EFFECTS OF ANTICELLULAR SERUM ON PHAGOCYTOSIS AND THE UPTAKE OF TRITIATED THYMIDINE AND URIDINE BY HELA CELLS*, ‡

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Anticellular antibody in the presence of complement alters permeability of the plasma membrane and causes cell destruction (2). The effect of anticellular antibody on cultured cells in a system free of complement varies with the concentration of antibody present. High levels of antibody agglutinate cells and interfere with growth. Lower concentrations do not interfere with growth of cells or induce morphologic changes detectable by light microscopy (3–5) but do block infection by certain viruses (4–7). This inhibition of virus infection by anticellular antibody suggested that the antibody might alter specific functions of the cell.

In the present study, HeLa cells were incubated with anticellular serum free of complement activity. The phagocytic ability of these cells was greatly impaired but the uptake of tritiated thymidine and uridine and their incorporation into nucleic acids of the cell was unaltered as evaluated by autoradiography.

Materials and Methods

HeLa Cell Cultures.—The cells were obtained originally from Microbiological Associates Bethesda, Maryland, and cultured as monolayers in Eagle's minimal essential medium (MEM) (8) with 10 per cent calf serum. Stock cultures were grown in bottles and transferred to plastic

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tissue culture dishes[1] or small plastic chambers. The cultures were incubated at 36°C in an atmosphere of 5 per cent CO_2 for 24 or 36 hours prior to use. Cells examined by electron microscopy were grown in tissue culture dishes and cells examined by light microscopy were grown in chambers. The chambers were constructed of coverslips fixed to frames of plexiglas[2] with acryloid B-72[3]. They had a capacity of 0.7 ml and the area of the coverslip on which cells grew was 1.13 cm^2. The chambers were sterilized under an ultraviolet lamp and kept in sterile Petri dishes.

Anticellular Serum.—The antiserum was prepared in rabbits. The antigen was a suspension of intact HeLa cells. The HeLa cells, grown in MEM, were freed from the glass with a rubber policeman or trypsin (0.25 per cent), washed 3 times with saline, and suspended in saline at a concentration of 10^6 cells per ml. Blood for controls was obtained from each animal prior to immunization. The animals received a minimum of 9 intravenous injections of 5 ml of the suspension of cells during a period of 3 weeks. Serum was separated from the blood obtained by cardiac puncture 5 to 7 days after the last regular injection and after booster injections. The sera were stored at −20°C. Prior to use all sera were heated at 56°C for 30 minutes and then diluted in MEM containing inactivated calf serum. Concentrations of anticellular serum used in these experiments produced minimal or no agglutination of the established cultures but did induce necrosis in 95 to 100 per cent of the cells of a duplicate culture when complement was added (Figs. 1 to 3). The complement was added to duplicate cultures as reconstituted guinea pig serum[4] (final concentration 5 per cent) or normal rabbit serum (final concentration 10 per cent). Sera added to supply complement had no cytotoxic activity when added to normal cells without anticellular serum. All cultures were incubated at 36°C in an atmosphere of 5 per cent CO_2.

Phagocytosis of Colloidal Gold.—Anticellular sera from 5 rabbits were pooled and added to the monolayer of HeLa cells cultured in plastic dishes. Control cultures were incubated with similar concentrations of pooled normal sera from the same animals. Eight hours later colloidal gold[5] was added to the cultures in a final concentration of 0.05 mg per ml. Twenty-four and 72 hours after addition of gold the cells were fixed, stained, and examined with the electron microscope using procedures reported previously (9).

Phagocytosis of Staphylococci.—HeLa cells cultured on coverslips were incubated with 0.5 ml of the diluted normal or anticellular serum for 6 to 8 hours. One tenth ml of a suspension of *Staphylococcus aureus* was added to each culture and incubation continued for 18 hours. The cells attached to the coverslips were rinsed with MEM to remove extracellular cocci, fixed in methanol, and stained with Giemsa. They were examined with the light microscope using an oil emersion lens (× 1000). Cells were considered to contain bacteri when the cocci were seen within the boundaries of the cell in the same plane of focus as the nuclei.

