. 1	5 $\times$ 10^6	0
Exp. 2	5 $\times$ 10^6	16
Thoracic duct lymphocytes	3 $\times$ 10^6	0
Peyer's patch	20 $\times$ 10^6	7
	15 $\times$ 10^6	21
	10 $\times$ 10^6	0
	5 $\times$ 10^6	0

It can be seen in Table I that most lymphoid cells showed little or no plaque forming capacity in the open CMC microcultures after 24 hr of incubation. It must be pointed out that an activity of 20 PFC/10^6 cells detected with the open technique represents an activity of circa 4–5 PFC/10^6 with the standard Jerne method. This is significantly higher than the usual background found after 1 hr incubation in agar, and it is thus possible that a slight stimulation in vitro was achieved in some experiments, but the minimal plaque numbers stand in marked contrast to the results with PC.

Cell to Cell Interactions.—In many in vivo (6) and in vitro (7) immune responses, cell to cell interactions appear to be of importance. We thus wondered whether the stimulation of plaque-forming activity in PC required the trigger-

ing of only one individual cell, or if cooperation between two or more cells were required. Such interaction could be of two types: interaction between two essentially different cells (macrophage–lymphocyte or thymus–bone marrow) or between two or more cells of the same class. We have approached the first type of interaction by reconstruction experiments and fractionation procedures, and the second by variations in concentration of the population of PC in the incubation system.

TABLE II

Plaque-Forming Capacity of a Mixture of Peritoneal Cells and of Lymphoid Cells from Various Sources. Young Male Mice, Open System

Source of cells	Concentration of cells	Number of plaques normalized to expected peritoneal cell performance
		%
Peritoneal cells plus	5×10^6	80
Spleen	5×10^6	
Peritoneal cells plus	5×10^6	51
Thymus	5×10^6	
Peritoneal cells plus	5×10^6	51
Bone marrow	5×10^6	
Peritoneal cells plus	5×10^6	80
Mesenteric node	5×10^6	
Peritoneal cells plus	3×10^6	52
Thoracic duct cells	3×10^6	
Peritoneal cells plus	5×10^6	35
Peyer's patch cells	5×10^6	
		Number of plaques/10^6 cells
Thymus cells plus	5×10^6	0
Bone marrow cells	5×10^6	
Mesenteric node cells plus	5×10^6	0
Bone marrow cells	5×10^6	

Reconstruction Experiments.—Mixtures of PC and of lymphoid cells from different sources have been made and these mixtures submitted to incubation in CMC following the standard procedure. The results of these experiments are shown in Table II. It can be concluded that no increased plaque-forming activity could be induced in the PC population by addition of spleen, thymus, bone marrow, mesenteric nodes, or thoracic duct cells. On the contrary, a slight inhibition of the normal expected activity of the PC alone is observed. This effect was investigated by mixing a constant proportion of PC (5 × 10⁶ cells/ ml) with an increasing proportion of spleen cells which are not, by themselves, plaque-forming. It can be seen, in Table III, that an increase in the proportion of spleen cells in the total cell population results in a marked reduction of the relative plaque-forming activity ascribed to the PC. Since the total cell con-

centration was not kept constant, it was not possible to distinguish between a specific inhibition of the PC activity by the spleen cells or a nonspecific reduction resulting from the excess of total cell concentration, such a nonspecific effect being due to an insufficient supply of gases or of metabolites for the cells.

These experiments show that the various lymphoid organs studied are not sources of cells which can readily be shown to interact with PC with resultant plaque formation. They do not approach the question of whether heterogeneous elements within the PC population may interact. This is best studied by fractionation of the PC population. One of us has shown in a previous study (8) that the removal of the large majority of the macrophage-like population (cells attaching to glass) from the peritoneal suspension does not reduce, and sometimes increases, the plaque-forming activity of the remaining population. It can be concluded that a large majority of the "macrophage-like" cells, plays no role in the process of stimulation of plaque formation taking place in vitro.

TABLE III

Effect of Increased Concentration of Non–Plaque-Forming Cells (Spleen Cells) on Plaque Formation by Peritoneal Cells. Young Male Mice, Open System

Concentration of peritoneal cells	Concentration of spleen cells	PFC peritoneal cells ± sᴇ
$10^6/ml$	$10^6/ml$	
5	0	3100 ± 550
5	5	2150 ± 193
5	10	930 ± 170
5	15	585 ± 117

This is not to say that a few macrophages are not still required in the process of stimulation, since our fractionation procedures have, by no means, removed 100% of the macrophages from the initial cell population.

