

## IN VITRO STIMULATION OF ANTIBODY FORMATION BY PERITONEAL CELLS

### I. PLAQUE TECHNIQUE OF HIGH SENSITIVITY ENABLING ACCESS TO THE CELLS\*

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Previous reports from this laboratory (1, 2) have described a phenomenon of hemolytic plaque formation by peritoneal cells from unimmunized mice placed in short term tissue culture in a medium containing carboxymethylcellulose (CMC).<sup>1</sup> The basic findings have been confirmed by Bendinelli (3) who added the observation that cells harvested from the pleural cavity behaved similarly. In these experiments, thin layers of a mixture of peritoneal cells (PC), sheep erythrocytes (SRBC), guinea pig complement, and CMC medium were placed on microscope slides, covered with a cover slip, sealed with paraffin wax at the edges, incubated at 37°C, and examined at intervals for appearance of hemolytic plaques. It was found that plaques, with a lymphoid cell at the center of each, began to appear after about 1 day of incubation, and their number rose to peak levels by 3 days. A number of arguments were advanced in favor of the view that the cells were making hemolytic antibody (1-3).

This system differed from other reported systems of the induction of immune responses in vitro (4-6) in at least five ways: (a) unimmunized lymphoid cells from more conventional sources, such as spleen and lymph node, did not produce plaques; (b) the apparent number of antibody-forming cells reached very high levels, at times approaching 0.5% of cells cultured; (c) the reaction was exceptionally rapid, levels just below maximal being reached within 2 days of incubation; (d) the phenomenon appeared not to involve cell division, as mitotic poisons failed to affect it; (e) lytic plaques could be obtained with only one type of erythrocyte, that of the sheep, whereas when preimmunized spleen cells were used, the CMC method was efficient at plaque revelation with all erythrocytes tested.

In the present papers, we report results with a modification of the former, "closed" culture system. The methods here reported allow access to the plaques,

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<sup>1</sup> Abbreviations used in this paper: CMC, carboxymethylcellulose; PC, peritoneal cells; SRBC, sheep erythrocytes.

and the cells within them, by micromanipulation. The new "open" system of CMC microculture has yielded still higher plaque numbers. In this first paper, we describe the method; present proof that the plaque is due to this lymphoid cell in its middle; show evidence that the hemolytic factor made is not a complement component; give experiments that argue against the phenomenon being merely a high level of "background" antibody formation; and demonstrate a remarkable enhancement of activity in mice that have been pregnant.

#### *Materials and Methods*

*Mice.*—The majority of the experiments were performed on CBA mice obtained from the Central Animal Facility, Centre National de la Recherche Scientifique (C.N.R.S.), Gif sur Yvette, France. Germ-free mice were obtained from two sources, The Gustave Roussy Cancer Research Institute, Villejuif, France, through the courtesy of Dr. J. C. Salomon, and from the Ciba Research Institute, Basel, Switzerland, through the courtesy of Professor Fischer and of Dr. Gisler.

Germ-free mice were used immediately on receipt, as were conventional mice received in parallel with germ-free mice. The mice from C.N.R.S., used in most experiments, fell into two groups, which we shall call "young males" and "retired breeders". Male weanlings were received aged 6 wk and were kept for a further 5–10 wk, thus being used as young males aged 11–16 wk. The other main type of mouse used were females that had given birth to several litters and were received at age 9–10 months. These were used within 3 wk of receipt. This group is referred to as retired breeders although a proportion of the mice were actually pregnant at the time of killing.

*Preparation of Peritoneal Cells.*—Techniques for preparation of peritoneal cells, tris-(hydroxymethyl)amino methane (Tris)-buffered Eagle's medium, CMC-gum, SRBC, and sealed monolayer cultures were all as previously described (1). This older method will be referred to as the closed system.

*Complement.*—Pretesting of complement was found to be an important factor leading to more reproducible results in this system. Guinea pigs were exsanguinated and serum was prepared from each. A large batch of peritoneal cells representing a pool from 10 to 20 young male mice was prepared. Open microcultures (see below) were prepared of these cells using each complement in turn, and the numbers of plaques appearing after 24 hr of incubation were scored. In a typical experiment, 5 to 7 out of 10 guinea pig sera gave satisfactory plaque numbers in the vicinity of 2000 to 5000 plaques/ $10^6$  peritoneal cells. The remaining sera were unsatisfactory for one or both of two reasons: hemolytic activity producing background lysis of the SRBC monolayer, or failure to yield satisfactory plaque numbers (due possibly to a cytotoxic component). Sera of the latter type were discarded. Sera of the former type were held with an equal volume of packed sheep erythrocytes for 18 hr at 7°C. This absorption procedure was found to reduce the complement titer of the sample by from 30 to 50%. In preliminary experiments, peritoneal cell microcultures were set up with varying concentrations of absorbed or nonabsorbed complements and concentrations optimal for plaque appearance were determined. Consistently satisfactory results were obtained with either 10% (v/v) of nonabsorbed or 20% (v/v) of absorbed complement, and these were used in all present experiments. The concentration of 10% absorbed complement used in much of the earlier work with the closed system (1, 2) was, in fact, found to be marginal, being satisfactory for some guinea pig sera but grossly suboptimal for others. This, together with the presence of some "toxic" complements, may have magnified individual variability from experiment to experiment in previous work.

*Open System of Peritoneal Cell Microculture.*—Peritoneal or other lymphoid cells at concen-

trations varying from  $2 \times 10^4$  to  $2 \times 10^7$ /ml, but usually between  $5 \times 10^5$  and  $5 \times 10^6$ /ml, were mixed (as for the closed system) with complement (see above), SRBC ( $5 \times 10^8$ /ml), CMC (1.54 mg/ml) and Tris-buffered Eagle's medium. Using a graduated 0.1 ml pipette, a volume between two graduations appearing to be 0.01 ml was expelled onto a  $22 \times 22$  mm cover slip. As the CMC medium is extremely viscous, a considerable amount of the mixture was left behind in the pipette and the actual volume expelled could be calculated to be 7.3 and not 10  $\mu$ l. A second cover slip was applied to the expelled droplet or "bead" of medium, and gentle pressure was applied to spread the medium between the two cover slips till it covered a circular area about 12 mm in diameter. Each cover slip was then grasped by its edge between thumb and forefinger of each hand, and with a slow, even horizontal pulling motion, the two cover slips were drawn apart. This left each cover slip with an adherent, thin, even layer of medium. With practice, it was not difficult to ensure (a) that each of the two cover slips held approximately equal volumes of medium and (b) that preparations of equivalent thickness were prepared from experiment to experiment. The cover slips were then rapidly placed under paraffin oil to prevent evaporation of the thin layer of cells and medium. The microcultures were incubated in one of two types of specially constructed containers (Fig. 1). The first type (Fig. 1 A), used when micromanipulation was not required, consisted of a base of a large glass microscope slide ( $7 \times 7$  cm), four sides of plexiglass of 2 mm thickness, and a covering of two large cover slips. The microcultures lay on the base, with the CMC medium facing upwards. The whole remaining volume of the chamber was filled with liquid paraffin, but a slight gap was left between the two cover slips which formed the lid, so as to allow exchange of gases. These chambers allowed excellent visualization of the monolayers with either a conventional or an inverted microscope. Such chambers hold four microcultures. The second type of chamber, used for micromanipulation, is basically similar (Fig. 1 B); it is somewhat deeper, the height of the walls being 3 mm, and one of the four walls is missing to permit entry of a micropipette. The only factor preventing spilling of the liquid paraffin and emptying of the chamber, with consequent immediate evaporation of the culture, was the viscosity of the paraffin. It is thus preferable to use a rectangular glass slide to form the base of the chamber, and to leave off one of the shorter walls. It is also important to ensure that such chambers are held strictly horizontal at all times.

