PRIMARY ANTIBODY RESPONSE IN VITRO IN PERITONEAL CELLS*

By ALAIN E. BUSSARD, Ph.D., and MELVYN LURIE

(From the Department of Cellular Immunology, Institut Pasteur, Paris, France)

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Since the early days of immunology many workers have tried to initiate and maintain in vitro an immunological response leading to the formation of antibody by cells from nonimmune animals (1, 2). This quest has been revived in the last decade, since immunologists felt the need of such an approach at a cellular level. Various laboratories have succeeded ir maintaining tissues and cells actively producing antibody in culture for various lengths of time (3), while others have been able to bring about antibody production in quiescent tissues and cells previously stimulated in vivo by in vitro contact with specific antigen i.e., a "secondary response" in vitro (4,5). Another line of investigation was to stimulate uncommitted cells in vitro before transferring them to incompetent recipients whose presumed role would be to sustain the life of the "grafted" antibody-producing cells ("immunocytes") (6, 7). A closer approach to the complete in vitro conditions was achieved by using peritoneal chambers in recipients that played the role of "living incubator" for the immunocytes (8, 9). In recent years, some authors have been able to initiate a complete in vitro stimulation of tissue fragments (10–13) or of isolated cells (14–18), from nonimmune animals. In the latter case the antibody production was demonstrated either by detection of antibody activity in the culture fluid (14–16) or associated with the cells (17, 18). This last system, using a method developed in our laboratory (19) for the detection of immunocytes by local hemolysis in gum (L. H. G.), will now be described in detail.

TERMINOLOGY

We feel that the term of immune response is clearer when applied to a whole organism than when applied to cells; for cells we rather use the phrase "cells from an immunized animal." If the cells are shown to produce antibody, we propose the term "immunocyte." Thus a *primary* immune response would be the reaction of an organism subjected experimentally for the first time to an antigenic stimulation. This definition, purely operational, eliminates the usually unsolvable question of whether an animal is uncommitted towards an antigenic determinant or a cross-reacting structure.

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Material and Methods

Preparation of Cells.—Female CBA mice of more than 8 weeks' age were sacrificed by cervical fracture or exsanguination. The abdominal skin was retracted, and 2 ml of Eagle-Tris culture medium was injected into the peritoneal cavity. The peritoneum was then slit, and the liquid withdrawn with a Pasteur pipette, and put into siliconized centrifuge tubes. An aliquot was withdrawn for cell count and differential count, and the suspension of peritoneal cells (PC)[1] centrifuged for 20 min at 500 *g* at 4°C. Other strains of mice have been tested: C_3H, T_6, C_{57} Bl, and AK.

Cell Enumeration.—The aliquot from the pooled peritoneal fluid was allowed to remain in the hemocytometer for 30 min at room temperature. The cells were then counted by phase contrast microscopy and differentiated into three groups: small lymphocytes, large cells remaining free, and large cells attaching on the glass.

The large cells made up between 30 and 50% of the cell population, 10–30% of them being attached. Approximately 5×10^6 cells were obtained per mouse.

Procedure for Demonstrating the Immune Response.—Five million cells were added to 1 ml. of a preparation containing 0.7 ml of a thick solution of 2.2% carboxymethyl cellulose (CMC) made in an Eagle–Tris buffer (see reference 19), 0.1 ml of a 50% suspension of sheep red blood cells (SRBC), and 0.1 ml of absorbed (with SRBC) guinea pig serum (GPS) as a source of complement (C′). The final preparation consisted of 1.54% CMC, 5×10^6 leukocytes per ml, 5×10^8 SRBC, and 10% GPS. In other experiments a final concentration of 5×10^6 SRBC was used. The preparations were then plated according to the method of Ingraham and Bussard (19). Plaques of hemolysis appeared between 15 and 48 hr of incubation at 37°C.

This procedure is the "one-step" system, in which the phase of stimulation by the antigen and the expression phase (formation of plaques of lysis) take place successively in the same sealed system. One of the drawbacks of such a technique is that the complement is a limiting factor. The amount of complement still active after 48 hr at 37°C is not sufficient to reveal all the potential antibody-producing cells.

A "two-step" procedure was also employed. The peritoneal cells (5×10^6/ml) were first incubated at 37°C in siliconized glass tubes, either in Eagle-Tris culture medium or in the CMC gum, under nitrogen in presence of SRBC (5×10^6 or 5×10^8 per ml) and 10% heated GPS. After 24 or 48 hr the cells were incorporated in gum (in the case of incubation in liquid medium) and 0.03 ml of C′ was added before plating on microscope slides under cover slips. The preparation was then sealed with vaseline and incubated to allow the plaques to develop.

Preparation of Anti-CBA Spleen Cell Serum.—Male mice of the BALB C strain received intraperitoneally suspensions of CBA spleen cells at days 0, 5, and 60, with 16, 35, and 125 million cells/mouse respectively. Blood was taken from the carotid on day 64 and the serum obtained was decomplemented at 56°C for 30 min.

Assay of Colchicine's Anti-Mitotic Action on Mouse PC.—Bottles containing 2.9×10^6 peritoneal cells, the majority of which were small lymphocytes, and 3 ml of Eagle-Tris culture medium containing 10% calf serum were incubated at 37°C for 68 hr. The medium was carefully removed with a curved-tip Pasteur pipette so as not to disturb the cells resting on the glass cover slip in the incubation bottle. Fresh medium containing 10^{-9} g of colchicine per ml was added. This concentration is known to inhibit mitosis in vitro (32). The cover slips were removed at 3.5 and 25 hr. The cells were fixed with 99% methanol for 5 min and stained with Giemsa (R. A. L. Giemsa R), then observed under oil immersion.