Effect of Concentration of Cells.—If cell to cell interaction between two (or more) cells of the initial population is required for the phenomenon of plaque formation to take place, the plaque number ought to be dependent on the concentration of the cells in the incubation system. Experiments using concentrations of PC of from 0.12×10^6 to $10 \times 10^6/ml$ have been performed using both the open and the closed CMC systems, and both young male and retired breeder mice. Some representative results are presented in Tables IV and V. As expected, plaque-forming activity (PFC/10^6 cells) of a population of spleen cells from immunized mice was relatively independent of cell concentration over the whole range studied, though some variation, not statistically significant, was encountered, especially with the open technique. This is not the case for peritoneal cells from unimmunized young mice. Three groups differ significantly in their plaque-forming activity ($P < 0.05$). At low concentrations (0.6–1 million cells/ml) a small or medium activity is observed (9–32% of

PFC/10^6); for medium concentrations (2.5–10 million cells/ml) maximal activities are recorded; at high concentration (10 million cells/ml) the activity is significantly decreased. With retired breeder female mice, somewhat similar results have been recorded, though individual variability between day to day experiments was more marked (Table V). In addition it should be pointed out that, for very low concentrations (0.1 × 10^6 cells/ml and below) individual

TABLE IV

Effect of Concentration of Cells on the Relative Plaque-Forming Activity (PFC/10^6) of Immune Spleen Cells or of Nonimmune Peritoneal Cells. Young Male Mice, Open Systems

Concentration of cells (10⁶/ml)	Percentage of maximum ± SE			
	Spleen cells from immunized mice		Peritoneal cells from unimmunized mice	
	Closed technique	Open technique	Closed technique	Open technique
10	87 ± 2.5	73 ± 4.5	67 ± 5	52 ± 6
5	95 ± 4	70 ± 6	79 ± 9.5	100 ± 12
2.5	100 ± 9.5	88 ± 7	100 ± 15	19 ± 7
1.25	86 ± 8	100 ± 8	32 ± 8	9 ± 3.5
0.61	88 ± 8	82 ± 6	24 ± 4.5	—

TABLE V

Effect of Concentration of Cells on the Relative Plaque-Forming Activity (PFC/10^6) of Nonimmune Peritoneal Cells. Retired Breeder Mice, Open System

Concentration of cells	PFC/10^6 ± SE	
	Exp. 1	Exp. 2
10⁶/ml		
2	—	28,100 ± 910
1	58,700 ± 10,800	40,200 ± 4,150
0.5	43,000 ± 5,100	53,000 ± 4,900
0.25	47,300 ± 7,700	53,600 ± 8,500
0.12	28,000 ± 7,700	1,530 ± 840

variations from preparation to preparation from the same initial suspension, were so high that these results could scarcely have been considered as falling within a normal distribution. Nevertheless, in Experiment 2, recorded in Table V, and in five other experiments of similar design, a striking and abrupt fall-off in expected plaque-forming ability occurred at a certain threshold in concentration. For example, in Experiment 2, the individual microcultures with the lowest cell concentration contained 456 cells per culture. Had its plaque-forming capacity been as expected, a mean of 24 plaques per cover slip would have been found, yet the actual mean was < 1. Unfortunately, there were three other experiments, of identical design, which (as in Experiment 1 of Table V)

failed to show this dilution effect. Cell dilution methods were checked with ^{51}Cr-labeled cells and found to be valid. No satisfactory explanation for this experimental variation is available. As in all experiments with retired breeders, the detailed pregnancy history, not avilable to us, may have been a significant variable.

In order to determine whether the decrease in plaque formation resulting from decrease in cell concentration could be due to nonspecific factors, such as some nutritional effect akin to a "feeder layer" function, experiments were performed in which PC were mixed with spleen cells in such a fashion that the PC concentration varied from 0 to 5 × 10^6/ml but the total cell concentration remained constant at 5 × 10^6/ml. The results (Table VI) show that with a falling PC concentration, suboptimal plaque formation ensues. It is unlikely that the reduction of plaque numbers by 60–80% of optimal levels could be due

TABLE VI

Effect of Concentration of Peritoneal Cells, at Constant Overall Cell Concentration, on the Formation of Hemolytic Plaques. Young Male Mice, Open System

Concentration of peritoneal cells	Concentration of spleen cells	PFC/10^6 peritoneal cells ± SE	
		Exp. 1	Exp. 2
10^6/ml	*10^6/ml*		
5	0	2210 ± 225	4770 ± 571
2.5	2.5	1250 ± 204	4620 ± 1,100
1.25	3.75	678 ± 115	2170 ± 121
0.61	4.39	440 ± 146	2030 ± 530
0	5	0	0

solely to an inhibitor produced by spleen cells, as an addition of 5 × 10^6/ml of spleen cells to a preparation containing 5 × 10^6 PC/ml only causes a fall of 20–30% in the expected plaque count (Tables II and III).

Thus the fact that the activity of a population of cells from an immunized animal is independent of concentration can be interpreted as a demonstration that antibody formation, at this level, is an independent phenomenon for each individual cell. In contrast, the concentration dependence of the phenomenon of plaque formation by normal peritoneal cells, shown here, with a new technique of incubation and for young, as well as for old donors of cells, imply that cell to cell interaction is probably involved in some stages at least of the development of this phenomenon. Whether this interaction requires the actual contact of cells or whether diffusible factors are involved cannot be decided in the light of the experiments described here. It can only be mentioned that cell to cell contact can take place in the CMC. This semisolid medium does not constitute an immobilizing environment for motile cells, as can be seen by time-lapse photography. This technique has allowed us to see that most cells from

the peritoneal cells suspensions are moving (in the preparations). Lymphoid cells are endowed with peristaltic, nontranslational movements and wandering cells, with typical macrophage motility, are also to be seen in the preparations. Actual fleeting contacts between cells, and specially between a PFC and a macrophage, have been observed more than once. Whether these contacts have any significance is not known, but they are mentioned only in order to show that such contacts are possible.