Suspensions of *Staphylococcus aureus* were prepared from cultures grown in tryptose broth for 18 hours. The bacteria were sedimented, resuspended in MEM, and diluted so that 0.1 ml contained a number of bacteria which favored phagocytosis of staphylococci by 85 to 95 per cent of cells of control cultures. Tetracycline was added to the medium in bacteriostatic concentrations (50 μg per ml) instead of penicillin and streptomycin. This concentration of tetracycline inhibited extracellular growth of staphylococci but some intracellular growth of bacteria may have occurred during the period of incubation. These studies were repeated with heat-killed organisms and the per cent of cells which had phagocyted bacteria were essentially

[1] 60 × 15 mm plastic tissue culture dishes, Falcon Plastics Division of B.D. Laboratories Inc., Los Angeles, California.

[2] Plexiglas frames are 2 × 2 × 0. 6 cm with a center hole 1.2 cm in diameter, Gladwyn Plastics Inc., Atlanta 3, Georgia.

[3] Acryloid B-72, Cope Plastics Co., Godfrey, Illinois.

[4] Markham Laboratories, Chicago.

[5] Inactive colloidal gold, Abbott Laboratories, Chicago.

the same in all experiments, although the cells incubated with viable staphylococci appeared to contain more bacteria than cells incubated with the heat-killed organisms.

Autoradiography by Electron Microscopy.—HeLa cells cultured in Petri dishes were incubated with normal or anticellular serum for 8 hours. Tritiated thymidine[6] (3 c per mmole) was added in a concentration of 1 μc per ml. The cells were incubated with the thymidine for 2 hours, fixed, sectioned, and subjected to electron microscopic autoradiography by methods previously reported (10).

Autoradiography by Light Microscopy.—HeLa cells cultured on coverslips were incubated with 0.5 ml of the diluted normal or immune serum for 6 to 8 hours. Tritiated thymidine as above or tritiated uridine[6] (3 c per mmole) was added to the cultures in a final concentration of 1 μc per ml. After incubation with the tritiated compounds for 4 hours, the cell monolayers were washed with culture medium, fixed with methanol, and stained by the Feulgen method. The coverslips were coated with Ilford K-5 emulsion (11), exposed for 7 days at room temperature, and developed with Eastman D-19 developer. Only slides with minimal background over cell-free portions of slides were included in the results. Cells were considered to contain tritiated compounds when 5 or more activated grains were present over the nuclei.

TABLE I

Inhibition of Phagocytosis of Colloidal Gold with Anticellular Serum

Serum	Dilution	Duration of incubation	
		24 hrs.	72 hrs.
Normal	1:2	46/107 (43)*	73/109 (67)
Normal	1:6	41/95 (43)	56/104 (54)
Immune	1:2	0/101 (0)	0/120 (0)
Immune	1:8	Not examined	0/73 (0)

* Numerator, number of cells containing colloidal gold; denominator, number of cells examined; numbers in parentheses indicate per cent.

TABLE II

Inhibition of Phagocytosis of Staphylococci with Anticellular Serum

Serum	Dilution of serum	
	1:5	1:10
Normal	1243/1335 (93)*	1135/1228 (92)
Immune	71/1380 (5)	138/1214 (11)

* Numerator, number of cells containing staphylococci; denominator, number of cells counted; numbers in parentheses indicate per cent.

RESULTS

Phagocytosis of Colloidal Gold.—Thin sections of HeLa cells were examined for intracellular gold with the electron microscope. Gold particles were present in vacuoles or free in the cytoplasm in 52 per cent of 415 sections of control cells

[6] Schwartz Bioresearch Labs., Inc., Orangeburg, New York.

examined (Fig. 4). Two hundred-ninety four sections of cells incubated with anticellular serum and gold were examined and these cells did not contain particles which could be identified definitely as colloidal gold (Table I).

Effect of Anticellular Serum upon Phagocytosis of Staphylococci.—The inhibition of phagocytosis by anticellular serum was tested further with HeLa cells and staphylococci. Coverslip cultures of HeLa cells incubated with serum and staphylococci were examined with the light microscope and the number of cells containing bacteria determined by a direct count of the stained preparations. The effects of individual sera from 3 rabbits and pooled sera from 5 rabbits

TABLE III

Concentration of Antiserum Inhibiting Phagocytosis and Causing Necrosis

Dilution of serum	Phagocytosis		Necrosis
	Normal serum	Immune serum	Immune serum plus complement
1:3.5	251/280 (92)*	9/267 (3.4)	++++‡
1:7	266/290 (91)	21/243 (8.6)	++++
1:14	190/209 (91)	67/215 (31)	++++
1:21	190/214 (90)	85/182 (48)	++++
1:28		180/208 (88)	++++
1:35			+++
1:42			++

* See Table II.