Microcinematographic Recording.—Microcinematographic observation of the cells while

[1] The following abbreviations are used in this paper: PC, peritoneal cells; CMC, carboxymethyl cellulose; SRBC, sheep red blood cells; GPS, guinea pig serum; PFC, plaque-forming cells.

they were producing hemolytic antibodies was performed. The optical setting used a Zeiss objective, phase contrast, dry, with a ×40 power. One frame every 8 sec was taken; when the film was projected at 24 frames per second, the final acceleration was about 200 times. The specimen under observation was kept at 37°C in a constant temperature chamber. Recording was carried on for 24 or 48 hr.

RESULTS

In Vitro Stimulation during One-Step Experiments.—The stimulation of antibody production has now been achieved in more than 60 different experiments, each involving four to five CBA mice. Of this number, six experiments were negative or gave so few plaque formers that the results were not significant. Thus 90% of experiments yielded positive results, that is to say, the peritoneal cells from nonimmunized mice were induced to produce antibody after incubation of more than 24 hr with sheep erythrocytes.

TABLE I

Stimulation In Vitro of Peritoneal Cells

Number of experiments*	Amount of complement	Inhibitors	PFC‡ ± SD
13	15% GPS§	—	304 ± 89.5
12	10% "	—	150 ± 122
15	10% heated GPS	—	0
2	10% GPS	Dinitrophenol 10^{-3} M	3
1	"	Puromycine 10 μg ml	3
1	"	10% serum anti-CBA spleen cells	0

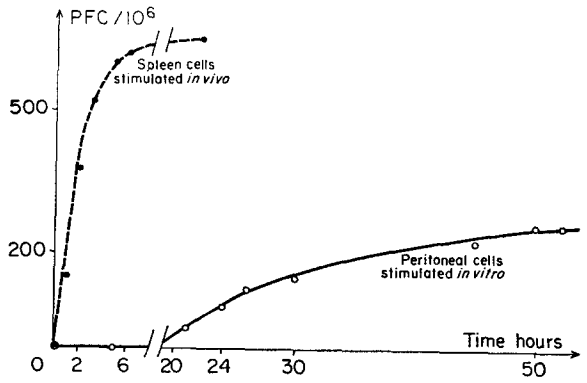
* Each experiment involves 5 mice.

‡ Mean number of plaque-forming cells.

§ GPS, guinea pig serum.

The results of two series of experiments are reported in Table I: significant activity was reached after 72 hr of incubation at 37°C. This activity is at least 100 times greater than that obtained in spleen or lymph node cells from nonimmunized mice (19–21) ("background activity"). Furthermore this background is maximum after 5 hr at 37°C after plating and will not increase further. The behavior of the peritoneal cells is quite different: no plaque of hemolysis ever appeared before 20–24 hr after the beginning of incubation (no immediate "background") in the case of the one-step procedure. The maximum was never obtained before 48–72 hr, this limit being probably due to factors (such as complement decay) not inherent to the cells. In fact, apparent activity of the peritoneal cells stimulated in vitro is of the order of magnitude of that obtained with mouse spleen or lymph node cells from an immunized mouse, 5 days after one injection of SRBC.

To determine whether we were dealing with a bona fide immunological phenomenon different control experiments were undertaken. The replacement of fresh guinea pig serum (active complement) by heated guinea pig serum (56°C for 30 min) gave without exception complete suppression of plaque formation. Metabolic inhibitors, such as dinitrophenol or puromycin also suppressed the phenomenon (see Table I). Since the formation of plaques is correlated with the life of the leukocytes present in the preparation, heating the peritoneal cells at 60°C for 30 min before their incorporation in the gum totally suppressed the formation of plaques. Microcinematographic observation of the cells producing antibody in the plaque of hemolysis always showed a correlation



TEXT-FIG. 1. Kinetics of plaque formation by cells from immunized mice or by cells from non immunized mice stimulated in vitro.

between the life of the cell (ascertained by its movements) and the increase in size of the zone of hemolysis. Finally, incorporation of the anti-CBA spleen cells serum (obtained in BALB C mice) also resulted in inhibition of plaque formation, though this serum was not toxic in itself (since it did not prevent plaque formation in rabbit spleen cells).

Kinetics of Plaque Formation.—The kinetics of plaque-formation allows the distinction between cells stimulated in vivo and peritoneal cells stimulated in vitro. Text-fig. 1 shows that a population of spleen cells 4 days after primary injection produced a maximum number of plaques after 6 hr. The immunological capacity of most immunocytes is not exhausted by this time, as can be seen by the fact that the size of most of the plaques increased continuously up to 24 hr after the beginning of plating.

Peritoneal cells from uninjected mice behaved quite differently; no plaques were seen before 20–24 hr after the beginning of plating, in the one-step method. The number increased readily to attain its maximum 48 hr later.

