THE INTERACTION OF MAMMALIAN CELLS WITH ANTIBODIES. I*

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Plates 66 and 67

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Development of quantitative methods for measurement of colony formation by single mammalian cells (1) makes possible accurate tracing of antiserum effects on inhibition of cellular reproductive capacity. In an earlier paper the quantitation of such action was demonstrated (2) and the high degree of sensitivity obtainable by the utilization of a small number of cells rather than a large inoculum was shown. The present report is the first in a series of studies undertaken to analyze in detail the reactions of antibodies with various types of mammalian cells grown *in vitro* and *in vivo*.

Methods and Materials

Tissue culture cells employed in this study included:—

(*a*) The S3 HeLa cells, a hyperploid, aneuploid, clonal strain of human carcinomatous origin (1, 3).

(*b*) Cells from the ovary of the Chinese hamster (CHBO-Cl), cultivated as described elsewhere (1 *b*, 5). These cells differ from normal human cells in a tendency to display a small degree of non-disjunction, which produces aneuploid chromosome numbers in part of the population (3, 4).

The cell plating procedures by which clonal colonies are formed from aliquots of monodisperse cell suspensions pipetted in culture medium into Petri dishes, have been described elsewhere (1, 5). All platings were performed in replicate. Colony counts on duplicate plates generally agreed within ±15 per cent. Antisera were prepared by three intramuscular injections of rabbits at weekly intervals with a saline suspension containing 1 cc. of 10^7 washed cells plus 1 cc. of Freund complete adjuvant (Difco). At the 4th week, the rabbits were bled by heart puncture and the serum collected and stored at −30°C. Antisera were heated to 56°C. for 30 minutes to inactivate their complement.

Guinea pig serum was used as a complement solution. The serum was freshly drawn by heart puncture and added to the reaction vessel to the final concentration indicated.

Cell Growth Media and Conditions.—Unless otherwise indicated, the antisera and growth

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media were heated as routine to 56°C. for 30 minutes to inactivate their contained complement. Experiment established that this treatment of the medium does not inhibit cell growth, and indeed in certain circumstances may augment it. It was also established that this heat treatment did not affect the specific antibody titre of any antiserum used in this study. For the S3 cell, the N16HHF medium as previously defined was employed (1*a*), while F4FC (1*b*) was used for the Chinese hamster and normal human cells. In some special experiments, as indicated, S3 was also grown in the other media. All plates were incubated at 37.0 ± 0.3°C. for 9 to 11 days in incubators in which the temperature, CO_2 content and relative humidity were precisely controlled as described. Fixation was effected by 10 per cent formalin, and Giemsa was used to stain the resulting colonies as described (1*a*, 1*b*).

Throughout this paper the term, cell killing, will be used to denote cessation of an isolated cell's ability to reproduce sufficiently to form a macroscopic, glass-attached colony. In all the experiments here reported the controls were treated in a fashion identical with that of the test plates, except that the highest concentration of antiserum employed was replaced by normal rabbit serum. In every case, the colonies resulting on such plates were identical with those in which no rabbit serum of any kind had been added. Hence, the results reported are due to the antibody content of the immune sera.

EXPERIMENTAL RESULTS

Cell Killing in the Presence of Complement—Limits of Sensitivity.—Mountain (6) and other workers have discussed the need for complement to produce cytopathogenic action of cell antibodies in massive culture situations. A typical result of experiments with single cell plating is presented in Table I A and Table I B. In these experiments, the indicated concentrations of heated antiserum and complement were added directly to Petri dishes containing the cell inocula and heated growth medium, which were then incubated for 9 days. The appearance of typical plates, after fixation and staining, has been described elsewhere (2) and is also illustrated in Fig. 1 A to 1 D. These experiments indicate that when single S3 cells are incubated together with specific antibody and complement: (*a*) the extent of killing is a direct function of both antibody and complement concentrations; (*b*) in the presence of 5 per cent complement, as little as 0.25 per cent antibody can be reliably detected; (*c*) along with the killing of cells, there is a decrease in colony size among the survivors—thus, the average number of cells per colony among the survivors of exposure to 0.5 per cent antibody and 3 per cent complement was only 60, as compared with a value of 1240 in an identical vessel without antibody, or 300 in an identical vessel with antibody but without added complement; (*d*) even in the absence of added complement, both cell killing and growth lag in the survivors occur, provided the antibody concentration is greater than 1 to 2 per cent, and (*e*) the killed cells lose their stainability by dyes like Giemsa, an indication of cytolysis described by other workers (6).