The microcultures in the open system exhibit the following features. Each has a volume of 3.65  $\mu$ l and normally contains 1,825 to 18,250 white cells. The cultures are only about 12  $\mu$  thick, and thus both SRBC and white cells form essentially a monolayer. On low power examination, all the cells are in one focal plane, although at 500-fold magnification it can be seen that the medium is so viscous that gravity does not cause the cells to settle on the cover slip and slight variations of focal level become manifest. The SRBC are spread more evenly and contiguously than in the closed system. It is our routine to count the numbers of plaques using a compound microscope and a magnification of 30-fold, dark ground. The optical qualities of the plaques compare favorably with those of the Cunningham liquid monolayer system (7) and are superior to those of either the former closed CMC system or the Jerne agar gel system (8), even in its microvariants (9). Some typical plaques are shown in Fig. 2. With practice, it is possible to detect plaques at very early stages of their formation. We now have no difficulty in recognizing, even at low magnification, plaques with no more than 5-7 lysed SRBC. It must be stressed that first class optical conditions are essential for optimal plaque counts. It is particularly important that red cell ghosts are rendered visible. Occasionally small artefactual irregularities occur in the SRBC monolayer, but these are readily distinguishable from plaques by the absence of ghosts and of a central lymphoid cell. We have found the most convenient optical conditions to result from the use of a Zeiss phase contrast condenser with the phase ring in position 1 together with a Plan-Achromat scanning objective,  $\times 2.5$ . This gives a brilliant dark ground effect, and small or doubtful plaques can

quickly be checked by swinging into position at  $\times 16$  long working distance phase objective without changing the condenser position.

Young male mice yielded a mean of  $2.3 \times 10^6$  cells per peritoneal cavity (range 1.1 to  $5.3 \times 10^6$ ) and as most experiments required a greater total number than this, pools of cells were frequently used. Retired breeder mice gave a mean of  $8.5 \times 10^6$  cells (range 4.7– $13 \times 10^6$ )

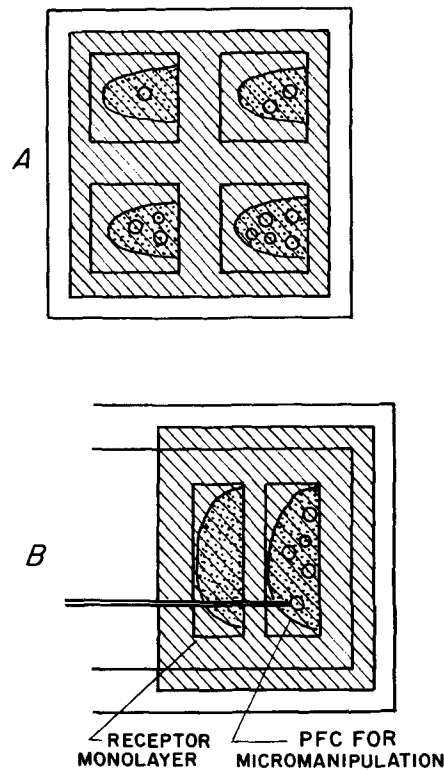


FIG. 1 A. A closed oil chamber with four preparations on glass cover slips containing some plaques of hemolysis. B. An oil chamber open on one side to allow micromanipulation. Two preparations on large cover slips are shown. A micropipette is shown in contact with the second preparation on the right.

and thus frequently, though not always, experiments were carried out on cells from a single animal. Unless otherwise stated experiments were carried out with a concentration of  $5 \times 10^6$  cells/ml of medium for young males, and 0.5 or  $1 \times 10^6$  cells/ml for retired breeders.

*Other Plaque Detection Systems.*—In several experiments, the liquid monolayer method of Cunningham (7) and the modified Jerne plaque method (9) were used. Two experiments were performed in which peritoneal cells were maintained in culture for 24 hr in an agarose gel medium. These were similar to the open microculture method described above, except that agarose at a concentration of 0.7% was substituted for CMC.

*Liquid paraffin.*—Several varieties of oil were tried including a light mineral oil termed Mayoline, several silicones, and a variety of commercial preparations of vaseline oil, or medici-