An index of the kinetics of plaque formation is given by the time required to

obtain 50% of the total number of plaques attainable. This time varies significantly, as can be seen in Table II, in the one-step and the two-step methods. This means that a previous contact of the peritoneal cells with the antigen reduced the time necessary for the appearance of plaque during the expression phase. Furthermore, there is also a certain inverse proportion between the time of pretreatment and the time required to get the 50% effect. If this curve is plotted, as in Text-fig. 2, one can see an obvious effect of the pretreatment on the decrease of the time necessary to obtain 50% of the maximum of plaques, this effect being more marked for the first 24 hr of incubation. The curve flattens after more than 48 hr of incubation, leaving a minimum period of 5 hr. This period is longer than the equivalent period (60–90 min) that cells from immunized animals require to attain the 50% plaque index, perhaps because peritoneal cells need a minimum adaptation period before they start to express their immunological activity. Such an adaptation period is not necessary for spleen cells or lymph node cells from immunized animals, since they start to produce antibody as soon as they are in the gum.

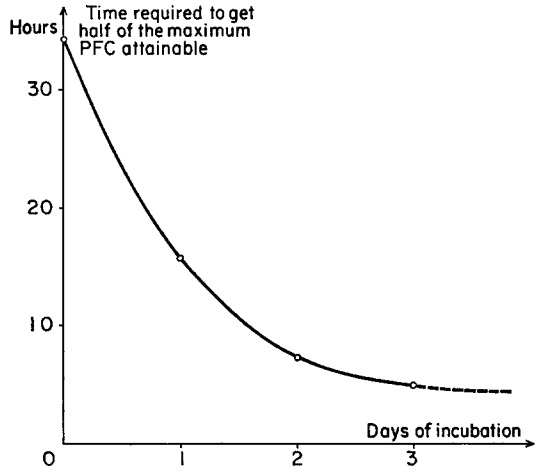
TABLE II

Differences in the Time Required for the Formation of a Certain Number of Plaques (50% of the Maximum) between Cells Plated Immediately and Cells Incubated with the Antigen before Plating

Immediate plating (One-step procedure)		Plating after incubation in gum (Two-step procedure)			
PFC/10^6 at 50% maximum	Time required to get this N°	Antigen (SRBC/ml)	Incubation	PFC/10^6 at 50% maximum	Time required to get this N°
	hr		*hr*		*hr*
95	32	5×10^6	24	60	18
200	44	"	"	15	10
32	40	"	"	18	18
150	28	5×10^6	48	110	6
122	36	5×10^8	41	120	8
80	28	"	42	50	4

The maximum number of plaques produced per million cells plated is highly variable, depending on complement quantity and activity, peritoneal cell concentration, and age of mice. Even when all the known components of the system are standardized, individual variations arise in the response. This implies that we are dealing with still unknown parameters in this highly complex, and probably unstable system.

Considering the effect of the strain of mice, our best results were with C_3H and CBA mice. Strains AK and C57 Black gave very poor results and T_6 mice variable ones.



TEXT-FIG. 2. Influence of the time of preincubation of the cells with antigen, on the time required, after plating, to obtain half of the maximum number of plaques attainable.

TABLE III

Influence of Serum during Incubation on the Viability and Immunological Activity of Peritoneal Cells

Incubation*		Viable cells, in per cent of initial population	Activity in PFC/10^6 viable cells
Duration	Conditions (Eagle-Tris)		
hr			
22	—	35	17
"	+ 10% heated GPS	20	610
"	+ 10% GPS	18.5	34
24	—	26	40
"	+ 10% heated calf serum	53	432
"	+ 10% heated GPS	50	488
24	+ 10% heated GPS	32	202
48	"	31.5	145
72	"	31	50

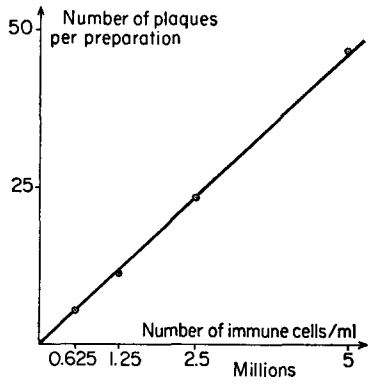
* Incubation in siliconized tubes at 37°C.

Apparently an important factor is the age of the peritoneal cells donor. Mice younger than 8 wk yielded cell populations of very low activity, whereas older mice gave much better results; mice of 6–8 months were very good donors. Sex is irrelevant in this matter.

When pretreatment is introduced before plating the cells, the conditions for

this incubation have a great influence on the final result of plaque formation. It can be seen in Table III that the presence of serum in the incubation medium is very important: though the viability index is not necessarily improved by serum, the immunological activity of the population is much greater in the case of cells incubated with GPS or calf serum than it is with cells incubated without serum.

Type of Plaque-Producing Cells.—The type of cells that produce antibodies in our system is difficult to ascertain since we are dealing only with vital observation under phase contrast microscopy or microcinema. The whole classification of leukocytes is based on the morphology of cells freshly taken from the animal (not after culture) and observed after fixing and specific staining. This makes it difficult to find homologies between these classical categories and the



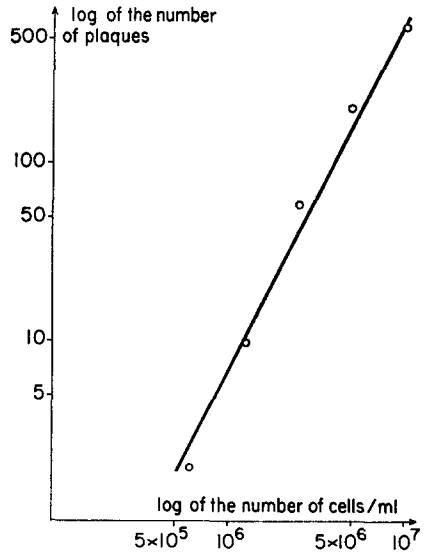
TEXT-FIG. 3. Effect of dilution of spleen cells from an immunized animal, by spleen cells from a nonimmunized one.