The normal growth medium, N16HHF, contains 20 per cent human serum plus 10 per cent horse serum. Comparison of the rate of killing of S3 cells by S3 antiserum added directly to this medium without heating demonstrated a

killing effect equivalent to the addition of 3 to 5 per cent complement to the heated medium.

TABLE I A

Colony Counts Obtained with Various Concentrations of Anti-S3 Serum and Complement Incorporated into the Medium in Plates Seeded with 100 S3 Cells

The colony counts indicated are equal to the plating efficiencies. The results shown are averaged from two separate experiments carried out on different days.

Antiserum concentration, *per cent*.....	0	0.25 per cent	0.5 per cent	1.0 per cent	2.0 per cent	5.0 per cent
Complement concentration						
per cent						
0	96.5	91.0	91.0	86.5	44.5	0
0.1	113.5	113.0	94.5	97.0	50.0	0
0.5	117.0	118.0	90.0	84.0	21.0	0
1.0	98.7	95.8	88.7	8.0	0	—
3.0	100.0	96.5	30.5	0	0	—
5.0	86.0	29.0	5.0	0	—	—

TABLE I B

Average Number of Cells per Colony Achieved after 9 Days of Incubation among the Survivors of Treatment with Antibody and Complement in a Typical Experiment like That Shown in Table I A

The numbers here shown were obtained by averaging approximately 20 colonies on each plate, and are to be regarded as a rough index of the growth delay produced even where the affected cells were not killed.

Antiserum concentration, *per cent*.....	0	0.25 per cent	0.5 per cent	1.0 per cent	2.0 per cent	5.0 per cent
Complement concentration						
per cent						
0	1200	—	300	140	30	0
0.1	1220	620	230	130	26	0
0.5	1280	670	170	92	24	0
1.0	1280	510	250	15	0	—
3.0	1240	290	60	0	—	—

Whether the cell killing which occurs in higher antibody concentrations in the absence of complement indicates an ability of antibody to kill cells by a different mechanism or whether the killing is due to a small residue of complement activity not completely eliminated by the heating cannot be determined from these experiments alone. An indication supporting the former possibility is the

fact that cells killed by antibody alone do not lose their stainability by cytoplasmic dyes.

It was of interest to determine the maximum sensitivity for antibody detection permitted by this technique for use in titration of antibody activity in experiments on kinetics. In the standard serum-containing growth medium, definite S3 antibody activity could usually be detected in concentrations as low as 0.25 per cent. However, when the defined medium described earlier (7) (which contains 2 purified proteins and synthetic micromolecular constituents) was substituted for the serum-containing medium, it was found that antibody concentrations as low as 0.03 per cent (*i.e.*, a dilution of 1:3000 in the reaction vessel) were readily detectable (Table II).

TABLE II

Demonstration that in Defined Medium (7), Anti-S3 Serum Can Be Detected in Concentrations as Low as 0.03 Per Cent in the Reaction Tube, in the Presence of 2 Per Cent Complement

100 S3 cells were plated in every case.

Concentration of antiserum	No. of colonies scored	Average No. of cells per colony among survivors
per cent		
0.33	0	0
0.11	2.5	90
0.03	23.5	330
0 (but containing 0.33 per cent normal rabbit serum as a control)	67	850

That the antibody activity is limited to γ-globulin was demonstrated by tests with fractionated serum.