‡ Estimated percentage of necrotic cells in the culture: ++++, 95 to 100; +++, 75 to 94; ++, 50 to 74; +, 15 to 49; 0—none.

on phagocytosis of staphylococci are summarized in Table II. Numerous bacteria were present in the majority of HeLa cells incubated with normal serum but only a few cocci were seen in an occasional cell incubated with the anticellular serum (Figs. 6 and 7).

The phagocytosis of staphylococci by normal HeLa cells was influenced by several factors; the number of bacteria present, duration of incubation, age of the HeLa cell culture, and the uniformity and compactness of the monolayer. Young cultures in smooth, relatively loose monolayers were more active in phagocyting staphylococci. Only those experiments in which phagocytosis of bacteria occurred in 85 to 95 per cent of the cells of corresponding control cultures were included in the results.

Comparison of Cytotoxic Concentration of Antiserum with the Concentration Inhibiting Phagocytosis.—Several coverslip cultures of HeLa cells containing similar numbers of cells were incubated with serial dilutions of serum for 8 hours. Staphylococci or complement were added to duplicate cultures and incubation continued for 18 hours. The occurrence of phagocytosis or necrosis in the appropriate cultures was evaluated.

Maximal inhibition of phagocytosis required concentrations of antiserum greater than those needed to induce necrosis in the presence of complement (Table III). Partial inhibition of phagocytosis occurred with the intermediate concentrations of antiserum. The minimal concentration of antiserum which produced necrosis in 95 to 100 per cent of the exposed cells in the presence of complement did not inhibit phagocytosis of staphylococci.

TABLE IV

Reversal of Inhibition of Phagocytosis

Time after addition of rabbit serum	Serum	Dilution	Group 1, continuous incubation with rabbit serum		Group 2, rabbit serum removed after 6 hours and replaced with medium	
			Phagocytosis	Necrosis	Phagocytosis	Necrosis
hrs.						
6	Normal	1:7	279/307 (91)*	0*	221/246 (90)	0
	Normal	1:14	286/322 (89)	0		0
	Immune	1:7	14/318 (4.4)	++++	20/312 (6.4)	++++
	Immune	1:14	29/289 (10)	++++	108/320 (34)	++++
24	Normal	1:7	239/269 (89)	0	240/267 (90)	0
	Immune	1:7	16/255 (6.3)	++++	130/272 (48)	++++
	Immune	1:14	41/276 (15)	++++	253/285 (89)	+++
30	Normal	1:7	201/223 (90)	0	190/216 (88)	0
	Immune	1:7	18/268 (6.7)	++++	251/293 (86)	+++
	Immune	1:14	51/282 (18)	++++	180/212 (85)	+
48	Normal	1:7	169/193 (88)	0	116/132 (88)	0
	Immune	1:7	20/246 (8.3)	++++	124/145 (86)	+
	Immune	1:14	66/275 (24)	++++	97/116 (84)	0

* See Tables II and III.

Reversibility of the Inhibition of Phagocytosis by Anticellular Serum.—HeLa cells cultured in chambers were incubated with normal or anticellular serum for 8 to 12 hours. The cultures were divided into 2 groups. Cultures in group 1 were incubated continuously with either normal or anticellular serum and served as controls for group 2. The fluids containing the rabbit serum were removed from the cultures in group 2. The cell monolayers were washed with MEM to remove unbound antibody and then incubated with MEM containing inactivated calf serum only. This procedure was repeated twice every 24 hours. At each change of the culture medium the phagocytic activity of representative cultures from each group was tested by the addition of staphylococci to the cultures. The persistence of antibody was detected by the occurrence of necrosis after addition of complement.