type of cells we observe in our system. Nevertheless, we can say that the active cells found in the hemolytic zone of our slides fall in two categories. The first can be seen in Fig. 1. It is a large cell with large nucleus and a vesicular cytoplasm. These cells contain usually round refringent bodies. The second type of cells is smaller, with a small nucleus and an apparently homogeneous cytoplasm, as can be seen in Fig. 2. Figs. 3 and 4 show two cells of different sizes in the center of a plaque; these cells are in contact in Fig. 3, a situation which is very frequently found in plaques. In many cases (such as in Fig. 3) a rosette of lysed red cells accompanies the active leukocyte.

Cinematographic observation of the cells during antibody production shows that they continually contracted during the whole period of plaque enlargement and that they were still living (as ascertained by their movements) 42 hr after incorporation in the gum. The viscosity of the medium did not prevent the movement of wandering cells. In some instances two cells attached and sepa-

rated, then reattached to each other for periods of approximately 10 sec two or three times before departing. Thus possibly an actual transfer of substances from cell to cell takes place readily during the phases of stimulation and expression. These findings raise the question of whether the phenomenon of plaque formation herein described requires the cooperation of two or more cells.

When spleen cells from immunized animals are diluted with unstimulated cells to maintain the concentration of leukocytes, there is a linear relationship between the number of plaque-forming cells (PFC) and the number of immunized cells plated (Text-Fig. 3). This proves that the formation of antibody

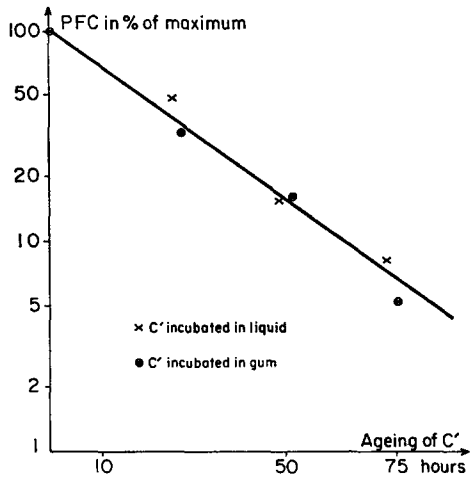


TEXT-FIG. 4. Effect of dilution of normal peritoneal cells on the activity of the population stimulated in vitro.

by immunized cells is not a cooperative process; rather each immunocyte is determined before plating, its activity being the result of an individual achievement.

The situation is quite different in regard to the population of normal peritoneal cells; in this case the relative activity (PFC/10^6 peritoneal cells) is largely dependent on the concentration of the cells. Within a certain range (from 6×10^5 to 10^7 cells/ml) the final activity rises considerably with concentration. Text-fig. 4 shows one of the experiments, in which a linear relationship between the logarithm of the number of plaques and the log of the number of cells/ml can be seen. This finding implies that the over-all process involved in the ultimate appearance of a plaque required the intervention of more than one cell.

True Activity of the Population.—The question can be asked whether the number of PFC, found when the plateau has been reached, measures the true activity of our population. Leaving aside the problems raised by the presence of more than one cell per plaque and by the sensitivity of the method (minimum amount of hemolysin necessary to bring the formation of a "noticeable" plaque), which are inherent in all methods of plaque formation (19–23), we must investigate the reasons for this plateau. One obvious reason is the decay in complement activity. Whereas it has been proven with short term experiments on plaque formation by cells from immunized animals (19) that in 5 hr



TEXT-FIG. 5. Influence of aging complement, at 37°C, on the plaque-forming capacity of spleen cells from immunized mice.

the detection system was still in complement excess, this is not true at the end of an incubation period of 72 hr at 37°C.

Model experiments of complement inactivation, under the same conditions used during plaque formation, were conducted with a suspension of cells of a known plaque-forming capacity (cells from immunized mice) as an index. It was found (Text-fig. 5) that at 37°C, complement decayed rapidly. After 50 hr the complement lost 85% of its initial activity. Therefore it is highly probable that the number of immunocytes after 3 days of incubation was much greater than the number of plaques formed. The effect of complement on plaque formation is further complicated, in the one-step system by the stimulation of the cells, since complement may play a role in the processing of the particulate antigen. The intervention of complement at two levels makes a quantitative analysis of its effect almost impossible, as long as the two phases of the over-all process cannot be separated experimentally.

Influence of Cell Division on the Phenomenon of Plaque Formation.—Inhibitors of cell division, or of DNA duplication, have been assayed in our system to see whether they have any effect on plaque formation. It can be seen in Table IV that fluorodeoxy-uridine, supplemented by uracyl, in a concentration which is known to completely inhibit DNA duplication has no appreciable effect on the activity of the peritoneal cells. Colchicine, added to the reaction mixture at a concentration of 10^{-3} μg/ml, which did inhibit mitosis in macrophage and small lymphocytes from the mouse peritoneum, had no effect on plaque formation by these cells.