Effect of Inhibitors.—Different inhibitors have been studied, either by mixing the reagent in the whole incubation mixture, or by micromanipulation of active cells from a normal monolayer culture to one containing an inhibitor.

Antimouse Immunoglobulin Sera.—Incubation of immune spleen cells (taken 4 days after one intraperitoneal injection of 5×10^8 SRBC) and of normal

TABLE VII

Effect of Antimouse Immunoglobulin Sera on Immune Spleen Cells or on Normal Peritoneal Cells. Young Male Mice, Closed System

Serum concentration	Percentage of activity related to controls					
	Anti-IgM serum No. 1		Anti-IgM serum No. 2		Anti-IgG serum	
	Immune* spleen cells	Peritoneal cells	Immune spleen cells	Peritoneal cells	Immune‡ spleen cells	Peritoneal cells
10^{-2}	46§	5	21	0	1550	108
10^{-3}	71	40	39	58	84	89
10^{-4}	86	112	93	190	89	160

* 4 day primary response.
‡ Secondary reaction.
§ Very small plaques.

peritoneal cells have been performed, in CMC, with different concentrations of specific rabbit anti–mouse globulin antisera. Since normal rabbit serum has a slight inhibitory effect on plaque formation by peritoneal cells, the effects of antisera are calculated as a percentage of the controls containing 1% normal rabbit serum. The plaque counts were done after 4 hr and 24 hr of incubation with the spleen cells and after 24, 48, and 72 hr of incubation with the peritoneal cells. The end points (24 hr for the spleen cells and 72 hr for the peritoneal cells) only are reported.

The pooled results of five experiments are given on Table VII. It can be seen that the anti-IgM sera are more inhibitory on the peritoneal cells than on the spleen cells. These results do not give a complete picture of the phenomenon since, after 4 hr of incubation the spleen cells were markedly more inhibited by the anti-IgM sera than after 24 hr. It can be said that the plaque-forming activity of the spleen cells "overcame" the inhibitory effect of anti-IgM molecules after 4 hr in such a way that a large number of plaques will be seen at 24

hr which are not present at 4 hr. This underlines the interest of long term incubation methods, such as the CMC one, for local hemolysis in gel studies. This presumably means that the inhibition of plaque formation is due more to a direct effect of the anti-IgM molecules on the local hemolysis of red cells than by an effect on the physiology of the antibody-producing cell. Anyhow, "one-step experiments" such as the one reported here, do not allow one to distinguish, experimentally, between the two inhibitory mechanisms suggested.

The anti-IgG serum manifests its usual amplification effect on immune spleen cells in a secondary immune response. This confirms the production of IgG by
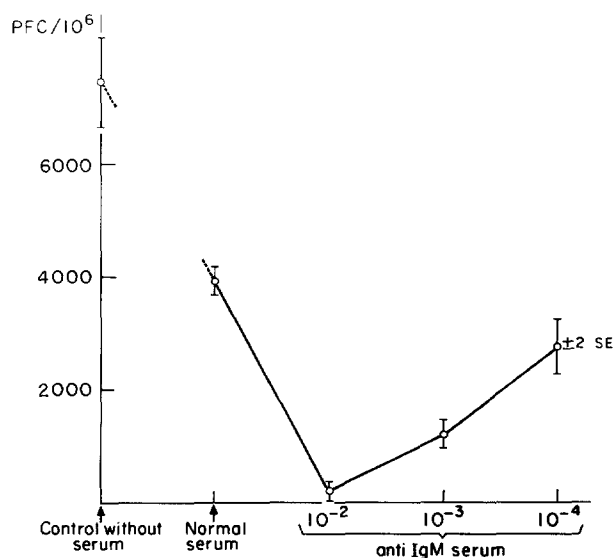

FIG. 1. Inhibition of plaque formation with various concentrations of rabbit anti-mouse IgM serum incorporated into the culture medium. Peritoneal cells in CMC open monolayer cultures, plaque counts read at 24 hr.

these cells. Peritoneal cells are essentially unaffected by the presence of anti-IgG antibody. The slight increase found with low concentration of anti-IgG is probably not significant.

The results of a typical experiment using the open system are given in Fig. 1. Again, there is a profound inhibitory effect of the anti-IgM serum over the control with normal rabbit serum (96% reduction). As in the closed system, anti-IgG sera had no greater inhibitory effect than did normal rabbit serum. The inhibitory effect of different normal rabbit sera varied greatly and presumably reflected the presence of some cytotoxic factor.