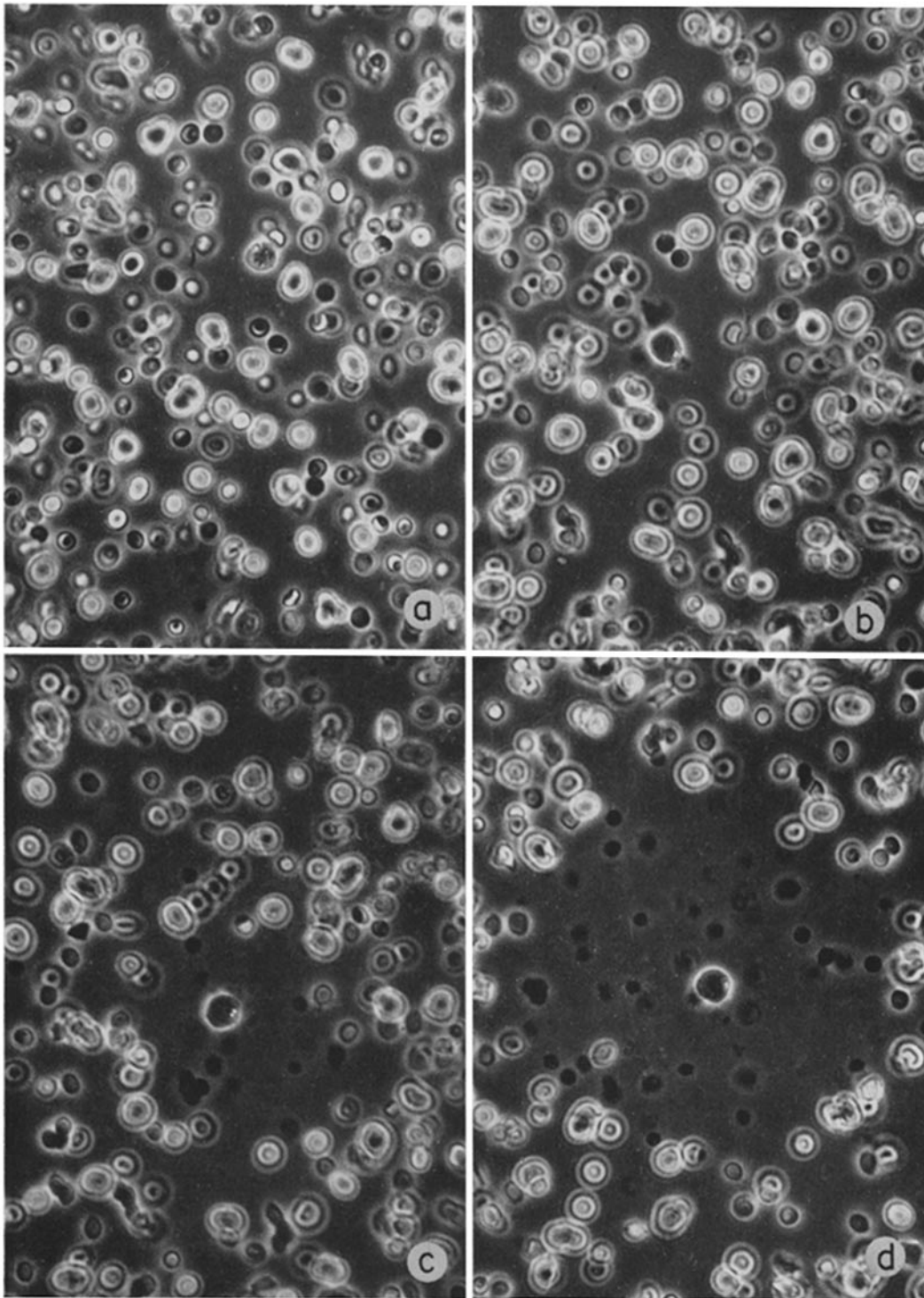


FIG. 2. Typical open system plates: (a) immediately after transfer; (b) 6 min later, 3 red cells have lysed; (c) at 15 min, a small plaque has appeared; (d) at 45 min.  $\times 400$ .

nal paraffin oil. The silicones were unsatisfactory, possibly because of diminished permeability to gases, and the numbers of plaques appearing in peritoneal cell cultures were markedly reduced. Mayoline and some samples of vaseline oil were hemolytic under the conditions used. The most satisfactory preparation was Pasteur Institute heavy medicinal paraffin oil. Contrary to what is generally believed, this and other mineral oils are not totally hydrophobic. In fact, over a 24 hr period, slight but significant desiccation of cultures through the paraffin was observed. Accordingly, paraffin was maintained over water in stoppered flasks at 37°C prior to use. Even so, cultures deteriorated after 36–48 hr, at least in part because of drying. This remains an unsolved problem of micromanipulation.

*Micromanipulation to Test Plaque-Forming Capacity of Cells at Center of Plaques.*—An essential feature of the present experiments was to test the capacity of cells that were found to be present at the center of a plaque to reform plaques after micromanipulation to a fresh monolayer, which frequently contained an inhibitor to be tested. For this, a Leitz micromanipulator and hand-drawn micropipettes with an orifice of about 12  $\mu$  were used. Somewhat surprisingly, the very viscous nature of the medium did not impede effective micromanipulation using conventional techniques. The plaque-forming cell (PFC) to be transferred was drawn into the pipette under 200-fold magnification (phase contrast) so that it was trapped just inside the orifice. It was then expelled into a premarked area of a fresh monolayer. This receptor monolayer sometimes contained all the elements of the revealing system (i.e., SRBC, complement, CMC, and Tris-buffered Eagle's medium); and sometimes it either lacked a component or contained a specific inhibitor. In early experiments, the PFC was washed in microdroplets of liquid medium prior to transfer into a new gel, but this was soon shown to be unnecessary as the tiny amount of hemolysin transferred from the original plaque together with the cell was not sufficient to cause observable lysis. It was important to perform the micromanipulation sufficiently carefully so as not to transfer red cell ghosts with the PFC. The micromanipulator, microscope, and chamber were enclosed in a plastic incubator maintained at 37°C so that cell metabolism was not interrupted at any stage. Accordingly, the time of appearance of the new plaque made by the transferred cell could be accurately assessed. To a degree, the length of the latent period depended on the care with which the transferred cell was observed. With continuous observation the first SRBC could often be seen to be lysed within 5 min after transfer. In practice, however, 20 or more PFC were usually transferred one after the other into premarked areas of the receptor gel, and examined at 10 min intervals for the formation of unquestionable plaques. The plaque diameter was measured with the aid of an eyepiece vernier. In typical experiments, PFC were examined frequently over the first 5 hr, and on one final occasion, about 20 hr after transfer.

*Time Lapse Microcinematography.*—This was performed on PFC using  $\times 16$  or  $\times 40$  phase objectives, a Vickers camera and Scientia film. Frames were taken each 8 sec and projected at 16 or 24 frames/sec. In several films, cinematography was commenced immediately after micromanipulation transfer of a cell.

## RESULTS

### *Comparison of Four Systems for the Detection of Anti-SRBC Antibody-Forming Cells.*—

In Table I, we present results of the numbers of PFC found, using different plaque revealing systems, in the spleens of mice that had been injected 4 days previously with  $5 \times 10^8$  SRBC. Such animals are known to be at the height of the primary IgM response to SRBC. In each case, reading of plaque numbers was performed after cells had been allowed to form antibody for 2 hr.

It is striking to note that the open CMC technique using ultrathin monolayers revealed over four times as many antibody-forming cells as the microvariant of the classical Jerne system, and over twice as many as the liquid monolayer or the closed CMC system. It can be assumed that the sensitivity factor for the conventional Jerne system performed on Petri dishes and read with a colony

TABLE I  
*Relative Sensitivities of Various Local Hemolysis Techniques for Detection of Plaque-Forming Cells from Spleen of Mice Immunized with SRBC 4 Days Previously*

Test system	No. of experiments	Mean sensitivity ratio $\pm$ SE
Jerne agar gel system micro variant	4	1
Cunningham liquid monolayer system	13	1.7 $\pm$ 0.18
Closed CMC system	16	1.8 $\pm$ 0.16
Open CMC system	16	4.1 $\pm$ 0.36

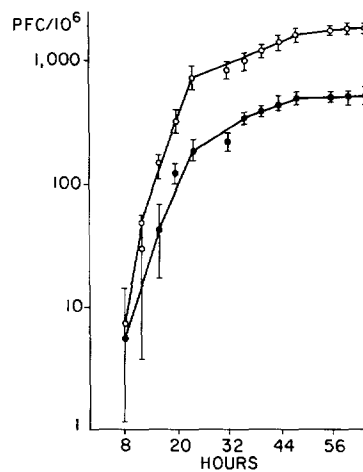


FIG. 3. Kinetics of plaque formation with the closed technique.  $\circ$ — $\circ$ ,  $5 \times 10^6$  cells/ml.  $\bullet$ — $\bullet$ ,  $2.5 \times 10^6$  cells/ml. Brackets indicate  $\pm 2$  SE.

microscope would have been still lower. These substantial sensitivity differences should be borne in mind when comparing results from different laboratories using hemolytic plaque systems.

*Comparison of Open Versus Closed System of Peritoneal Cell Microculture.*—In numerous experiments, peritoneal cells from the same suspension were set up in both open and closed systems for comparison. Details of a typical experiment are shown in Fig. 3 and summarized results of 35 experiments are given in Table II. It is evident that, in the open system, the latent period is much shorter

and the rate of ascent to peak levels more rapid. Furthermore, the final value of peak plaque numbers is over twice as high for the open system. Whereas this in itself could be explained by a difference in the sensitivity of the revealing systems (see Table I), the kinetic features could not be explained in this fashion

TABLE II  
*Greater Rapidity of Appearance and Higher Total Number of Hemolytic Plaques in the Open Versus the Closed System of Peritoneal Cell Microculture*

No. of experiments	Ratio 24 hr plaque counts open/closed	Ratio peak* plaque counts open/closed
35	$5.0 \pm 0.64\ddagger$	$2.1 \pm 0.3\ddagger$

\* Peak counts were achieved at 24–30 hr in the open system and at 72 hr in the closed system.