TABLE IV

Influence of Mitotic Inhibitors on Stimulation of Peritoneal Cells In Vitro

Conditions of experiment	Inhibitor used	Activity in PFC/10^6
Immediate plating	—	18
Immediate plating with inhibitors	FUDR* 4 μg/ml-Uracyl 2 μg/ml	19
Preincubation in gum 39 hr at 37°C	—	140
Preincubation in gum, inhibitor from the beginning	—	120
Preincubation in gum, inhibitor added after 39 hr	FUDR 4 μg/ml-Uracyl 2 μg/ml	111
Immediate plating	—	2370
Immediate plating with inhibitor	Colchicine 10^{-3} μg/ml	2097
Immediate plating	—	507
Immediate plating with inhibitor	Colchicine 10^{-3} μg/ml	525

* 5-Fluorodeoxyuridine.

Type of Antibody Formed.—An important question is the type of antibody involved in plaque formation. There is no direct, easy approach to this problem in our system: the use of mercaptoethanol as an inhibitor of IgM is barely possible as it rapidly lyses the SRBC in the gum. Furthermore, some IgG molecules could also be denatured by this reducing agent. The use of specific antisera gives another clue. We used rabbit antiserum against purified CBA mice IgG. This serum, incorporated in the gum, had a great enhancing activity on the number of plaques given by spleen cells from immunized mice in a stage of secondary reaction, confirming the results of previous authors (24, 25). That the number of PFC formed by peritoneal cells was unaffected by the incorporation of this serum in the gum supports the view that the antibody formed by the cells is an IgM. If this is the case, it would be a reason to believe that we are

dealing with a true primary stimulation, but obviously this simple negative proof is not sufficient to demonstrate the IgM nature of the antibodies.

Effect of Actinomycin D on the Response In Vivo and In Vitro.—Another line of investigation was the study of the influence of actinomycin D on the immune response in vitro. When actinomycin is present in the medium containing spleen cells from immunized mice, there is little or no effect on the plaque-forming ac-

TABLE V

*Influence of Actinomycin D on the Formation of Plaques by Spleen Cells of Immunized Mice**

PFC/10^6		Reduction in activity
Controls	With actinomycin (1 μg/ml)	
		%
160	72	61
728	718	1.4
526	555	0
296	225	24

* Primar y immunization. Cells taken 5 days after one intraperitoneal injection.

TABLE VI

Influence of Actinomycin D on the Formation of Plaques by Peritoneal Cells Previously Stimulated In Vitro

Period of stimulation without actinomycin	Activity* of cells immediately incorporated in gum	Activity of cells preincubated		Reduction in activity
		Controls	Actinomycin in gum (1 μg/ml)	
hr				%
24	—	83	14	83
24	65	35	12	65
41	247	245	70	72
48	1300	76	0	100

* In PFC/10^6.

tivity of the population (Table V). This confirms the demonstration that actinomycin does not suppress the formation of antibody by cells previously stimulated in vivo. We tried to establish a model system which would, in a sense, reproduce the situation mentioned above. Peritoneal cells from immunized mice were incubated in liquid medium, with SRBC, for periods varying from 24 to 48 hr. They were, afterwards, incorporated in a gum containing actinomycin D (1 μg/ml). Viable cell counts showed that there was no significant difference, in control experiments, between cells incubated for the same length of time with or without actinomycin. As can be seen in Table VI, the

presence of actinomycin in the gum, after the period of stimulation, does not always completely suppress the expression of immunological activity by the cells. It is true though, that the reduction of activity is more marked than in the previous system (Table V). This may be due to a specific toxic effect of actinomycin on the cells, weakened by their incubation in vitro.

TABLE VII

Influence of Actinomycin D on Peritoneal Cells, from Normal Mice, Incubated with Antigen

One-step system

Amount of actinomycin in gum	Activity in PFC/10^6		Reduction in activity
	Controls	Cells with actinomycin	
			%
1 μg/ml	109	1	99.2
"	189	0	100
2 μg/ml	1340	6	99.5
"	310	0	100

TABLE VIII

Influence of Actinomycin D on Peritoneal Cells, from Normal Mice, Incubated with Antigen

Two-step system

Period of incubation with actinomycin D (1 μg/ml)	Activity remaining* relative to controls	Activity‡		Reduction in activity
		Controls	Actinomycin	
hr				%
18	38	41	0	100
24	62.5	118	0	100
24	—	83	0	100
26	—	178	0	100

* Number of PFC/10^6 in a population preincubated in CMC and plated in CMC, compared to the number of PFC/10^6 in a population plated immediately.

‡ In PFC/10^6.

Nevertheless, the situation is different when actinomycin is present during both the phase of antigenic stimulation and the phase of expression. As shown in Tables VII and VIII, actinomycin completely suppresses plaque formation under these conditions. This is true both for the one-step procedure and for the two-step procedure.

DISCUSSION AND CONCLUSIONS

The interpretation of our experimental findings must be considered at different levels.

First, it must be ascertained that we have not been describing simple artefacts (such as contamination with hemolytic bacteria) that bring formation of plaque of hemolysis in the gum. The following considerations eliminate this possibility:

(*a*) Foci of bacteria were never found in the plaques (our medium contained antibiotics).

(*b*) If plaque formation was related to bacterial contamination, the number of plaques would not reach a plateau after 72 hr of incubation, but would rise continuously since the nutrient factors are not exhausted by this time.

(*c*) The presence of active complement is an absolute requisite for the formation of plaques, which would not be the case if we were dealing with a production of hemolytic factors by bacteria.

(*d*) The sensitivity of the plaque-forming centers to anti-leukocyte serum—which is not directed against microorganisms—is a proof that the leukocytes present in the plaques are responsible for their formation.