Antimouse Immunoglobulin Sera Effects as Judged by Transfer Technique.— Micromanipulation of plaque-forming cells was performed as described in the

previous article (1). Cells in the center of a plaque of hemolysis were taken and transferred into a new layer of CMC, normal or containing the proper reagent. Plaque formation by the transferred cells was observed repeatedly over 20 hr after the transfer. Effect of anti-IgM or anti-IgG sera was first assayed on immunized spleen cells (primary response). Transfers were made 1–3 hr after initial culture of the cells (Table VIII). The "plating efficiency", after one transfer, was found to be 87% in controls (transfer from normal medium to normal medium). Anti-IgG serum at 1:100 final concentration in the recipient medium did not affect the plating efficiency. Anti-IgM serum (No. 2) at 1:500 final concentration reduced the plating efficiency to 40% and anti-IgM serum at 1:100 final concentration brought it down to 25%. This reduction in plaque-forming activity of immunized spleen cells (primary reaction) transferred to a

TABLE VIII

Effect of Anti-Immunoglobulin Antisera on Appearance of Plaques After Micromanipulation Transfer of 4 Day Immunized Spleen Cells

Inhibitor	No. of Plaques No. Tested		Mean Diameter of Plaques
		%	μ
Control:	26/30	87	198
1:100 anti-IgG	15/15	100	166
1:500 anti-IgM	6/15	40	53
1:100 anti-IgM	5/20	25	129*

* This included one very large plaque of 347 μ. The mean diameter of the other four plaques was 75 μ.

medium containing anti-IgM is quite comparable (Table VII) to the reduction found in plaque-forming capacity of immunized spleen cells incorporated in an anti-IgM-containing medium.

Micromanipulation transfer of plaque-forming peritoneal cells was then undertaken at times varying from 3 to 24 hr after initial culture. It can be seen, in Table IX, that the plating efficiency was 71%, in control transfers. At 1:100 final concentration, a polyspecific rabbit antimouse globulin serum suppressed all activity in the transferred cells. Anti-IgM at 1% or 0.2% concentration was also completely inhibitory. Anti-IgG serum, on the contrary, showed no inhibitory activity, the plating efficiency of the transferred population being the same as that of the controls. A mixture of 0.5% anti-IgG and 0.5% anti-IgM is completely inhibitory. This may be considered as an indication that no double producers (IgM and IgG produced by the same cell) could be found among the 100 cells transferred.

Thus, the results of inhibition by transfer experiments confirm completely the results of inhibition by incorporation of the cells in a gum containing the

antisera. It leads us to conclude that peritoneal plaque-forming cells are IgM producers such as are spleen cells from a mouse 4 days after the first injection of sheep erythrocytes.

In order to see if inhibition of plaque formation by the transferred peritoneal cells was achieved by an irreversible damage of the cell, a second transfer of these cells was attempted. PC cells were first allowed to make plaques, then transferred to a monolayer containing anti-IgM, and, after 1 to 2 hr, were retransferred into a normal monolayer. The results of such experiments are reported in Table X. It is interesting to see that, although the cells have been

TABLE IX

Effect of Inhibitors on Plaque Formation after Micromanipulation Transfer of Peritoneal PFC

Inhibitor in receptor gel	No. of plaques / No. of cells Transferred	Plaques	Mean plaque diameter
		%	μ
Controls (No rabbit serum or 1% normal rabbit serum)	60/85	71	173
1:100 rabbit anti-mouse globulin serum	0/20	0	—
1:100 or 1:500 rabbit anti-mouse IgG serum	37/50	74	167
1:100 or 1:500 rabbit anti-mouse IgM serum	0/50	0	—
1:200 anti-IgM + 1:200 anti-IgG	0/100	0	—

TABLE X

Reversibility of Inhibition of Plaque Formation by Anti-IgM Sera

No. of PFC tested on double transfer*	No. forming plaques in 1:100 anti-IgM	No. forming plaques on retransfer to normal medium
20	0	14

* See text.

submitted twice to the hazards of micromanipulation, they have survived in a high proportion to give a final plating efficiency (14/20) of the same order as that obtained after one transfer.

It can then be concluded that the suppression of plaque formation by anti-IgM antibody is not due to an irreversible cytotoxic effect on the hemolysin-producing cell. It is not possible to know yet if the inhibition effect is due to a blockade of the hemolytic process (as currently seems most likely), to a reversible switching off of the biosynthesis of antibody by the cell, or to a combination of both effects.

Effect of Actinomycin D on Plaque Formation.—Spleen cells from immunized mice have been shown (9) to be essentially insensitive to actinomycin D at concentrations from 1 to 10 μg/ml in terms of their plaque-forming activity

with the closed technique. Peritoneal cells, on the other hand, have been shown (9) to be very sensitive to actinomycin D, with the closed system of incubation. The action of actinomycin D was assayed with our new (open) technique on PC from young mice or from retired breeders.

Effects of actinomycin D are different whether one considers peritoneal cells from young mice or from old breeders, as can be seen from Table XI, which gives the results of two representative experiments. At concentrations as low as 10^{-2} μg/ml, actinomycin suppresses 95% of the plaque-forming activity of PC from young male donors while, at such a concentration of inhibitor, 55% of the cells from old breeders remain active. Even at a concentration of 10 μg/ml of actinomycin, 17% of the cells from old mice are still active.