10 cc. of serum (anti-S3 or normal rabbit serum, respectively) were added to 5 cc. of saturated $(NH_4)_2SO_4$ at 2.0°C. After 10 minutes, the mixture was centrifuged at 2000 R.P.M. (Spinco L type ultracentrifuge, rotor No. 40) for 5 minutes. The supernatant liquid and the pellet were collected separately, dialyzed in the cold 3 times against 600 cc. portions of buffered physiologic saline (solution 2, of reference 1*b*), and sterilized by millipore filtration.

The precipitate, containing the γ-globulin fraction, when prepared from a cell antiserum, revealed a specific activity equivalent to that of the original serum, when computed on the basis of the γ-globulin constituting the only active component. The supernatant had no activity. When the same fractionation procedure was carried out on normal rabbit serum, neither fraction showed any activity.

Effect on Glass Attachment.—Experiments were performed to determine whether the action of the antibody affects the ability of the cells to attach to

glass. The data of Table III show that antiserum alone, even in 4 per cent concentration, does not affect glass attachment by the cells, but antiserum plus complement can inhibit this reaction almost completely.

This result made it necessary to test whether prevention of colony formation by antibody and complement under the present experimental conditions might

TABLE III

Demonstration That a 10-Minute Exposure to Antiserum Alone Does Not Prevent Glass Attachment by HeLa cells, But Antiserum Plus Complement Strongly Inhibits the Reaction

S3 cells were resuspended to a concentration of 10^5/cc. in heated growth medium plus antiserum, with and without complement added in the concentrations shown. The reaction vessels were maintained at 37°C. for 10 minutes, then the medium was diluted 1:10 with heated growth medium to slow down the reaction, and 1.0 cc. of cells was plated in Petri dishes for measurement of glass attachment. Another aliquot was diluted by a further factor of 100, and an inoculum of 100 cells plated for colony formation in heated growth medium. The per cent of cells attached was assayed by counting glass-attached cells through a microscope, on the Petri dishes fixed and stained in the standard manner, after 6, 12, and 22 hours of incubation. All three measurements yielded identical results within the limits of uncertainty indicated. Colony formation was scored on the plates seeded with 100 cells after 9 days of incubation.

Cell treatment for 10 min. at 37°C.	Per cent of cell inoculum (10^4 cells) which became attached to glass in 6 to 22 hrs.	Per cent of cell inoculum (100 cells) which formed visible colonies in 9 days
3 per cent heated antiserum + 5 per cent complement	10.8 ± .4	9.7
3 per cent heated antiserum alone	66. ± 7*	92.0
4 per cent heated antiserum + 5 per cent complement	1.6 ± 0.6	3.3
4 per cent heated antiserum alone	69.0 ± 12*	94
Control: 4 per cent normal rabbit serum + 5 per cent complement	70.0 ± 1.6*	94.5

* These values are appreciably less than 100 per cent, a fact probably attributable to the relatively short incubation times employed, and to the large amount of crowding obtained with such massive inocula.

be only a reflection of release of the cells from the glass surface, rather than of more profound metabolic changes. Single cells were allowed to attach to glass Petri dishes in growth medium and incubated for 4 days, to permit 3 to 4 cell divisions. The average number of cells per colony was counted at this point, and then 1 per cent antiserum was added to the unheated growth medium. Incubation was continued for periods varying from 3 to 48 hours in successive dishes, after which the antibody-containing medium was removed, the cells washed and new, unheated growth medium added. After further incubation for a total of 9 days in growth medium (either heated or unheated) all the cells

were fixed and stained and the colonies counted. Typical data are shown in Table IV. They indicate: (*a*) Cells already attached to glass are not released by treatment with antiserum in the presence of complement. (*b*) Removal of the antiserum and replacement by growth medium does not reverse the cell-killing action. (*c*) Throughout several days of incubation in growth medium following treatment with the antiserum and complement, the number of cells per colony did not change. Hence, no further cell division occurs after an exposure of 3 hours or more to antiserum in the presence of complement nor do the killed cells appear to be released from the glass. Results essentially identical were obtained with a variety of antiserum concentrations, ranging from 1 to

TABLE IV

Demonstration That Treatment of Glass-Attached Cells with Antiserum in the Presence of Complement Does Not Effect Their Removal from the Glass; That the Cell Killing Is Not Reversible When the Antiserum Is Replaced by Normal Growth Medium, and That No Cell Growth Occurs after Exposure to Antiserum

Single cells in complement-containing growth medium were allowed to attach to glass and multiply for 4 days. Then 1 per cent antiserum was added. After various incubation times, the antiserum-containing medium was removed, normal medium (either heated or unheated) was replaced, and the cells incubated for a total of 9 days after which they were fixed and stained, and the average number of cells per colony counted.