‡ SE of the mean.

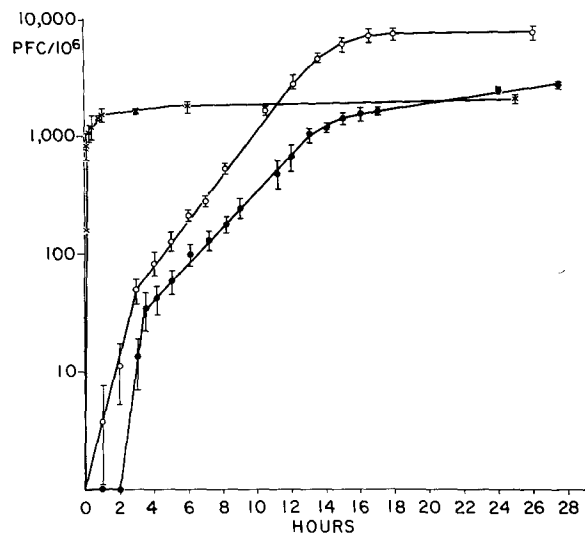


FIG. 4. Kinetics of plaque formation with the open technique. X—X, spleen cells from immunized mice (4 days after one injection of SRBC). Peritoneal cells from young mice in two separate identical experiments, O—O and ●—●. Brackets indicate  $\pm 2$  SE.

and must represent a different behavior of the PFC themselves. In all other experiments reported below, the open system of culture has been used.

*Detailed Kinetics of Plaque Appearance in the Open Peritoneal Cell Culture System.*—In several experiments, hemolytic plaque counts were performed at frequent intervals on up to 16 replicate samples of peritoneal cell microcultures to obtain a detailed kinetic curve of plaque appearance. In Fig. 4, the results of two typical experiments on young male mice are compared with similar



counts made on spleen cells from a mouse immunized 4 days previously with SRBC and placed in open culture. With the immunized cells, the first plaques appeared within 5 min and plaque counts rose rapidly to near peak levels by 1 hr. With the peritoneal cells, the kinetics were quite different. Plaque counts at 1 and 2 hr were negative or very low. From about 3 to about 13 to 14 hr there was a strictly exponential phase, plaque counts doubling about every 2 hr. By about 12 hr of culture, the plaque counts had reached half their final value. After 14 hr the rate of rise of plaque counts slowed down considerably, and peak values were reached by 20 to 24 hr.

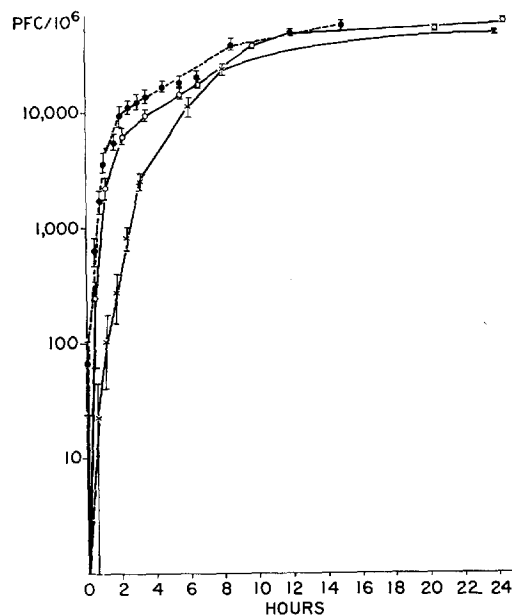


FIG. 5. Kinetics of plaque formation by peritoneal cells from retired breeder mice. Brackets indicate  $\pm 2$  SE.

A chance observation showed us that retired breeder mice (see Materials and Methods) yielded greatly increased plaque numbers in peritoneal cell culture. This ability was not shared by old male mice nor by old or young virgin female mice; it was specifically associated with the reproductive process. Fig. 5 gives the kinetics of three typical experiments on plaque appearance in open peritoneal cell cultures of retired breeder mice. There was more individual variation from experiment to experiment, but two constant features were more rapid plaque appearance over the first 4 hr and much higher final plaque numbers. In these experiments, it was hardly possible to speak of a latent period. A few plaques had appeared by 30 min and by 4 hr the plaque number was around

10,000 per  $10^6$  cells cultured, this being about 10% of the final figure achieved. In two of the three experiments shown, there was a tendency for exponential kinetics between 2 and 9 to 12 hr with a doubling time of about  $3\frac{1}{2}$  hr, but this was not as obvious a feature as with the young male mice. Again, plaque counts at 12 hr were about half those finally achieved, the rise being a slow one over the final 12 hr.

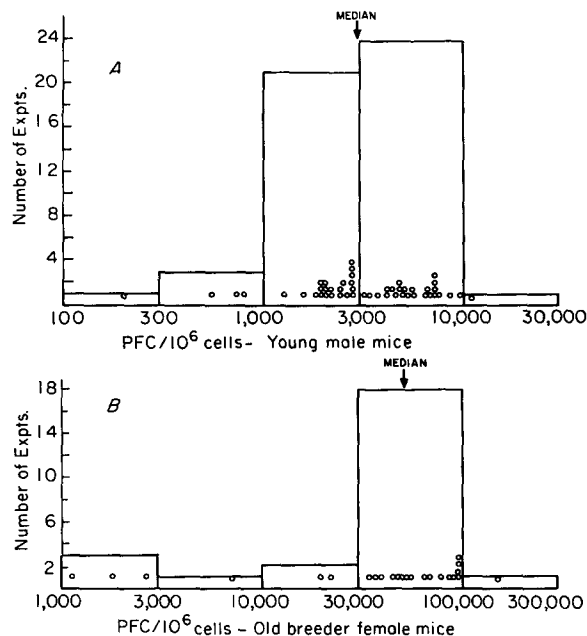


FIG. 6. Normogram summarizing 50 experiments with peritoneal cells from young males and 25 experiments with old breeders.

The overall pattern of plaque appearance with these retired mice is thus somewhere in between that of immunized spleen cells and young male peritoneal cells. However, in no case did the 1 hr plaque count with the breeder peritoneal cells exceed 3% of the final total achieved, and thus 97% or more of the plaques that appeared were not behaving like classical antibody-forming cells harvested from an immune animal.

*Reproducibility of Results from Experiment to Experiment in Open Peritoneal Cell Microcultures.*—When due attention is given to the complement source, reasonably satisfactory reproducibility can be attained in the open peritoneal cell culture system. Fig. 6 gives the results of 75 successive experiments, (a) 50 on young males and (b) 25 on retired breeders. With the young males, 90% of the experiments gave plaque counts between 1000 and 10,000 per million,

the median figure being 3000 and the mean  $3.980 \pm 375$ . Three of the four "failed" experiments with plaque counts less than 1000 came within the first 11 experiments, there being only one such in the remaining 39. Approximately similar variation was encountered with the retired breeders, where 72% of the results fell between 30,000 and 100,000 plaques per million cells, and 84% gave  $> 10,000$  plaques per million. The median value was 52,000 PFC/ $10^6$  and the mean  $56,200 \pm 700$ . The difference in the mean between young and breeder mice was significant at a level of  $P < 0.001$ .