Another interesting phenomenon in retired breeders is the fact that actinomycin, at low concentrations, seems to accelerate the appearance of PFC in

TABLE XI

Effect of Actinomycin D on Plaque Formation by Peritoneal Cells from Nonimmunized Mice

Actinomycin μg/ml CMC	Young Mice		Old breeders	
	PFC/10^6 ± SE	Control	PFC/10^6 ± SE	Control
		%		%
10	91 ± 25	1.9	8,040 ± 1,000	17
1	64 ± 15	1.4	7,370 ± 1,120	15
10^{-1}	23 ± 10	0.5	12,800 ± 1,500	27
10^{-2}	246 ± 37	5.2	26,600 ± 2,500	55
10^{-3}	3,060 ± 620	65	33,400 ± 1,270	70
Controls	4,740 ± 380	100	48,000 ± 1,020	100

the first hours of incubation. After this initial acceleration of plaque formation by the actinomycin, the speed of formation of new plaques decreases in comparison with controls and, at the end, the final activity of the control group is higher than the activity of the actinomycin group even for the lowest concentration of inhibitor (10^{-3} μg/ml). This effect of low doses on the kinetics of plaque formation by peritoneal cells has been further investigated and will be discussed in the following article of this series. In one experiment in which 1 μg of actinomycin had caused mild depression of final plaque numbers, we measured the diameters of 50 randomly chosen plaques from each sample. We found that the plaques from the actinomycin group were much smaller (mean diameters: controls, 292 μ; actinomycin group, 120 μ). This suggested that the RNA molecules responsible for hemolysin production by the cell had a life span of 24 hr. This action of actinomycin D on peritoneal cells from young donors observed with the new open technique, confirms our previous results obtained with the closed technique. In view of what has been found with immunized spleen cells and with peritoneal cells from old donors, this effect can hardly be

ascribed to an overall cytotoxic effect (since the activity of these cells are not much affected by actinomycin D), except if one postulates a profound difference in susceptibility between peritoneal cells from young mice and peritoneal cells from old mice or immunized spleen cells.

In itself, the effect of actinomycin D is interesting and may lead to the same interpretation for the behavior of peritoneal cells from young or old donors. If actinomycin D acts on our peritoneal cells at the same molecular level as it does generally, inhibition of DNA transcription in messenger RNA (m-RNA), we may interpret our results in considering that immune cells and some of the peritoneal cells from old breeders contain enough stable m-RNA involved in production of specific IgM so that antibody production can take place in the absence of a new formation of specific m-RNA.

Effect of Colcemid on Plaque Formation.—The addition of Colcemid at various concentrations was found not to inhibit the formation of plaques by

TABLE XII

Effect of Colcemide on Plaque Formation; Young Male Mice, Open System $(PFC/10^6)$

Control	Colcemide (μg/ml)		
	10^{-2}	10^{-3}	10^{-4}
2733 $\pm$ 396*	2265 $\pm$ 310	1935 $\pm$ 328	2455 $\pm$ 348
6126 $\pm$ 406	4575 $\pm$ 330	4510 $\pm$ 360	5410 $\pm$ 310

* 2 SE

peritoneal cells from young mice (Table XII). These results confirm our previous work (4) which showed that cell division was not required for the development of plaque-forming cells among the peritoneal cells incubated. In view of the very rapid rise of PFC in our new technique (open system), it was hardly to be expected that cell division was involved in the development of the plaques of hemolysis.

Effect of Puromycin on Plaque Formation.—The effect of puromycin, a known inhibitor of protein synthesis, on plaque formation was assessed by incorporating the drug at a concentration of 10 μg/ml in the culture monolayers. Progressive plaque appearance in cultures, with or without puromycin, was assessed using our three standard cell sources: spleen cells from in vivo immunized mice, and PC from unimmunized young male or retired breeder mice. The results at two of the time points monitored are given in Table XIII. The effects on immune spleen PFC are, at first sight, surprising. The plaque number was reduced only by a factor of 2, despite a concentration of drug which should have been highly inhibitory to protein synthesis. The result is more readily appreciated when one bears in mind the great sensitivity of the present plaque detection method. It is known that antibody-forming cells store in their cytoplasm an amount

equal to about 30 min worth of production (10), and thus secretion of stored product alone (presumably not immediately affected by puromycin) would release sufficient antibody to give a plaque in many instances. Accordingly, we preincubated cells from immunized spleens for $\frac{1}{2}$ to 3 hr in a liquid medium containing puromycin, and only then incorporated the cells into a puromycin-containing gel. Reductions of plaque counts down to 5% of control levels was readily achieved.

The effect of puromycin on PC of young male mice (Table XIII) was much more marked than that on immunized spleen cells. At 3 hr, three-quarters of the plaques had failed to appear in the presence of the inhibitor, and, most

TABLE XIII

Effect of Puromycin on Plaque Production by Spleen Cells from Immunized Mice or by Peritoneal Cells from Nonimmunized Mice

Nature of cells	Type of mice	Puromycin concen-tration	3 hr culture		22 hr culture	
			PFC/10⁶ ± SE	Control	PFC/10⁶ ± SE	Control
				%		%
Spleen cells	Young male mice immu-nized 4 days previously	0	4,810 ± 510	—	4,730 ± 558	—
		10 µg/ml	2,640 ± 313	55	2,330 ± 136	49
Peritoneal cells	Nonimmunized young male mice	0	290 ± 35	—	4,210 ± 633	—
		10 µg/ml	75 ± 14	26	73 ± 20	1.7
Peritoneal cells	Nonimmunized retired breeder mice	0	10,400 ± 769	—	49,300 ± 5,130	—
		10 µg/ml	8,440 ± 842	82	9,330 ± 948	19

significantly, no new plaques appeared over the next 19 hr, whereas in control cultures the plaque count rose nearly 15-fold over this interval. As in the actinomycin D experiments, the degree of inhibition achieved by puromycin was less for plaque formation by PC from retired breeders than for PC from young males. However, with retired breeders the plaque count also did not rise significantly after 3 hr in the presence of puromycin.