Second, we are led to ask whether the phenomenon of plaque formation expresses a vital activity of these leukocytes.

(*a*) A first evidence for this is that heating of the peritoneal cells (60°C for 30 min) before their incorporation in gum suppresses all plaque formation.

(*b*) The best criterion for determining cell viability is motility. Microcinematography has shown that in all developing plaques leukocytes were actively moving. End of motility (death of the cells) always coincided with end of the growth of the plaque.

(*c*) The influence of anti-leukocyte serum also favors the idea that the leukocytes should be living if plaques are to be found.

Third, we believe that we are describing an immunological phenomenon. The fact that C′ is necessary for the formation of plaques is, by itself, completely demonstrative.

(*a*) As far as we know, no hemolytic process, other than an immunological one, requires C′ for its manifestation.

(*b*) The inductive characteristics of the reaction require a phase of stimulation before the phase of expression. This stimulation is manifested by a time-lag found in the one-step procedure. Pretreatment of the leukocytes with the antigen leads to reduction of this lag.

(*c*) The fact that this phenomenon is due to the activity of reticuloendothelial cells, known for their role in immunological processes, though not a proof, is strong evidence that we are observing an immune reaction, i.e., a synthesis of antibody.

The next question then is whether we are dealing with a true primary stimulation triggered in vitro. It is always difficult to make sure whether one is using truly "naïve" cells with no past experience with the antigenic determinants injected, the more so if these antigenic patterns are very common in nature, as the ones we used (antigen of the red cells surface). Under these conditions

there is no hope of demonstrating that the cells' ancestors, or the cells we actually worked on, have never encountered the antigens used in the system, or a cross-reacting structure. Nevertheless, we have some arguments in favor of the primary nature of our immune reaction.

(*a*) The findings that anti-IgG serum has no effect on plaque appearance pleads in favor of the IgM nature of the hemolytic antibodies. This, in turn, favors the idea of a primary response.

(*b*) The kinetics of the response in vitro is quite different from the kinetics of plaque formation by cells previously immunized. In the latter case these cells completely expressed their activity in 5 hr. This is also the case for the few plaque-forming cells found in spleen from nonimmunized mice ("background" activity). In our system we always found, on the contrary, a lag of 24 hr before the beginning of plaque formation. This lag can be reduced by a previous incubation of the cells with the antigen.

(*c*) The influence of actinomycin in the complete in vitro system brings additional evidence for the primary nature of stimulation. From what has been shown in vivo (26), and from what we demonstrated in vitro with cells stimulated in vivo (27, 28), the fact that actinomycin acts only on the primary response should be interpreted as a proof that antibody synthesis is governed by the action of a rather stable RNA messenger. When a certain amount of this messenger has been synthesized (which cannot take place in presence of actinomycin) it can work repeatedly, for a certain period of time. During this phase the system should be relatively insensitive to the presence of actinomycin. The great sensitivity of the cells during the stimulation period, compared with the decreased sensitivity of these cells during their phase of expression, could be interpreted as a proof that we are observing a true primary response. Obviously, other simple explanations should be investigated, such as a difference in the speed of penetration of actinomycin in unstimulated cells and in cells from an immune population. Finally, it requires the definite demonstration, at the molecular level, that actinomycin has been acting in accordance with the classical scheme of molecular biology and not by another pathway (general cytotoxicity, for instance).

The fact that the activity of the cell suspension is highly dependent on concentration strongly favors the view that our phenomenon results from a cooperative action of two or more cells. Cinematographic observation has shown that cells do move readily through the gum and could contact each other many times during the development of plaques. Transitory contact between two cells in a plaque of lysis has been actually observed. The presence of two contacting cells in many plaque centers (at least 30%) was regularly observed. These observations at least establish the possibility of cell interaction, based on the effect of dilution on the cell suspension activity.

These findings would be in line with the scheme (15) proposing a multi-step sequence for antibody formation: a first step in which the antigen is taken and processed in the macrophage, followed by a transfer of information to another cell which is responsible for antibody production.

All our attempts to stimulate in vitro lymph node cells suspension, which contains few macrophages, were unsuccessful. With spleen cell suspension that contained a

small number of macrophages, it was possible to get a slight effect (but definitely higher than the background) of plaque formation.

An antiserum prepared against spleen cells blocked the appearance of PFC. Even if cytotoxicity is involved, it would be mainly directed against small lymphocytes. Anti-macrophages antibody may simply inhibit presumed phagocytosis of SRBC by macrophage (29), but would be poorly cytotoxic in regard to the known insensitivity of macrophage. If attempts to separate the phase of stimulation from the phase of expression in our system are successful, one could completely circumvent the effect of cytotoxicity on the serum's target cell (or cells) by incubation of cells with antibody, but without C′, followed by addition of C′ and plating to reveal the immunological activity of the cells. Despite the anticipated experimental difficulties, the anti-cell antibody approach should be invaluable in discerning the nature of the transfer of material from one cell type to another, a transfer which may be presumed through certain reports (15, 16).