TABLE III  
*Capacity of PFC to Form a New Plaque after Micromanipulation Transfer*

Type of PFC transferred			No. of Exp.	No. of new plaques No. of transfers	Suc- cess
Spleen cells from mouse immunized with SRBC 4 days previously and incubated for $\frac{1}{2}$ to 3 hr in CMC open system			6	85/99	% 86
Peritoneal cells from un-immunized mice	Young males	In CMC open system cultured for 4 hr	1	6/6	100
	" "	In CMC open system for 18-24 hr	8	78/128	60
	Old breeders	In CMC open system for 3-6 hr	3	45/56	80
	" "	In CMC open system for 18-24 hr	1	7/10	70

As we had no record of the breeding history of each mouse, it seems possible that this was one variable of importance and this is a factor engaging our present attention.

*Experiments Involving Micromanipulation Transfer of PFC Designed to Show that Hemolysis was Caused by a Product Secreted by the Cell in the Plaque.*—The question had been raised whether, in the closed system, the plaques of hemolysis might not have been due to some bacterial contaminant or to the release of catabolic enzymes from dying macrophages or other cells (10). Accordingly, cells were taken by micromanipulation from the center of plaques appearing in open cultures of peritoneal cells at times ranging from 3 to 24 hr of culture. With or without an intermediate step of washing in liquid culture medium, they were placed in a fresh culture monolayer containing all the elements of the first, except peritoneal cells. As a control, microcultures were prepared from immunized spleens and at 30 min to 3 hr of culture, PFC were transferred in an exactly similar manner. The results are given in Table III. It is seen that with

“authentic” antibody-forming cells from immunized spleens, a high transfer efficiency of 86% can be achieved, the 14% of cells which failed to make a plaque presumably representing cells that had degenerated prior to or immediately after transfer, or cells damaged by micromanipulation. When PFC from peritoneal cell cultures taken at an approximately equivalent time of culture (i.e. 3 to 6 hr) were transferred, an equivalent success rate was achieved, the mean from four experiments being 82%. When older cultures acted as the source of PFC for transfer, the transfer efficiency was somewhat but not drastically lower at 62% (mean of nine experiments). It was consistently noted that at such times readily detectable desiccation of microcultures had already commenced, and thus many cells may have been in poor health at the time of transfer. One single micromanipulation transfer did not exhaust the plaque-forming capacity of the transferred cells. In several experiments, as soon as a transferred cell had made a detectable plaque, we remanipulated it to a fresh gel, and a third plaque appeared with an equivalent latent period. This process could be repeated up to five times without apparent harm to the cell, and, while no systematic attempt has been made to exhaust the cells, the multiple transfer technique offers obvious possibilities for a study of the effects of various inhibitors on a single cell.

To investigate this question further, time-lapse microcinematography was performed on about 20 peritoneal PFC immediately after transfer. This revealed that some cells appearing on morphological grounds to be in good condition were, in fact, totally motionless and thus probably dead. Such cells consistently failed to induce plaque formation. In contrast, all cells exhibiting the normal motility of lymphoid cells as previously described (1, 2) lysed at least some red cells after transfer, though in one or two cases the filming was terminated before a true plaque had appeared. In several cases, the death of the cell could be observed in the film. When this occurred, and even if it was accompanied by explosive lysis of the PFC, the plaque stopped increasing in diameter. Taken together these results strongly suggest that the hemolysis is caused by some substance actively secreted by a living lymphoid cell rather than by something passively absorbed to the cell or leaking out from its interior, during and after its death. These results also eliminate the possibility that plaques were caused by bacterial contamination, as microorganisms were never seen nor transferred.

*Kinetics of Plaque Growth after Micromanipulation Transfer.*—A series of experiments was designed to determine whether transferred cells were actively secreting a hemolytic product into the surrounding medium, or whether they simply constituted a depot source of a hemolysin, as might have been the case for a cell with cytophilic antibody on its surface or a macrophage rupturing and releasing nonspecific lysins (10). Micromanipulation transfers into normal receptor monolayers were performed on (a) immunized spleen PFC, (b) peritoneal cell culture PFC, and (c) about  $10^{-8}$  ml of anti-SRBC serum from a hyperim-

munized rabbit. The diameter of resultant plaques were measured at frequent intervals over the succeeding 20 hr at 37°C. Representative results are shown in Fig. 7. With immunized spleen cells, a tiny plaque could usually be first seen 5 to 20 min after transfer. Then there was an approximately linear relationship between plaque diameter and the square root of the time of incubation, which lasted from 3 to 4 hr, after which the plaque diameter grew little or not at all. Kinetics of plaque growth after transfer with peritoneal cells was broadly similar. The new plaque was slightly slower to appear, grew perhaps somewhat more slowly but for a slightly longer period of about 5 hr, the final plaque diameters being much the same. The depot source of antibody, however, gave grossly different growth kinetics. The resulting plaque appeared with a delay

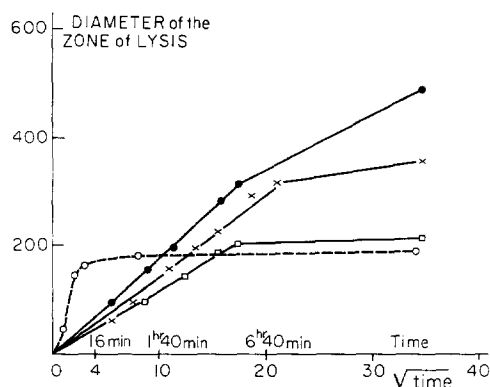


FIG. 7. Kinetics of growth by hemolysis zones formed by: a microdroplet of hemolytic serum, ○----○; three different peritoneal (PFC) cells after micromanipulation transfer, ●—●, x—x, and □—□.

of about 1 min, had reached a substantial size by 2 min, and showed relatively little further growth after 5 to 10 min. When the kinetics of growth of the hemolytic zone formed by a microdrop deviates from Fick's law (after 2 min) the initial volume of the drop is, at minimum,  $\frac{1}{10}$  of the volume of the zone of lysis. (Fick's law is based on the assumption that the concentration of the reagent in the initial reservoir is essentially constant.) This deviation from Fick's law takes place, for a plaque forming cell, when the cell volume represents, at maximum,  $\frac{1}{300}$  of the volume of the zone of lysis.

This comparison rules out, in our opinion, the possibility that plaques of lysis around cells could result from free diffusion of an antibody preformed in the cell. Such a lysis must result either from an active "pumping out" of preformed antibody by the cell or by an active synthesis. In both cases, a concentration gradient of antibody must be built up by the living cell.