Average No. of cells per colony at time of antiserum addition (4th day)	Duration of antiserum treatment	Average No. of cells per colony on the 9th day
	hrs.	
13.8	0 (*i.e.*, control)	1240
13.8	3	13.5
13.8	6	14.8
13.8	24	12.7
13.8	48	14.3

12 per cent. Even in the presence of the latter high concentration of antibody plus complement, detachment of the dead cells from the glass does not occur.

Specificity of the Reaction.—Species specificity has been reported for HeLa cells grown *in vitro* by massive cell culture techniques (10*b*). Experiment with the single cell methodology also demonstrated that the antibodies to S3 cells and CHBO-Cl cells exhibit little or no cross-reaction to each other, when single cell techniques are employed. The cell specificity of these two antisera is demonstrated in experiments in which each antibody was titrated against its homologous and heterologous cell. Typical results are shown in Table V. These experiments were carried out exactly as previously described, except that it was found always necessary to heat all rabbit sera employed for use with the Chinese hamster cells, because of the occasional presence of a heat-labile inhibitor in sera from both normal and immunized animals. Heat-labile cell

inhibitors in various sera have been described by several workers (8). The present one is of some interest because of its action on the Chinese hamster cell but not on S3. When such rabbit sera were heated to 60°C. for 5 minutes, the non-specific inhibition of Chinese hamster cells disappeared and the experimental results achieved the same high degree of reproducibility as with S3. Thus, for each antiserum there is a fairly considerable region of concentration in which the homologous cell is completely inhibited from establishing colonial growth while the heterologous cell grows as well as the control. Not until con-

TABLE V

Reciprocal Tests of S3 and CHBOCl Antisera on Each Cell Type in Presence of Complement

Concentration	Plating efficiency	
	S3 cells	CH-BOCl cells
per cent	*per cent*	*per cent*
A. Test of Anti-S3 serum		
0 (control)	75.0	60.0
0.3	26.0	—
0.5	3.3	60.0
0.8	0	59.0
1.0	0	54.0
5.0	—	61.5
B. Test of anti-CHBO-C1 serum		
0 (control)	76.5	45.0
0.1	—	42.0
0.2	—	1.5
0.3	—	0
0.5	—	0
2.0	81.0	—
5.0	85.5	—

centrations of 3 per cent or more of anti-S3 serum, and possibly 10 per cent of anti-CHBO-Cl serum are achieved, do non-specific cross-reactions begin to appear. In Fig. 1 is shown a set of experiments demonstrating how these antisera can exercise their selective action on a mixture of the two cell types seeded into the same plate: Addition of each antiserum alone to such a mixture results in wipe-out of all the homologous cells but none of the heterologous ones. Addition of both antisera kills all the cells on the plate.

Similarly, experiment demonstrated that only the homologous cells were able to adsorb appreciable cell-killing activity from cell antisera (Table VI).

Cytologic Effect of Exposure to Antiserum with and without Complement.—Cells growing on a glass surface and treated with specific antiserum and complement

TABLE VI

Demonstration of Antiserum Specificity by Means of Cell Adsorption Tests

Antisera were mixed with an equal volume of the appropriate cell suspension at a final density of about 5×10^7/cc., and kept at room temperature for 60 minutes with occasional shaking. The cells were then removed by centrifugation, and the supernatant collected and titrated by the standard procedure in the presence of 4 per cent complement.