The lack of dependence of plaque formation on mitosis presents another group of questions. In the view of many workers cell division and blast cell transformation are related to the immune response (4, 30, and footnote 2). But while having certain characteristics of immune response, such as recognition of foreigness, these reactions have been quantified by per cent of blast cells present, or uptake of radioactive DNA precursors, and not by either amount of antibody produced or per cent of cells producing antibody. Mitosis does not appear as a prerequisite for our type of immune response. But if DNA-mediated RNA synthesis (i.e., messenger RNA) is such a prerequisite (as suggested by the complete inhibition of the response by actinomycin D), how do cells stopped in mitosis make antibody, i.e. protein, since RNA production ceases during mitosis (31, 32)? There are several possibilities. The first is that an experimentally undetectable production of mRNA goes on through mitosis. The second is that the fraction of cells of each category apparently not arrested in mitosis by colchichine is physiologically different than its cytologically identical counterpart in two respects: (*a*) it, and only it, carries on the immune response; (*b*) it undergoes cell division rarely, if at all. The third possibility is that mast cells and polymorphonuclears in the PC population, although small in number, carry the immunological burden.

A complete demonstration that we got a true primary response is still lacking. This proof could be obtained from experiments that show the specificity of the antigenic sensitization during the phase of induction. For this purpose, induction phase and expression phase should be, experimentally, completely separated; this is not achieved yet. It would also require the use of two artificial, or at least well defined, non cross-reacting immunogens. It will then be easy to prove the primary nature of the stimulation, by showing that the pretreatment period results in the specific sensitization, followed by further expression of the cells towards the antigen to which they were presented and not towards the other one.

We believe that we have demonstrated the possibility of obtaining a true stimulation in vitro of isolated uncommitted cells. It may be asked why this

[2] Main, R., and L. Cole. 1966. Immunological interaction between allogenic and xenogenic rodent spleen cells in culture: stimulation of DNA synthesis. Personal communication.

opportunity was not discovered earlier, since attempts in this direction have been made for more than 60 yr. We will offer some reasons to explain our positive results.

The first is related to the nature of the cell population employed. The peritoneal exudate is a mixture of different cells, namely macrophages and cells of the lymphocytic series. If the immune process requires two phases, the first involving a macrophage and the second a lymphocyte or plasma cell, the peritoneal population should be well suited for this purpose. An other advantage of peritoneal cells is that they are probably well adapted to withstand the mechanical hazards implied by the preparation of the cells (washing, centrifugation, changes in temperature) and their sojourn in the in vitro conditions. This adaptation results from the very fact that these cells are "free cells" in the body (where they must probably sustain, there also, many agressions), much better prepared to resist deleterious environment than attached, or semi-attached cells, like spleen or lymph node cells. In fact, the viability index of peritoneal cells after in vitro incubation was much greater than the viability of lymph node or spleen cells suspension kept under the same conditions.

The nature of the medium, primarily tissue culture fluid but also the carboxymethyl cellulose gum, may be a second reason. The latter, a polysaccharide that has been used in tissue culture techniques for many years with great success, constitutes a physiological semisolid environment for the cells. This has been shown by the relatively long period during which the cells survive, as demonstrated by their mobility. Finally, another advantage of the CMC is its activity as an adjuvant of the immune reaction (33).

A last reason can be found in the special conditions under which the cells are living. Indeed, Mischell and Dutton have suggested (18) that one reason for their success in inducing primary response in vitro was that their cells were kept under semi-anaerobic conditions. It is most probable that our cells, enclosed in this sealed system, were also under more or less anaerobic conditions.

In fact, the recent demonstrations by different authors that in vitro stimulation could be obtained with both organ fragments and isolated cells (10–13) makes the weight of evidence required to prove our point much lighter than it would have been few years ago.

We must keep in mind that the in vitro stimulation described here may be of limited scope, i.e., may not be applicable to all manifestations of the immune response. It could be reasonably considered that the primary response to foreign cells, accompanied by the rapid production of highly hemolytic 19 S, is a relatively primitive immune response. This response would involve the participation of macrophages, with phagocytic action followed by a transfer of processed antigen(s) to the antibody producing cell(s). The fact that this relatively transient, primitive, and not highly specific response could be elicited in vitro does not imply that a production of 7 S antibody against soluble antigens could be stimulated in the same conditions.

Regardless of the exact significance of the immune reaction we obtained, its relevance to theories of antibody formation is obvious. We found that 1% of a population of cells may produce antibodies against a given foreign type of cells. It must be remembered that the size of this competent group of cells does not depend on cell division; the same proportion of competent cells found after stimulation was present

in the initial population, as freshly collected from the animal. If we are really dealing with a true primary response, these findings are very difficult to reconcile with a simple clonal selection view: how would it be possible to account for the large capacity of a normal animal to react to a great number of different immunogenic determinants (characteristics of the immune response) when the number of different cellular groups is so limited? The more so, if we consider that not all the cells of the population are potential producers: some types of cells may never become immunocytes, some others would be in a stage of maturation incompatible with any possible stimulation towards specific competence. This increases again the relative specific activity of the population after stimulation to a value obviously incompatible with any theory of selection at the cellular level. This view would clearly be much strengthened if we could show that this reasoning is valid for any kind of antigen chosen at random.

Even if we are observing some sort of occult secondary reaction, it is still highly improbable that a normal animal, not in the stage of immune response against an infection, would store such a high proportion of specific responders against a previously encountered immunogen (or cross-reacting one) in order to react immediately by triggering such a large amount of immunocytes; this would be rather uneconomical.

Within the limited scope of the system used, our results are difficult to reconcile with the simple explanation of the immune response by a selection of a genetically specialized clone. They are more in line with recent results which shed, for similar reasons, some serious doubt on the clonal selection theory (34, 35, and footnote 3).