The inhibition of phagocytosis was reversed completely by repeated incuba-

tions with culture medium free of anticellular serum (Table IV, Fig. 8). The phagocytic activity of the cells returned although sufficient antibody was present in the culture to induce necrosis when complement was added. Repeated experiments demonstrated that the time required for the antibody to elute from the cells varied with different sera and was influenced by the concentration and

TABLE V

Electron Microscopic Autoradiography of Cells Treated with Anticellular Serum and Exposed to Tritiated Thymidine

Serum	Dilution	Thymidine
Normal	1:6	13/111 (11.7)*
Immune	1:8	10/76 (13.4)

* Numerator, number of cells labeled; denominator, number of cells examined.

TABLE VI

Light Microscopic Autoradiography of Cells Treated with Anticellular Serum and Exposed to Tritiated Thymidine or Uridine

Serum	Dilution	Thymidine	Uridine
Normal	1:7	125/229 (55)*	160/261 (61)
Immune	1:7	124/240 (52)	207/318 (65)

* See Table V.

titer of the anticellular serum originally added to the culture. Inhibition of phagocytosis persisted for as long as 48 to 72 hours with selected concentrations of some sera. The supernatant from cultures previously incubated with antiserum was cytotoxic when added to normal cultures with complement indicating that some of the antibody eluted and retained capacity to induce necrosis in the presence of complement.

Uptake of Thymidine and Uridine by HeLa cells Incubated with Anticellular Serum.—The effect of anticellular serum upon the uptake of thymidine and uridine and their incorporation into nucleic acids was evaluated using tritiated compounds and autoradiography with electron and light microscopes. The sera and dilutions tested were those used in the study of phagocytosis. Concentrations of antiserum that had inhibited phagocytosis in the preceding experiments did not interfere significantly with the uptake and incorporation of thymidine or uridine by HeLa cells as the number of labeled cells were similar in both groups (Tables V and VI). No significant difference was noted in the number of activated grains over individual cells of cultures incubated with either normal or anticellular serum (Figs. 5, 9, and 10).

Morphologic Alterations in HeLa Cells Incubated with Anticellular Serum.—

Goldberg and Green (12) found that ascites tumor cells exposed to anticellular antibody developed a marked folding of the cell membrane. A similar change was noted in the membrane of many of the HeLa cells incubated with the antiserum and examined with the electron microscope (Fig. 11). Although similar foldings were seen in some cells incubated with normal serum it was not as marked nor as extensive as seen in the cells incubated with anticellular serum.

DISCUSSION

Anticellular serum inhibited the phagocytosis of colloidal gold and staphylococci. The same concentrations of antisera did not interfere with the transport of thymidine and uridine into the cells. These findings indicate that thymidine and uridine enter cells by mechanisms independent of phagocytosis or pinocytosis. Consistent with these findings are reports (13, 14) that anticellular serum did not interfere with uptake of amino acids in the absence of complement. It was not determined, however, whether the concentrations of anticellular serum were sufficient to inhibit phagocytosis.

The processes involved in pinocytosis and phagocytosis are similar; *i.e.*, pseudopodia form and fuse to enclose drops of medium or particles adhering to the cell surface (15). The morphologic changes induced in cell membranes of ascites tumor cells as noted by Goldberg and Green (12), and similar changes found in the HeLa cells suggest that the antibody agglutinates adjacent portions of the membrane. The agglutination of the cell membrane by antibody would be expected to interfere with the continuous evagination and invagination of the cell boundaries associated with active phagocytosis and pinocytosis. This concept is supported by studies of Easton, Goldberg, and Green (16) demonstrating ferritin-labeled antibody between the invaginated membranes of ascites tumor cells. If this is the manner by which anticellular antibody interferes with phagocytosis one might expect that the antibody would have to combine with a large portion of the cell membrane. The finding that inhibition of phagocytosis required higher concentrations of antiserum than that needed to produce necrosis in the presence of complement would be consistent with this supposition. Also in support of this theory is the observation that the phagocytic activity of the cells returned while there was still sufficient antibody in the system to induce necrosis when complement was added to the culture.