Final antiserum concentration in plating vessel	Plating efficiencies of cells in the presence of antisera at the indicated concentrations, after various adsorption procedures				
	2	1	0.5	0.25	0
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
A. Anti-S3 serum			*S-3 cells*		
Unadsorbed	0	0	0	52.5	85.5
Adsorbed with S3 cells	31.5	79.5	87.5	84.8	85.5
Adsorbed with CHBOCl cells	0	0	0	47.5	85.5
B. Anti-CHBOC1 serum			*CHBOCl cells*		
Unadsorbed	0	0	0	0	78.5
Adsorbed with S3 cells	0	0	0	13	78.5
Adsorbed with CHBOCl cells	10.0	63.0	77.5	80	78.5

TABLE VII

Demonstration That Maximum Cell Killing Occurs Only When Complement Is Present Simultaneously with or after the Addition of Antibody, But Not When It Is Only Present before Antibody Addition

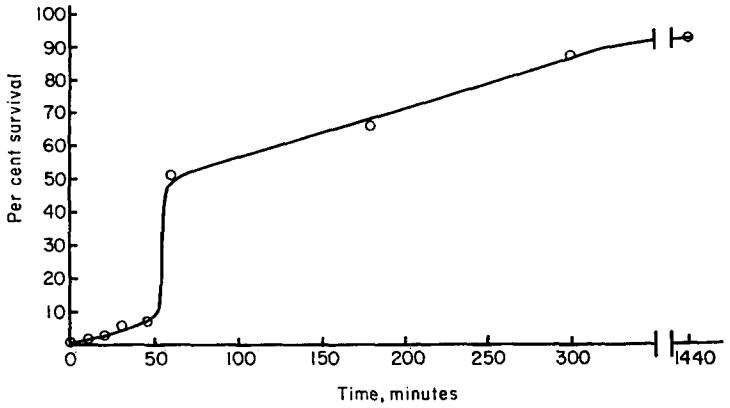
In a series of plates seeded with 100 S3 cells, in heated growth medium, antiserum, complement or both were added, and the plates were incubated for 6 hours. At the end of this time, the media were removed, and the cells washed 3 times with 3.0 cc. volumes of heated growth medium, after which a new addition of fresh growth medium was made. Then complement or antiserum were respectively added so that each plate received both agents in equal amounts, but in reverse order. The plates were incubated for 9 days, then treated with fixative and stain in the standard manner, and the resulting colonies counted. Appropriate controls were performed as indicated.

First addition	Second addition (after 6 hrs. incubation and thorough washing)	Plating efficiency
		per cent
None	None	73.5
1 per cent antiserum	None	67.5
1 per cent antiserum + 3 per cent complement	None	0
1 per cent antiserum	3 per cent complement	0
3 per cent complement	1 per cent antiserum	54
3 per cent complement	3 per cent complement	72

lose their ability to be stained by cytoplasmic reagents like Giemsa stain. This is a manifestation of the well known cytolytic effect of antiserum plus complement on mammalian cells (6, 9). S3 cells which have been killed by exposure to a high concentration of specific antiserum in the absence of complement, present

a very different appearance when fixed and stained by the standard technique. The cytoplasm becomes very heavily stained under these circumstances. These results suggest that the killing action of high antibody concentrations in the absence of complement proceeds by a mechanism different from that produced by antibody plus complement.

Time Relationships in the Action of Antibody and Complement.—As is the case in other immunologic systems dependent on complement action, the action



TEXT-FIG. 1. Demonstration that S3 HeLa cells, exposed for 1 hour to antiserum alone but no complement, progressively lose their sensitization if they are incubated at 37°C. before addition of the complement.

100 cells were added to each Petri dish together with 1.5 per cent of heated anti-S3 serum in heated growth medium. The plates were incubated for 1 hour at 37°C. to permit the cells to attach to the glass. Then the medium was removed, the cells washed, new, heated growth medium without antiserum added, and the plates reincubated. After various time intervals shown, complement was added to a final concentration of 3 per cent, and incubation was continued for 9 days. The number of colonies developing was counted and expressed as a per cent of the original cell inoculum.

of the antibody must precede that of complement as indicated by the data of Table VII if maximum cell killing is to occur.