*Complement-Dependence of Plaque Formation.*—The next point we wished

to prove was that the PFC from peritoneal cell cultures were forming antibody and not a complement component. The latter possibility, though remote, needs consideration as the culture system is undoubtedly complex. It contains guinea pig serum as a complement source which, despite absorption and testing, may contain sublytic concentrations of antibody against sheep erythrocytes. It also contains mouse peritoneal cells, some of which may be forming antibody with specificity for SRBC or may have such an antibody adsorbed to them (as cytophilic antibody). The following rather elaborate argument can then be constructed. SRBC become coated with antibody against them, either from the guinea pig serum or through a general formation of antibody by some non-PFC in the culture. Lysis does not occur because the CMC interferes in some way with the lytic system. As cultures deteriorate, the SRBC become nonspecifically more readily lysable. Some cells manufacture a complement component, and a plaque appears around them. A variation on this theme is that the PFC makes, or causes to be released, calcium or magnesium ions, that had been a limiting factor through some action of CMC on them.

First, if the PFC is causing a plaque because it is making a complement component, then authentic complement added to aging cultures should cause hemolysis. Accordingly, we took 24 hr peritoneal cell open cultures containing numerous plaques. To premarked areas of the monolayer that happened not to contain a plaque we micropipetted in fresh, unabsorbed, undiluted guinea pig serum in varying volumes. Additions estimated to be 10, 20, 50, or 100 times the volume of a PFC caused no detectable lysis. Volumes estimated to be 1000–10,000 times the volume of a PFC caused a disturbance of the monolayer and some irregularity of the SRBC. Within some minutes, light "background" hemolysis in the relevant region could be noted, but the appearance was totally different from that of a plaque. Absorbed guinea pig serum did not give this effect.

Secondly, we reasoned that if plaques were due to complement formers, this indicated a suboptimal complement concentration in the aging cultures, otherwise the whole monolayer would have become uniformly lysed, rather than displaying plaques. To show that this was not the case, we took PFC from 4-day immunized spleens, (generally believed to be antibody-forming cells) and transferred them by micromanipulation to marked, nonlysed areas of 24 hr peritoneal cell cultures. In four of five cases (80%) a new plaque appeared around the transferred cell. The rate of appearance, rate of growth, and final diameter of these plaques were no different from those observed after transfer of PFC to fresh monolayers. The experiment shows that if PFC from immunized spleens are indeed antibody formers, aging cultures still contain enough complement to support plaque appearance. A lack of complement is not apparent, so it seems unlikely that a complement-forming cell would make a plaque.

Thirdly, we tested directly the idea that the PFC was making a heat-labile

complement component. 10 PFC from 25 hr peritoneal cell cultures were transferred into a monolayer containing absorbed complement that had been heated at 56°C for 30 min. After 2 hr incubation, no plaques had appeared. Then, by micromanipulation a tiny droplet of unheated, absorbed complement was instilled into each area of the monolayer containing a transferred cell. Within minutes, plaques appeared around 7 of the 10 (70%) transferred cells. This result is strongly suggestive of antibody formation. Had the PFC been forming a heat-labile complement factor, a plaque should have appeared before the addition of unheated serum. Had the cell been making a heat-stable complement component, it is hard to know what one cell might have added to a monolayer already possessing a 10% saturation of the heat-stable components of guinea pig serum.

Finally, the experiment just described was performed on a mass scale. Peritoneal cells were incorporated in CMC medium containing SRBC and 10% guinea pig serum that had been heated to 56°C for 30 min. Such cultures did not give plaques, confirming in the open system the observation previously made on numerous occasions in the closed system (1, 2). After 24 hr of incubation these cultures were then slowly and gently covered with an undetermined amount of unheated guinea pig serum dispensed by micropipette. It did not prove possible to provide an even coverage of the monolayer, and thus exact counts of plaque appearance for comparison with control cultures could not be performed. However, it was clear that numerous plaques appeared within minutes after addition of unheated complement. This represents the formal equivalent of the classical Jerne technique, the validity of which as a test for antibody production seems not to have been seriously questioned.

A strong further argument for the antibody nature of the hemolysin produced by the PFC is provided by the effects of specific inhibitory antisera. These will be described in the accompanying paper, and suggest that the peritoneal PFC are all IgM producers.

*Experiments Designed to Prove that PFC are the Result of an In Vitro Inductive Process.*—Even if it is admitted that the PFC from peritoneal cell cultures are making antibody, it is still possible that this represents nothing more than the detection in vitro of a high number of cells of “background” PFC type, i.e., cells that had been stimulated in vivo by some antigen cross-reacting with SRBC and had been forming antibody at the moment of killing. The long latent period for the appearance of many of the plaques in culture might then be postulated to be due to the formation of antibody either in very small amounts or of very low avidity. In other words, some cells might have had to secrete their product over long periods (up to 24 hr) in order to accumulate enough antibody in their vicinity to lyse the required number of red cells. This possibility, which seemed reasonable to us a priori, could be destroyed readily by micromanipulation experiments of simple design. If a background PFC had

to "work" for 24 hr to produce the original plaque in a peritoneal cell open culture, then, in the absence of any change in the synthetic rate, it would require a further 24 hr to produce the same sized plaque after transfer to a new SRBC-complement-CMC monolayer. Accordingly, we performed careful kinetic studies of the rate of plaque appearance both in the original cultures and after micromanipulation transfer. Counts of plaque numbers were made at frequent intervals and care was taken to identify and score even very small plaques. The relevant data are given in Fig. 8 which should be compared with Figs. 4 and 5.

In Fig. 8, we present four curves. In each case, the plaque count at a given

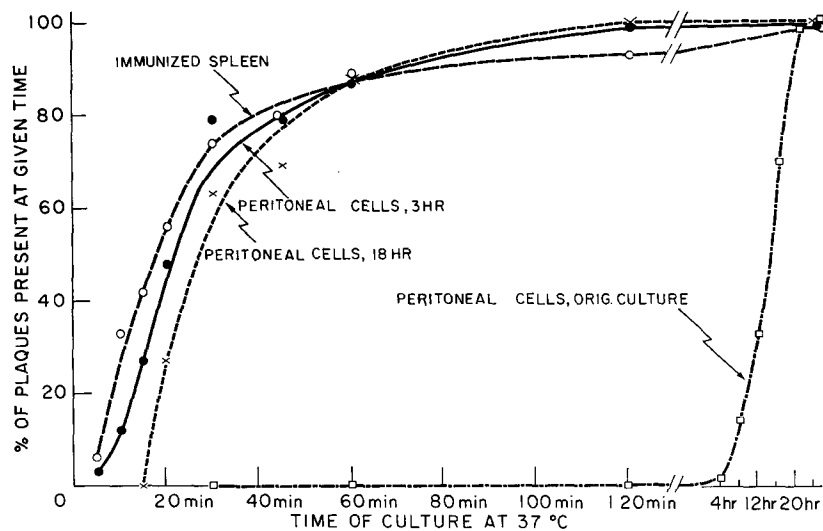


FIG. 8. Kinetics of plaque formation by immunized spleen cells,  $\circ$ --- $\circ$ ; peritoneal cells in original culture,  $\square$ --- $\square$ ; peritoneal cells transferred in fresh medium after 3 hr in original medium,  $\bullet$ --- $\bullet$ ; after 18 hr in original medium,  $\times$ --- $\times$ .

time is expressed as a percentage of the plaque count after 24 hr observation. The curve on the extreme right is data presented in more detailed form in Fig. 4, curves *a* and *b*. It is simply the rate of appearance of plaques in open cultures of peritoneal cells from young male mice. Note the break in the time scale; about half of the plaques have appeared by 12 hr. The next curve to its left represents the kinetics of appearance of plaques after transfer when PFC were micromanipulated from 18-24 hr cultures of young male peritoneal cells into a new gel. In fact, half of these donor PFC had taken somewhere between 12 and 24 hr to form their original plaque. After transfer, half of the cells capable of forming a second plaque had done so within about 25 min. The remaining two curves describe the behavior after transfer of PFC from 3 to 6 hr peritoneal cell cultures and  $\frac{1}{2}$ -3 hr immunized spleen cell cultures. With



these cells, the latent period before appearance of the second plaque was even shorter. The majority of the plaques had manifested themselves by 30 min. In all 3 cases, nearly 90% of the plaques which were to appear had done so by 1 hr after transfer.