Anticellular antibody might inhibit phagocytosis by mechanisms other than that suggested. The antibody may interfere with metabolic activities of the cell or may alter the chemical nature of the membrane and the physical forces influencing phagocytosis. Sbarra and Karnovsky have shown that sodium fluoride inhibited glycolysis and phagocytosis by leucocytes (17). However, Bickis, *et al.* (13) demonstrated that anticellular antibody did not interfere with glycolysis in ascites tumor cells in the absence of complement. Whether the presence of the antibody on the plasma membranes would alter the physical forces between cells and particles which favor phagocytosis is not known. Other investigators have shown that serum proteins in the medium stimulate

phagocytosis (15) and normal globulins enhanced the phagocytosis of staphylococci by leucocytes (18).

It has been reported that leucocytes (19) and ascites tumor cells (20) incubated with isoantibodies were still capable of phagocytosis and pinocytosis. Ascites tumor cells treated with antibodies prepared in rabbits did retain some phagocytic activity as Easton, *et al.* (21) demonstrated ferritin-labeled antibody lining the membrane of pinocytotic vesicles. Since our studies suggest that inhibition of phagocytosis by anticellular antibody is influenced by the number of antigenic sites reacting with antibody, the discrepancy between the findings of others and our results may be due to the concentration and titer of the antiserum studied and the fact that the antiserum to HeLa cells was prepared in rabbits. Although Stuart's studies (22) indicated that anticellular sera inhibited phagocytosis by leucocytes, complement was not eliminated from his system and cell destruction occurred.

Anticellular serum blocked infection of tissue cultures by many viruses (4–7). The mechanisms involved in this inhibition have not been defined, although it has been shown that antibody decreased the uptake of virus. Experimental evidence indicates that receptors must be available on the cell membrane for attachment of the viral particle if infection by certain intact viruses is to occur (23). It has been suggested that the attached viral particle is then taken into the cell by pinocytosis (23, 24). Electron microscopic studies of the early interactions between viruses and cells (25–27) support the theory that attached viral particles reach the cell interior through the process of phagocytosis similar to colloidal gold in our experiments. Studies comparing the entry and distribution of herpes virus and colloidal gold in HeLa cells are consistent with this supposition (28). Sodium fluoride which inhibits phagocytosis, decreased the uptake of vaccinia virus (29). Our findings suggest that anticellular serum inhibits viral infection by interfering with the phagocytic component of the penetration of virus into cells. The covering of receptors by anticellular antibody may be an additional significant factor.

SUMMARY

Anticellular serum inhibited phagocytosis of colloidal gold and staphylococci by HeLa cells. This inhibition of phagocytosis was reversed by conditions which allowed the antibody to elute from the cell. Concentrations of antiserum that inhibited phagocytosis did not interfere with the transport of tritiated thymidine and uridine across the cell membrane, and their incorporation into cell nucleic acids was unaltered as evaluated by autoradiography. These results indicate that thymidine and uridine were taken into cells independently of phagocytosis.

Morphologic changes induced in the cells by antibody suggest that the antibody agglutinates adjacent portions of the cell membrane. This agglutination of the cell membrane by antibody would be expected to interfere with the

continuous evagination and invagination of the cell membrane associated with phagocytosis.

The inhibition of virus infection by anticellular antibody may be a result of the effect of the antiserum upon phagocytosis.

All aspects of the use of experimental or laboratory animals have been in accordance with the recommendations of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D. C.