Thus, for the three types of cell populations considered, there is a compartment of PFC (about 50% for immunized spleens, 20% for retired breeder PC and 2% for young male PC) which is resistant to inhibition by puromycin and which expresses itself in 3 hr. The most likely explanation of this fraction is that they represent cells with stored antibody. Another possibility is that the rate of entry of puromycin into cells is, for some reason, low in the CMC system, thus leading to apparently inefficient inhibitory effects.

Time Lapse Microcinematography of Cells in Monolayers with and without
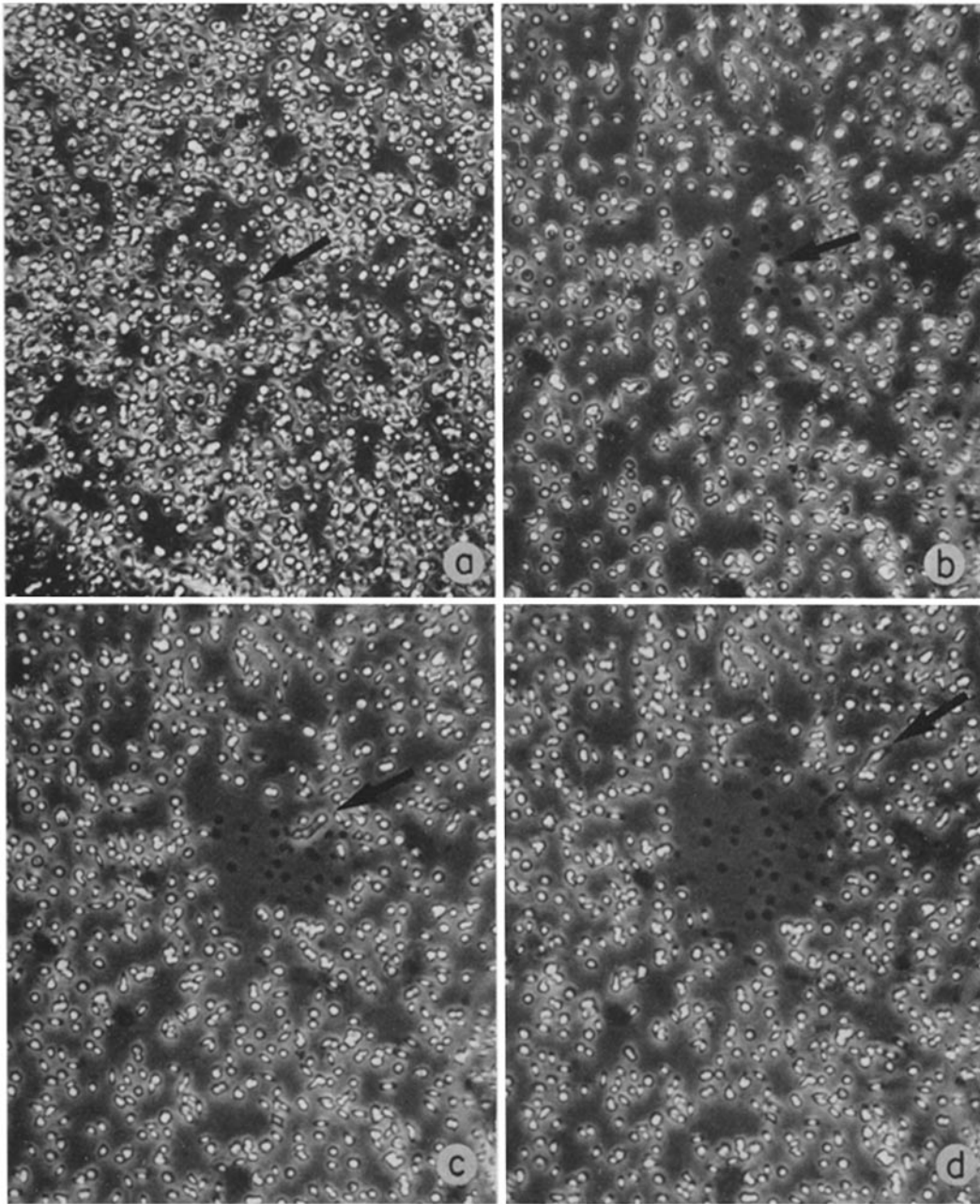
FIG. 2