Since complement can kill cells which had been previously exposed to antiserum, experiments were carried out to determine how long a period can intervene between these two treatments without loss of cell sensitization. Cells treated with 1.5 per cent antiserum for 1 hour were washed and then incubated for various times at 37°C., after which complement was added. Incubation was continued for 9 days after which the resulting colonies were fixed, stained, and counted. As shown in Text-fig. 1, unless the complement is added within 1 hour, sensitization of a large part of the cells is lost. The reaction responsible for loss of sensitization at 37°C. displays an induction period of about 45 minutes before significant loss of sensitization occurs. In preliminary

experiments carried out at 23°C., loss of sensitization of cells which had been treated with antibody did not occur after more than 3 hours, suggesting the participation of metabolic events in the densensitization process.

DISCUSSION

The experiments here described fall into two categories. The first, which includes demonstration of the need for complement for cell killing by antiserum *in vitro*, demonstration that the killing activity of antiserum is limited to the γ-globulin fraction, the demonstration of the high degree of species specificity obtainable with cell and antiserum preparations, and leakage of cell constituents attending treatment with antibody and complement, contains no new effects, but simply demonstrates how these well known actions can be studied quantitatively by means of the single cell plating technique. This method permits accurate titration of antibody activity in dilutions ten to fifty times higher than that commonly achieved by methods using massive cell inocula (6, 10). It also has demonstrated that treatment with antibody plus complement has two separate actions on cell growth—an irreversible killing of some cells plus a reproductive lag among the survivors. In this respect the actions of antibody plus complement resemble that of ionizing radiation (11).

The new finding here presented is that somatic cells which have been sensitized by adsorption of specific antibody can lose their sensitization when incubated at 37°C., unless complement is added within 1 hour. The nature of this reaction is of interest. The kinetics reveal that the reaction is reasonably complex, displaying a marked induction period, followed by a precipitous loss and a final tailing off of residual sensitization. It is conceivable that tissue cells may be able to phagocytize adsorbed antibodies and so neutralize them, although simple dissociation of the adsorbed antibody in the diluted solution has not yet been ruled out. Studies on the mechanism and kinetics of this reaction are continuing.

Work with these techniques is continuing in attempt to explore further the antigenic-specificity of normal cells from different tissues and in comparing antigenicities of normal and malignant cells. Some studies pertinent to the former point have already been described by Sato and his coworkers (12).

SUMMARY

The single cell plating technique has been applied to quantitation of the reproductive killing of mammalian cells by specific antibodies. This method confirms previous demonstrations by other workers of localization of all the killing activity in the γ-globulin fraction of specific cell antisera but not of normal sera; the need for complement for the killing action in low doses of antibody and the leakage of cell constituents attending cell killing under these conditions. In concentrations of 4 per cent or higher of heated antiserum cell

killing occurs without added complement. The cell plating technique permits highly reproducible quantitation of antibody action and demonstrates antibody activity in sera diluted 1:3000. It permits demonstration of very high degrees of species specificity as shown by virtually complete absence of cross-reaction between antisera to Chinese hamster and S3 HeLa cells, respectively. Somatic cells which have been sensitized by absorption of specific antibody lose their sensitization when incubated at 37° unless complement is added within 1 hour.