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EXPLANATION OF PLATES

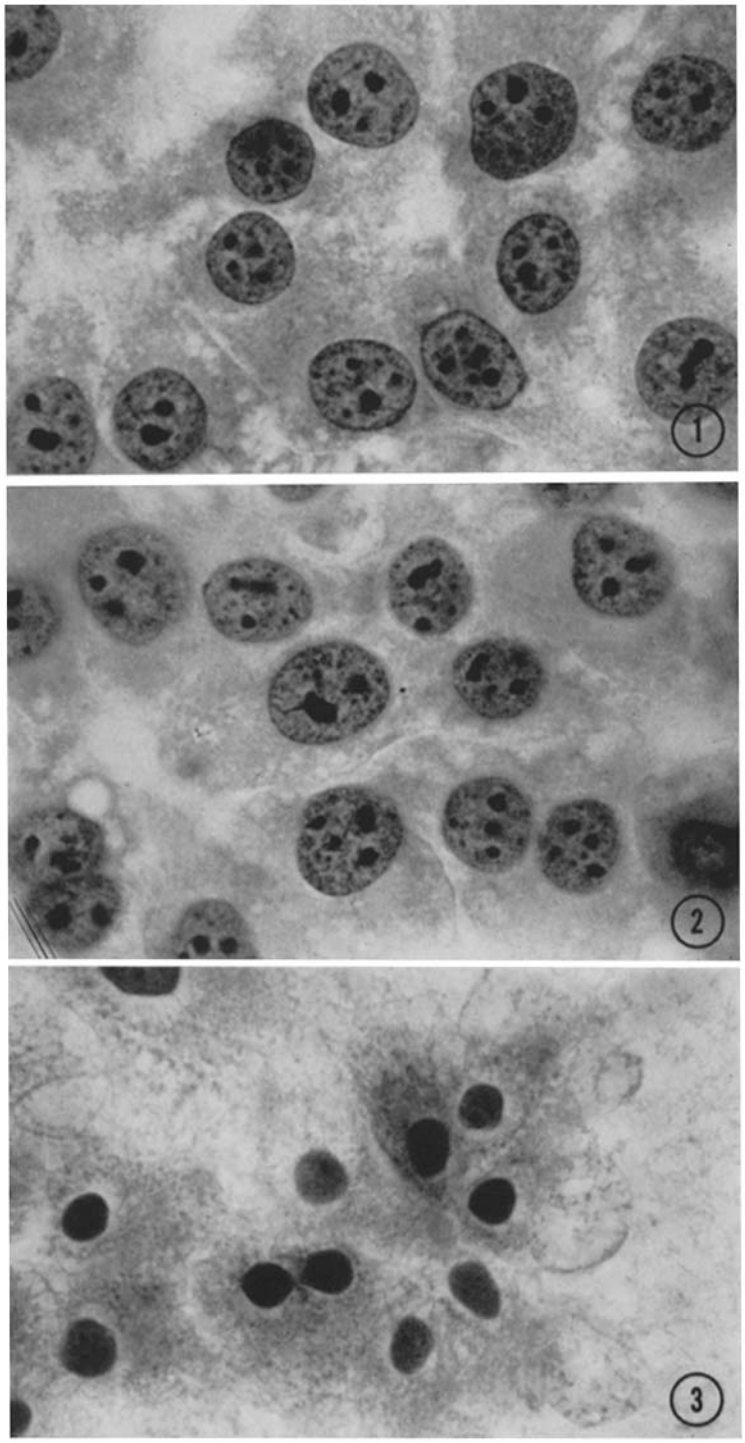
PLATE 77

The HeLa cells were cultured on coverslips for 24 hours and then incubated with 0.5 cc of a 1:5 dilution of heat-inactivated rabbit serum for 24 hours. Complement was added to the cultures in Figs. 1 and 3 as reconstituted guinea pig serum in a concentration of 5 per cent. The cells were fixed in methyl alcohol and stained with Papanicolaou stain. × 1000.

FIG. 1. Normal rabbit serum and complement.

FIG. 2. Rabbit anticellular serum without complement. The cells are essentially unchanged from those incubated with normal serum.

FIG. 3. Rabbit anticellular serum and complement. The cells are disrupted and the nucleic are pyknotic.