Inhibitory Sera.—A series of films were made of plaque formation by cells after micromanipulation transfer. The original plaque-forming PC was transferred into a monolayer culture containing 1:200 anti-IgM. After 1–4 hr time-lapse microcinematography, the cell was retransferred into a monolayer without inhibitor. After transfer into the IgM-containing monolayer, the cell continued to live, displaying its usual "peristaltic", nontranslational movement. No plaque appeared around the cell, although the films did reveal the lysis of 2–5 SRBC in the immediate vicinity of the cell. On retransfer to normal, noninhibitory monolayers, the cell engendered plaque formation without detectable lag. The rate of plaque growth was, in general, quite standard, showing that the synthetic capacity of the cell had not been affected by exposure to the anti-IgM serum. However, in two of eight film sequences run in this fashion, the PFC exhibited an apparent change in its movement pattern after exposure to anti-IgM. For 2–3 hr after retransfer to normal medium, it continued to behave normally, exhibiting peristaltic movement. Then, it suddenly displayed active translational movement typical of that of lymphocytes in nonviscous media. Long cytoplasmic processes were seen, and red cells became attached to their surface and were soon after lysed. Secretion of antibody continued while the cell was moving so that in these two cases a "snail-track" of lysis outlined the path of the cell, and a "satellite" plaque developed around the area, 100–200 μ away from its original location, where the cell finally settled down. Some features of this process are seen in Fig. 2. We interpret this to be an irritant effect of anti-IgM on the lymphocyte cell membrane, but have not yet performed an extensive series of control experiments involving other inhibitory antisera.

DISCUSSION

The chief findings of this paper can be summarized as follows:

(*a*) The appearance of hemolytic plaques in culture monolayers containing

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FIG. 2. Behavior of a peritoneal cell from a young male mouse taken from the center of a plaque 20 hr after the beginning of CMC open microculture, and transferred serially by micromanipulation into two CMC monolayers, the first containing 1:200 rabbit anti-mouse IgM serum, and the second being a normal SRBC-CMC-complement monolayer. Frames from time-lapse microcinematographic film. The arrow identifies the PFC. (*a*) 1 hr after transfer to monolayer containing anti-IgM. No plaque has formed. (*b*) 15 min after retransfer to normal monolayer. A small plaque is present, the cell is round and at the center of the plaque. (*c*) After 2 hr of culture in the normal gel. The cell has started to show active translational movement. This is unusual, and may or may not have been due to the irritating effects of anti-IgM on the cell membrane. SRBC are adhering to the PFC; they will shortly be lysed. (*d*) After 3½ hr culture in the normal gel. The PFC has moved right out of the original plaque and is beginning to cause lysis in another area. In 1–2 hr, a "satellite" plaque will appear here. More SRBC are attached to the surface of the PFC; they will also be lysed shortly. × 160.

PC can be specifically and reversibly inhibited by rabbit anti-mouse IgM serum. This strengthens the arguments already advanced for this response being due to antibody formation.

(*b*) The appearance of plaques is dependent on peritoneal cells in an optimal concentration. It cannot be mimicked by lymphoid cells from other sources; whatever mixtures have been tried are ineffective. The data, though incomplete, suggest that optimal plaque formation depends on some cell to cell interactions between PC which is neither a macrophage–lymphocyte interaction nor a non-specific feeder-layer effect.

(*c*) The induction of antibody formation in PC is entirely independent of cell division in vitro and only variably dependent on messenger RNA synthesis. Actinomycin D is a potent (98%) inhibitor of plaque appearance when young male mice are used, and is ineffective, as expected, in the case of preimmunized spleen cells. With PC from retired breeder mice, a substantial compartment of cells exists which can form plaques even in the presence of high concentrations of actinomycin D, suggesting that such cells contained the necessary messenger RNA at the time of killing of the animal.

Taken as a whole, the inhibitor experiments reinforce the view expressed in the first paper that we are dealing here with the induction of some kind of a typical immunological recall phenomenon. It now seems proven beyond reasonable doubt that the PC are forming hemolytic, IgM antibody against SRBC. The puromycin results suggest that most PC must synthesize this IgM *de novo* in cultures, an argument strongly against the notion of cytophilic antibody as the basis of this phenomenon. The actinomycin D experiments imply that new RNA synthesis is involved, presumably because antigen subserves some inductive or derepressive function. However, the speed of the induction and the total failure of mitotic poisons to inhibit it place the phenomenon in striking contrast to the usual systems of the induction of primary immune responses in vitro. A speculative explanation may be that the PC populations contain a novel kind of memory cell, stimulated in vivo by some cross-reacting antigen of food or autologous origin, which has the characteristic of being fired off quickly and without cell division in the CMC cultures. In that case, the higher reactivity, greater actinomycin D and puromycin resistance, and more rapid induction kinetics of the retired breeder mice could all be explained by postulating a greater degree of this hypothetical prestimulation.

As regards the cell to cell interaction work, clearly much remains to be done. For example, no experiments on neonatally thymectomized or irradiated and restored mice have yet been performed. In considering the immune response of the intact mouse to SRBC, it appears that a collaboration between thymus-derived and bone marrow-derived lymphocytes is required for optimal activity (3, 6), but this needs to be investigated as regards this PC inductive phenomenon. We hope to clarify this issue and also to embark on more definitive

biophysical separation techniques. Despite these limitations in our knowledge, we consider this dependence on cell concentration one of the strongest arguments against the view that our system is measuring a background phenomenon of little inherent interest.