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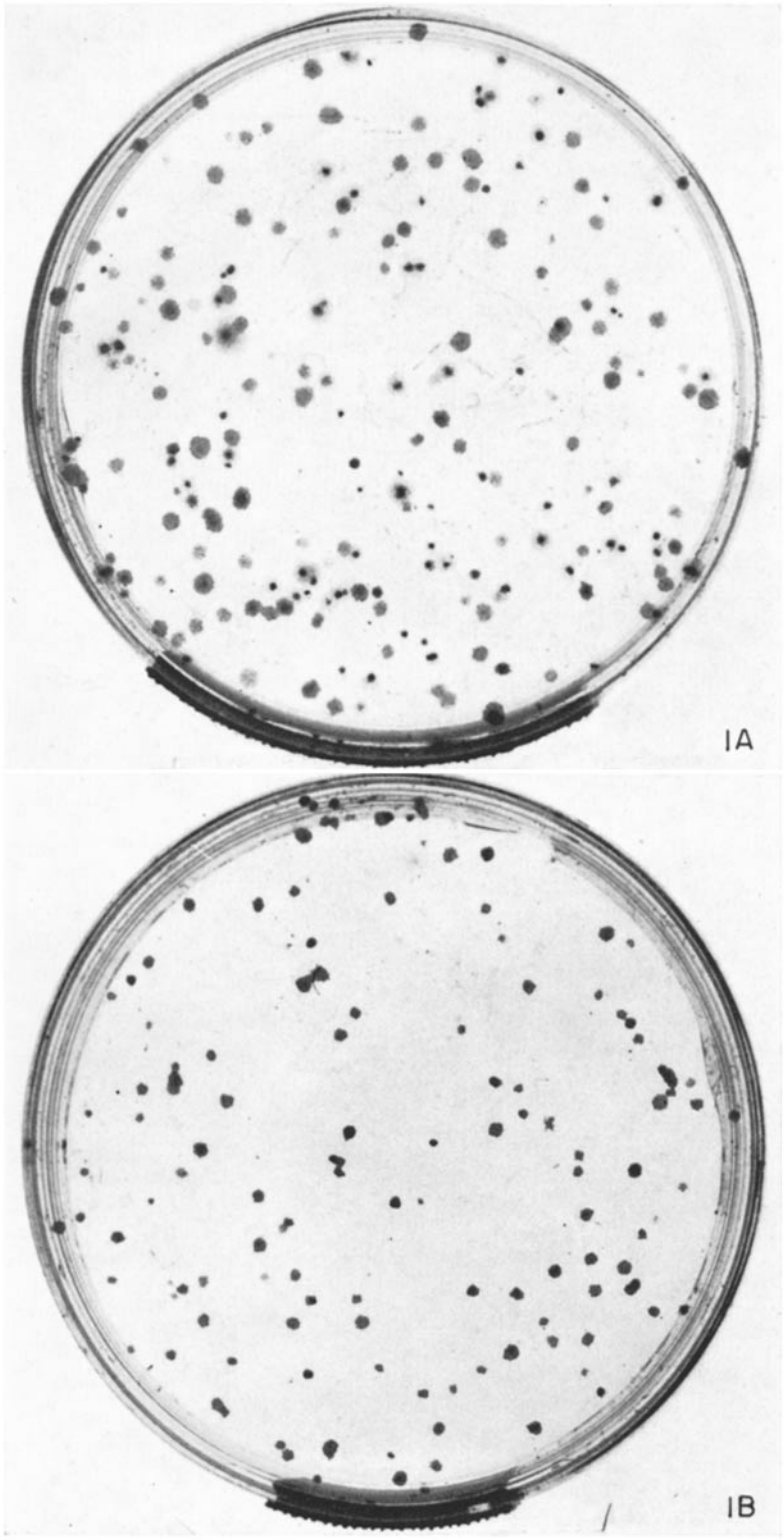
EXPLANATION OF PLATES

FIGS. 1A to 1D. Demonstration of the highly selective action of antisera to S3 cell and to the CHBOCl cells respectively. The colonial and cellular morphology of the clones formed by plating these two cell types are so distinctive as to permit absolute identification of their colonies by inspection. Colonies of S3 cells are compact and uniformly dense. Those of CHBOCl are wide and diffuse, often with a dense center, wherein the cells grow in a multilayered structure. (4) All plates received simultaneous inocula of 125 cells of each type, in a medium consisting of equal volumes of N16HHF and F4FC, plus complement. × 2.

PLATE 66

FIG. 1 A. No antiserum. This plate has 120 colonies of S3 cells and 74 of CBHOCl.

FIG. 1 B. 2 per cent anti-S3 serum. There are no S3 colonies and 82 CHBO-Cl colonies.