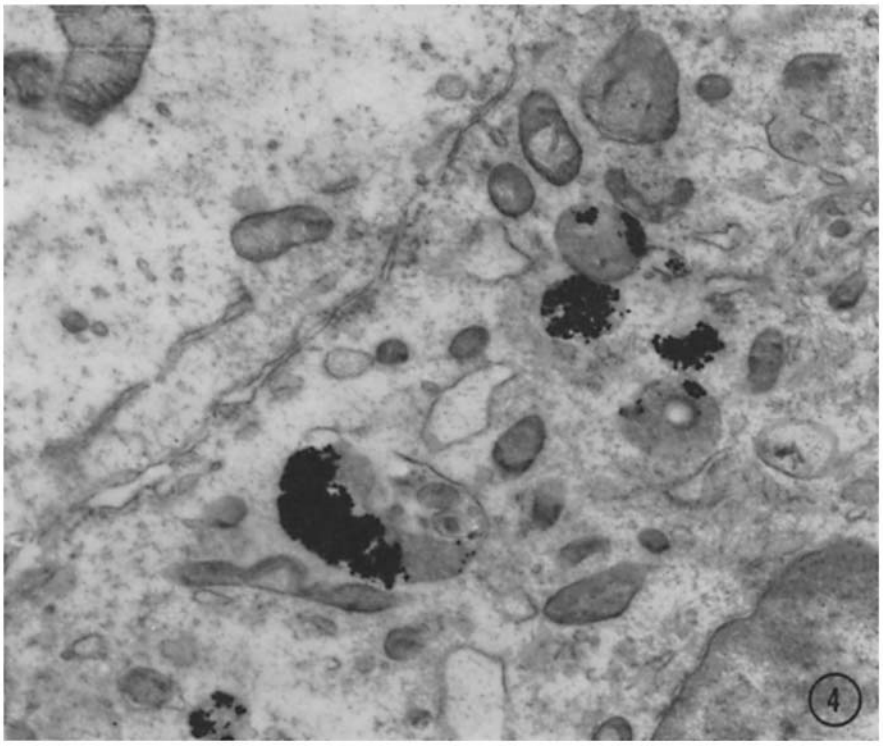


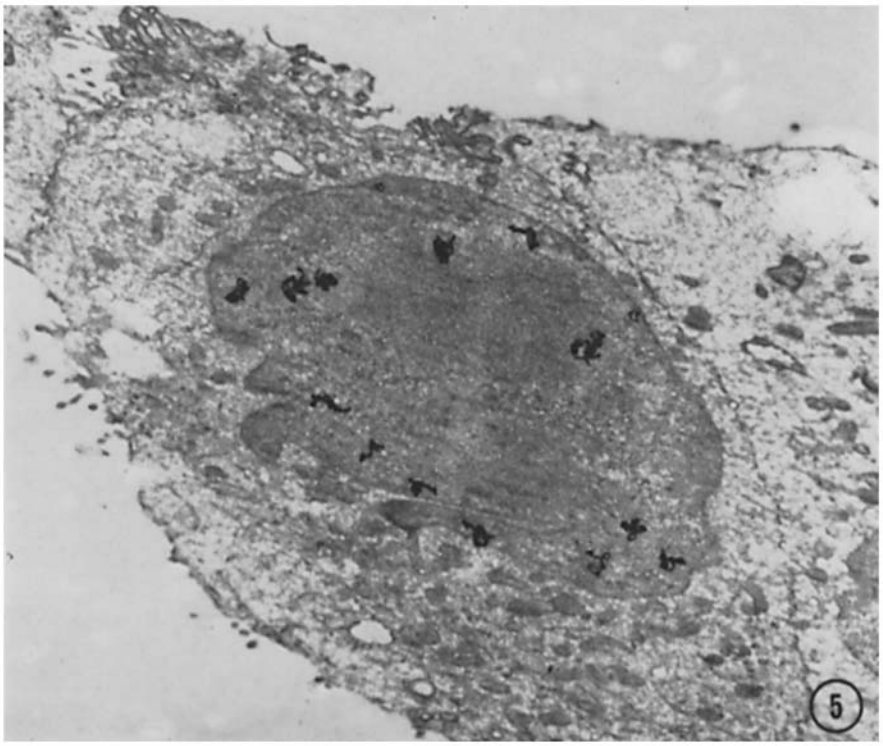
(Carey *et al.*: Anticellular serum and HeLa cells)

PLATE 78

FIG. 4. Micrograph of a portion of HeLa cell incubated with normal rabbit serum and colloidal gold for 72 hours. The gold is seen within inclusion vacuoles. Phosphotungstic acid. × 16,000.