SUMMARY

Peritoneal cells (PC), from nonimmunized mice, incubated in a carboxymethyl cellulose gum containing sheep red blood cells (SRBC) and guinea pig complement (following the technique of Ingraham and Bussard) start to produce plaques of hemolysis 20 hr after the beginning of incubation at 37°C.

In contrast, spleen cells from immunized mice complete their plaque-forming activity in 6 hr. The fact that formation of plaques by previously uncommitted cells is related to the life of the leukocytes, and is complement dependent brings evidence that we are dealing with an immunological phenomenon. Puromycin suppresses the formation of plaque. Previous incubation of the PC with SRBC in liquid medium, before incorporation in the detection system, reduces the lag in the production of plaques. This indicates that a phase of stimulation precedes the phase of expression (manifested by plaque formation).

The immunological activity of the peritoneal cell suspension is highly dependent on the concentration of the suspension, which indicates that this activity results from a cooperative process. Actinomycin D, which does not suppress the production of plaques by cells from immunized animals, stops completely the in vitro induction. It is concluded that we have probably observed a primary immune response induced in vitro.

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[3] Trentin, J., et al. 1966. Antibody production by mice repopulated with limited numbers of clones of lymphoid cell precursors. Personal communication.

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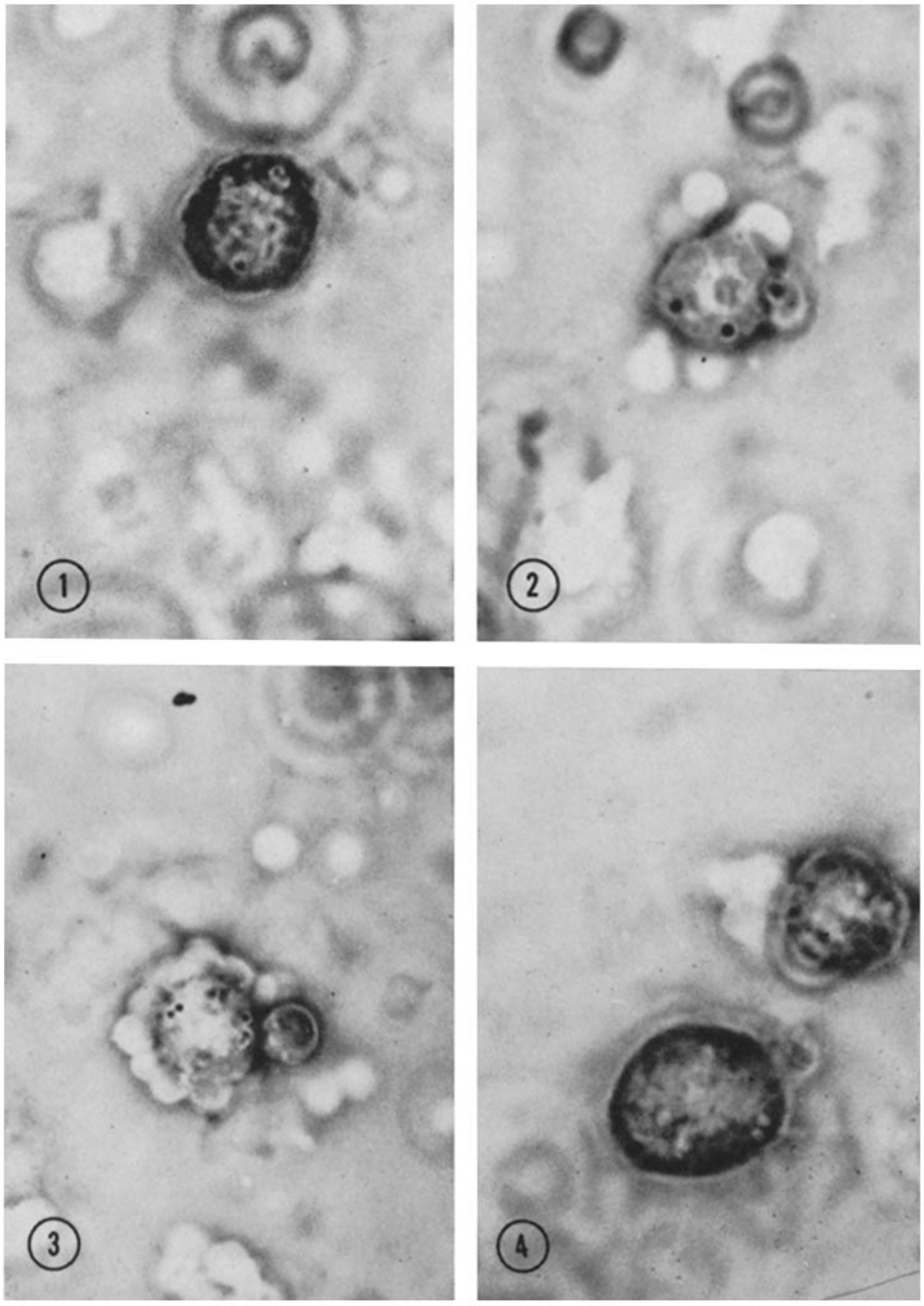
EXPLANATION OF PLATE 99

FIGS. 1-4. Immunocytes from a suspension of peritoneal cells from normal mice, stimulated in vitro. Microphotographs taken with phase contrast optics, × 1000, 48 hr after beginning of incubation.



(Bussard and Lurie: Primary antibody response in peritoneal cells)