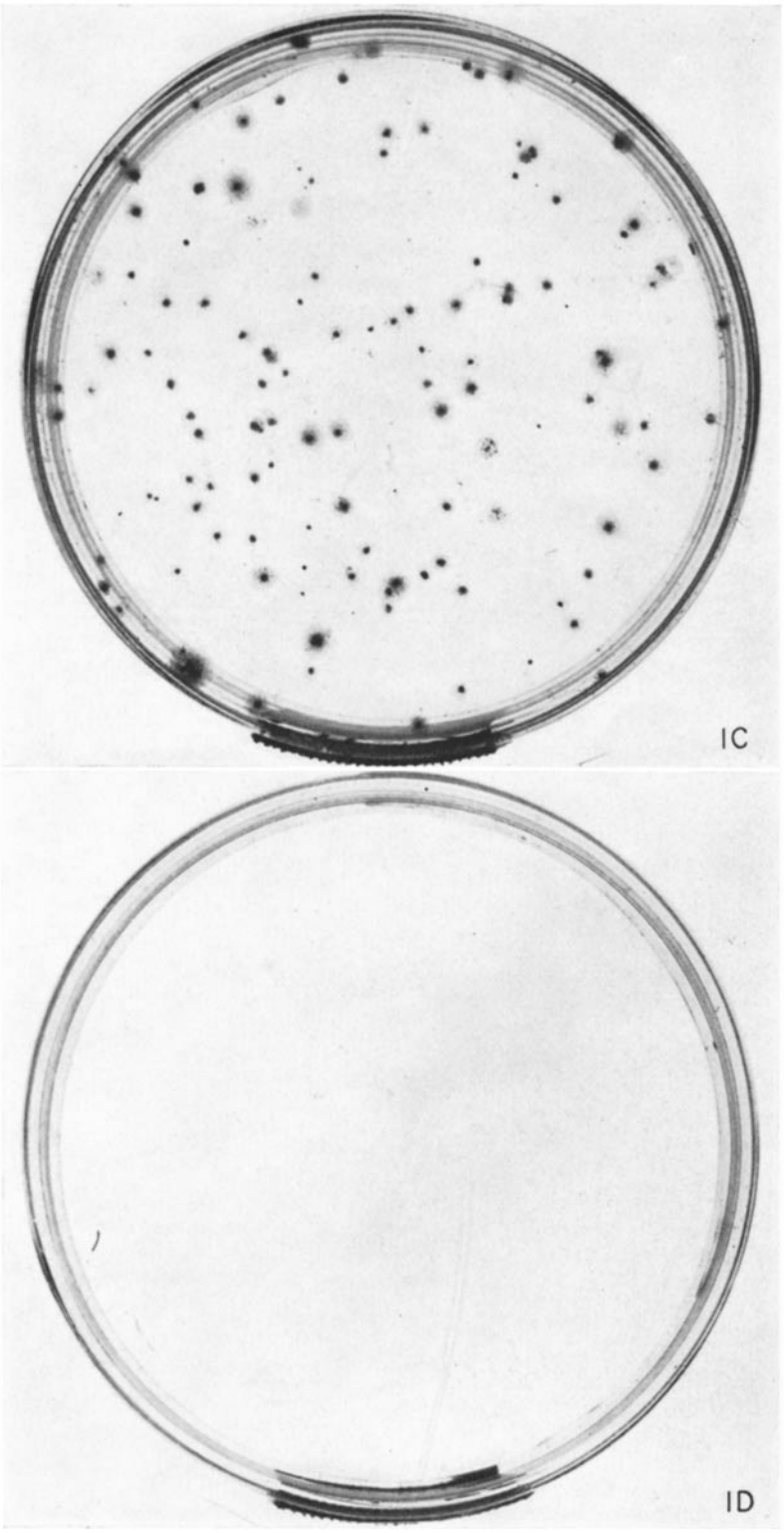


(Oda and Puck: Interaction of mammalian cells with antibodies. I)

PLATE 67

FIG. 1 C. 2 per cent anti-CHBO-Cl serum. There are 124 S3 colonies and none of CHBO-Cl.

FIG. 1 D. 2 per cent of each antiserum. No colonies whatever appear on the plate



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