The actual contact between a macrophage and a PFC seen from time to time in cinematographic observations proves that cell to cell contact is possible in our semisolid system. It does not, by any means, prove that this is the ordinary pathway for the immune response triggered in vitro, but, simply, that the physical conditions existing in the semisolid environment do not forbid cell agitation and contact between cells.

Finally, it is our impression that the system of ultrathin layer culture may be of great use for the study of different steps of the immune response at the cellular level. It allows fine observation of cell physiology, as well as actual manipulation of the active cell. By contrast with the Jerne or Cunningham techniques, which are believed to measure only those cells actually forming antibody in vivo at the moment of killing of the animal, the CMC system allows functional survival of cells for at least 24 hr, together with continuous monitoring of antibody-forming capacity. During this period it is evident that regulatory changes of a profound nature take place in the cell population. Whether these represent the beginning of a true immune induction in the usual sense of the word, or some derepression of cells already precommitted to the formation of antibodies which can lyse SRBC is not yet clear. In either case, the fact that lymphoid cells from normal mice can be induced to form up to 100,000 PFC/10^6 cells plated within 24 hr and without cell division, must henceforth be taken into account by all students of the immune response using this species and this antigen.

SUMMARY

Peritoneal cells (PC) from normal, unimmunized mice were placed in ultrathin monolayer cultures containing carboxymethylcellulose (CMC), sheep red blood cells (SRBC), and complement, and tested for the appearance of plaques of lysis. The behavior of PC from young male mice and from female mice that had given birth to several litters (retired breeder mice) was studied.

It was found that cells from spleen, mesenteric lymph node, thymus, bone marrow, thoracic duct lymph, or Peyer's patches could not form plaques in the CMC microcultures. Also, various combinations of these cells did not lead to plaque formation. When cells from any of these sources were mixed with PC, there was either no effect or an actual inhibition of plaque formation, the plaque counts being lower than would have been expected from the number of PC present in the mixture.

Optimal plaque formation by peritoneal cells was found to be dependent on an optimal cell concentration, this optimum being around 5×10^6/ml for young male mice and 0.5×10^6/ml for retired breeders. Inhibition of plaque formation

was found with either supra- or suboptimal cell concentrations. The inhibition by excess cell concentration may have been a simple nutritional or nonspecific overcrowding effect, as it could also be induced by an addition of an excess of spleen or lymph node cells. The failure of more dilute PC preparations to give adequate numbers of plaques appeared to be more specific, as plaque numbers could not be restored to normal by addition of spleen cells. The suggestion was that some cell to cell interaction between PC was involved. This dependence on cell concentration was not seen with immunized spleen PFC.

Plaque appearance could be specifically and reversibly suppressed by placing PC in a medium containing rabbit anti-mouse IgM serum. Anti-IgG serum had no such effect. These experiments strengthened our view, expressed in the accompanying paper, that plaque formation was due to the formation of IgM, hemolytic antibody to SRBC by the PC.

Metabolic inhibitors were incorporated into monolayer cultures and had different effects with the different types of PFC used. In the case of spleen cells from mice actively immunized against SRBC 4 days before killing, actinomycin D had no effect on plaque counts and puromycin reduced plaque numbers by a factor of 2. In the case of PC from young male mice, actinomycin D in concentrations above 0.01 μg/ml caused reductions down to < 2% of control values in plaque counts, and puromycin (10 μg/ml) had a similar effect. The PC from retired breeder mice occupied an intermediate position between the two cases just discussed. A compartment of cells, equal to about one-fifth of the total normal PFC compartment, was identified as resistant to high concentrations of either actinomycin D or puromycin, being similar in these respects to PFC from spleens of intentionally preimmunized mice. The mitotic poison, Colcemid, did not affect plaque counts in any situation tested.

The theoretical implications of these results are briefly discussed.

BIBLIOGRAPHY

1. Nossal, G. J. V., A. E. Bussard, H. Lewis, and J. C. Mazié. In vitro stimulation of antibody formation by peritoneal cells. I: Plaque technique of high sensitivity enabling access to the cells. *J. Exp. Med.* **131**:894.
2. Boak, J. L., and M. F. A. Woodruff. 1965. A modified technique for collecting mouse thoracic duct lymph. *Nature (London).* **205**:396.
3. Miller, J. F. A. P., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I: Hemolysis-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:801.
4. Bussard, A. E., and M. Lurie, 1967. Primary antibody response in vitro in peritoneal cells. *J. Exp. Med.* **125**:873.
5. Ingraham, J. S., and A. E. Bussard. 1964. Application of a localized hemolysin reaction for specific detection of individual antibody forming cells. *J. Exp. Med.* **119**:667.
6. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transpl. Rev.* **1**:3.

7. Dutton, R. W. 1967. In vitro studies of immunological response of lymphoid cells. *Advan. Immunol.* **6:**254.

8. Bussard, A. E. 1969. *In* Immunological Tolerance. M. Landy and W. Braun, editors. Academic Press Inc. New York. 201.

9. Bussard, A. E. 1968. Primary antibody response induced in vitro among cells from normal animals. *Cold Spring Harbor Symp. Quant. Biol.* **32:**465.

10. Nossal, G. J. V., and O. Mäkelä. 1962. Elaboration of antibodies by single cells. *Annu. Rev. Microbiol.* **16:**53.