For immunized spleen cells, the kinetics of plaque appearance after transfer is identical with that observed on original plating (Fig. 4). Here it is clear that the PFC were forming antibody at a brisk rate from the moment of explantation into culture. On the other hand, the transfer experiments prove that the PFC, while also forming antibody at a comparable rate at the moment of micro-manipulation, had not been doing so from the beginning. In other words, at sometime between first explantation and cell transfer, the rate of antibody synthesis by the PFC had been considerably accelerated. Whether this represents a true indication of synthesis *in vitro*, or merely a derepression of a cell that had been forming antibody *in vivo* but had stopped, for some reason, on explantation into culture, is not revealed by these experiments.

TABLE IV  
*Plaque Formation in Cultures of Peritoneal Cells with Small Starting Volume*

Volume of culture	No. of samples	Cell concentration	No. of peritoneal cells per culture	PFC/10 <sup>5</sup> ± SE
3.65 μl	8	5 × 10 <sup>6</sup> /ml	18,200	1,920 ± 198
0.456 μl	32	5 × 10 <sup>6</sup> /ml	2,280	2,810 ± 430

*Plaque Formation in Cultures of Reduced Volume.*—It was of interest to establish what was the minimum number of peritoneal cells that could be cultured and yield a standard proportion of plaque-forming cells. For reasons discussed in the second paper of this series, dilution of the number of cells per ml of culture medium caused less than the expected number of plaques to appear. Therefore, we set up cultures in which the cell concentration was held at normal levels, but in which the volume of culture medium was reduced. Because of the tendency for desiccation of microcultures, the smallest volume that proved technically feasible was 0.45 μl or one-eighth of the standard cultures. Table IV gives the results of a typical experiment on young male mice. It is obvious that cultures starting with as little as about 2000 cells gave adequate responses. Moreover, only technical considerations prevented cultures with even smaller cell numbers being set up.

A similar type of experiment was performed with peritoneal cells from retired breeder mice, where the cell concentration was 1 × 10<sup>6</sup> cells per ml and thus the starting cell number only 456 cells per culture. Such cultures gave a mean of 24.7 plaques each, yielding a perfectly normal proportion of 54,300 PFC/million cells cultured.

Thus it is clear that in this system, hemolytic plaque formation can be started with absolute numbers of cells far smaller than those needed to set off a classical Mishell-Dutton system (4) or a hemolytic focus in the spleen of an irradiated animal of Kennedy type (11).

*Plaque Formation by Peritoneal Cells from Germ-Free Mice.*—In view of the possibility of cross reactions between antigens on the surface of a sheep red blood cell and certain antigens of normal intestinal bacteria, it was of interest to test the behavior of open cultures of peritoneal cells from germ-free CBA mice. In this study, mice from two sources were used. The results are given in Table V. Clearly, the germ-free mice from the French source behaved in a completely standard fashion. The germ-free mice from the Swiss source gave somewhat

TABLE V  
*Plaque Formation in Culture of Peritoneal Cells from Axenic and Holoaxenic CBA Mice*

Nature of mouse	Source of mouse	PFC/10 <sup>6</sup> ± SE	
		Axenic CBA mice	Holoaxenic CBA mice
Young males Exp. 1	Institut Gustave Roussy	2,770 ± 261	—
Young males Exp. 2	“ “ “	4,900 ± 720	—
Retired breeder	“ “ “	31,200 ± 3,260	—
Young virgin female	“ “ “	1,400 ± 122	—
Young males	Ciba Research Institute	632 ± 37.4	892 ± 68.4
Young virgin females	“ “ “	976 ± 86.9	1,010 ± 73.1
Young virgin females	“ “ “	893 ± 75.3	880 ± 81.4
Young virgin females	“ “ “	1,100 ± 83	1,440 ± 173

poorer activities in PFC but, since the experiments were done in different conditions, these activities could be considered as reasonably high. Thus, the conclusion can clearly be drawn that the germ-free state as such does not affect the plaque-forming capacity of mouse peritoneal cells.

#### DISCUSSION

It can now be regarded as proven that the hemolytic substance responsible for plaque production by peritoneal cells placed in culture in the CMC medium is associated with the cell at the center of the plaque. Strong evidence has been presented in favor of the view that the hemolytic molecules are actively secreted by a living, healthy lymphoid cell. The complement dependence of plaque appearance, the specificity of lysis for SRBC, and the similarity in plaque appearance and growth kinetics between plaques made by peritoneal cells and those made by authentic antibody-forming cells from immunized spleens suggest that the hemolytic substance made by the PFC is complement-fixing antibody against SRBC. Still more persuasive evidence in favor of the view that the

peritoneal PFC are making IgM with specificity for SRBC will be presented in the second paper. In all, the evidence for the phenomenon being due to antibody production is now at least as strong as that for other accepted methods claiming to detect single antibody-forming cells.

The great sensitivity of the open CMC system in plaque detection warrants comment. In part, this is due to excellent optical conditions and a very even spread of a thin red cell monolayer, which favor detection of small plaques. A further factor may be the tendency for CMC to favor local accumulation of protein around the secreting cell, both by its effect on the viscosity of the medium and by actual reversible binding of protein. The faster kinetics of plaque appearance in open *versus* closed peritoneal cell culture systems are not so readily explained. Certainly, gas exchange between culture medium and the atmosphere was easier in open cultures, and, thus, in the initial hours the open system may have been a more physiological one, favoring whatever derepressive or inductive influences led to plaque appearance. At later stages, the open system also became unphysiological, owing to dessication, and this is a feature urgently requiring an improvement in methodology.

The exponential kinetics of appearance of plaques in culture observed over a critical 10 hr period is interesting, but totally unexplained. In many biological systems, exponential increases are indicative of the existence of self-replicating entities. In this case, the possibility of replication of cells can be ruled out. The possibility that a self-replicating entity (nucleic acid, episome) passes from cell to cell in our cultures has naturally occurred to us, but in the absence of evidence, further speculation is fruitless.

The issue of just what this peritoneal cell response actually represents will be engaging us repeatedly throughout the papers in this series. Basically, there appear to be four broad possibilities. (*a*) The phenomenon is some nonimmunological artefact of little biological interest. (*b*) It is a way of detecting background cells, i.e., cells forming antibody *in vivo* at the moment of killing of the animal which cross-reacts sufficiently with SRBC to cause a lytic plaque. (*c*) It is a primary immune response induced *in vitro*. (*d*) It is a secondary immune response *in vitro*, with certain unusual features.

The first possibility now seems very unlikely. The complement dependence, specificity, and cell transfer experiments essentially rule out the possibility of some contaminating bacterial lysis and the same data argue strongly against lysis being due to catabolic enzymes released from a dead or dying cell. A cytophilic antibody cannot be formally ruled out, but the slow growth of plaques over many hours after cell transfer speaks against this possibility, as does the lymphoid nature of the PFC and the absence of any preimmunization procedure involving adjuvants. Formation of a complement component is ruled out by arguments already cited. Formal, immunochemical proof of the IgM nature of the lysis is now being sought.

The second possibility must be fully discussed, especially for the retired breeder mice. At this stage, the definition of a background antibody-forming cell is a purely operational one. For the present purposes, it could be considered a cell harvested from an animal not intentionally preexposed to the antigen concerned and capable of forming a hemolytic plaque within 1–2 hr of incubation *in vitro*. It is widely assumed that cells with such characteristics represent antibody-forming cells arising in an animal in response to some naturally occurring antigen which cross reacts with the test antigen. On this general definition, less than 0.1% of the peritoneal PFC from young male mice, and less than 3% of the PFC from retired breeder mice could be regarded as background cells. As the micromanipulation transfer studies (Fig. 8) clearly show, a change in the rate of secretion of hemolytic product occurs in PFC at some time after initial incubation, i.e., an acceleration from some subthreshold to a threshold rate. The following argument then needs consideration. Let us suppose the peritoneal PFC are cells forming antibody *in vivo*, which shut down synthesis and/or secretion for a few hours after being placed in an artificial culture medium, but which recommence synthesis when habituated to the new environment. This theory is not without appeal. It would be greatly favored if experiments showed that the commencement of active *in vitro* antibody secretion was totally independent of the presence of antigen in the medium. For the moment, we have not been able operationally to separate the phase of “induction” from the phase of “expression”, but this should soon become feasible so that the above idea could be directly tested. This theory, however, leaves several important facts unexplained. As relevant data follow in the accompanying paper, further discussion will be reserved.

The third possibility is that of a primary immune response induced *in vitro*. Certainly, the response differs in both extent and tempo from our classical views of a primary response. However, the most potent argument against this view is the inability to evoke the phenomenon with other types of red cells, e.g., horse, pigeon, or even goat. From a formal point of view, this failure could be explained away by postulating that sheep cells present certain advantages in a lytic system where early antibodies of extremely low avidity are involved. However, other types of red cells have been found quite adequate for the detection of very early (< 48) *in vivo* immune responses in our CMC system<sup>2</sup> or for both early *in vivo* or *in vitro* responses using the Jerne system (4). It seems more likely that the special advantage of the sheep cell is that it presents surface antigens to which adult CBA mice have been, in some way, presensitized. If, however, it turns out eventually that this is a primary response, this would have grave implications for the clonal selection theory (12), as in the case of the retired

<sup>2</sup> Boris, S., S. Deutsch., G. J. V. Nossal, and A. E. Bussard. 1970. *In vitro* stimulation of antibody formation by peritoneal cells. III. Effect of active immunization on the subsequent *in vitro* performance of peritoneal and spleen cells. *Immunology*.

breeder mice, 1 cell in 10 or 20 can respond. Even if some "infection" of cells with a transmissible agent were involved, the fact that responses can readily be induced with cultures containing only about 400 cells would need to be explained.

This brings us to the fourth possibility, namely that the response is something akin to a secondary response. Here we must first discuss the nature of the possible prestimulation. A cross-reacting antigen from intestinal flora seems effectively ruled out by our experiments on germ-free mice. An antigen in food is a possibility, and this could explain why both germ-free and conventional mice from a particular source responded poorly. Specific investigations, which we will cite,<sup>2</sup> have not supported this view. Finally, we may be dealing with some autoantigen, for example a fetal mouse erythrocyte antigen, which cross-reacts with SRBC. In this case, the increased autoantibody reactivity associated with pregnancy would require especially careful investigation.

If mice are indeed presensitized to some SRBC antigen, we are still left with several puzzles, and in particular the rapidity of the *in vitro* response and the fact that only peritoneal cells and not lymphoid cells from other organs work in the system. It may be that several types of "memory cells" in fact exist. The classic type may be a small lymphocyte, not secreting antibody, and requiring to go through blast cell transformation and repeated division and differentiation after antigenic stimulation. Another may be the type behaving as a PFC in the present system. This would be a cell not actually forming antibody but ready to respond very quickly on antigenic restimulation, without intervening division. In fact, such a cell might have been an antibody former at some previous time in its life span. This hypothesis implies greater subtleties in the regulation of immunocyte behavior than previously suspected. That such subtleties exist will be shown in the accompanying paper.

Finally, perhaps the most striking finding of the present experiments was the extraordinarily heightened activity of peritoneal cells from retired breeder mice. We have not yet had the opportunity of establishing at what stage in the complex sequence of events entailed in pregnancy and lactation the heightened activity sets in, but such experiments are in progress. Three explanations offer themselves. In increasing order of likely importance they are: (a) mild mechanical irritation of the peritoneum by the growing uterus; (b) hormonal stimulation of the cells (13); (c) activation of the cells by some antigen of fetal or placental origin. Further work should clarify this issue, but for the moment the fascinating possibility exists that the peritoneal cells are forming some autoantibody of physiological significance in the reproductive process.

#### SUMMARY

An improved method for the short-term culture of mouse peritoneal cells in a medium containing carboxymethylcellulose (CMC), sheep erythrocytes

(SRBC), and guinea pig complement is described. It involves preparation of microcultures, of thickness 12–15  $\mu$  and volume 3.6  $\mu$ l, under paraffin oil. With such cultures, peritoneal cells from normal, unimmunized young male CBA mice give about 3000 hemolytic plaques per million cells cultured, this figure being attained within 24 hr. The plaque detection method is about four times as sensitive as the Jerne technique.

A method is described whereby such plaque-forming cells (PFC) can be transferred, by micromanipulation, to fresh monolayer cultures containing SRBC, CMC, and complement. In this fashion, the secretory capacity and susceptibility to inhibitors of peritoneal PFC can be tested in detail. Using this technique, evidence is presented that the hemolytic substance responsible for plaque formation is actually secreted by the cell at the center of the plaque, and is not a complement component but probably an antibody. Studies on the time of plaque appearance after cell transfer, and the subsequent growth rate of the zone of hemolysis, have been performed. They speak against the idea that the PFC is either a reservoir of cytophilic antibody or a "background" PFC. Rather they suggest that active antibody secretion is induced in the cell at some defined time point in culture.

Detailed kinetics of the rate of appearance of plaques in peritoneal cell cultures revealed an exponential phase lasting from about 3 to about 13 hr with a doubling time of 2 hr. The reasons for this are not known.

A greatly heightened reactivity was shown in peritoneal cells of mice that had been pregnant several times. Cultures of such cells showed more rapid plaque appearance and a peak activity about 20 times higher than with cells from young male mice. Cultures in which 1 cell in 10 formed a plaque were not infrequent. A series of experiments on germ-free mice showed reactivity similar to that of conventional mice from the same strain and source.

The significance of the findings for cellular immunology are discussed.

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