FIG. 5. Electron microscope autoradiograph of a HeLa cell which has been incubated with anticellular serum for 8 hours and tritiated thymidine for 2 hours. Uranyl acetate. × 5600





(Carey *et al.*: Anticellular serum and HeLa cells)

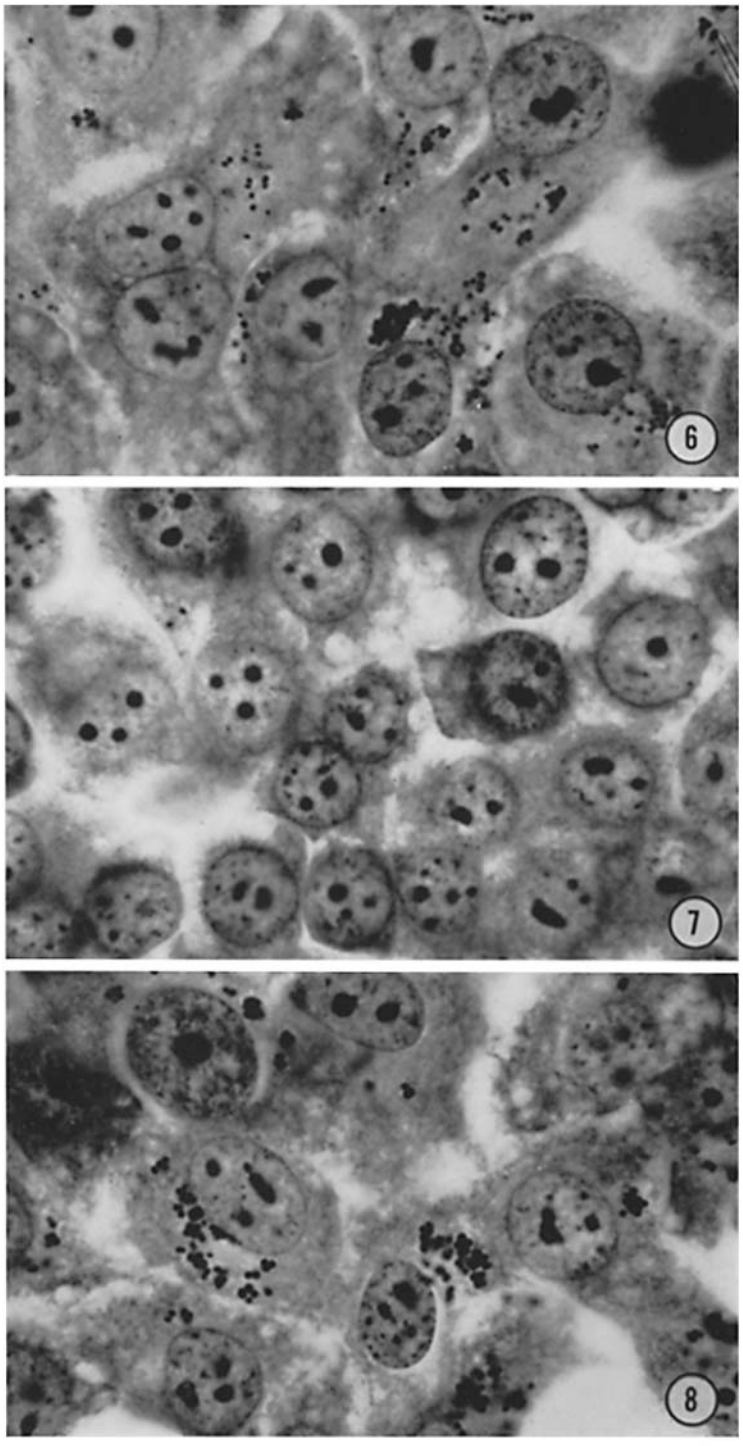
PLATE 79

FIGS. 6 and 7. The HeLa cells were cultured on coverslips for 24 hours and then incubated with a 1:7 dilution of rabbit serum for 8 hours. Staphylococci were added to the cultures and incubation continued for 18 hours. The cell monolayers were rinsed with growth media to remove extracellular bacteria, fixed in methyl alcohol, and stained with Giemsa. × 1000.

FIG. 6. Normal rabbit serum and staphylococci. Staphylococci are present in the majority of the HeLa cells.

FIG. 7. Rabbit anticellular serum and staphylococci. Phagocytosis of staphylococci was inhibited and only an occasional bacterium is seen in the cells.

FIG. 8. The cells are from a companion culture to those in Fig. 7. However, after 8 hours of incubation with the anticellular serum the antibody was removed and further incubation was carried out in medium free of anticellular serum. When staphylococci were added (as in Figs. 6 and 7), numerous bacteria were phagocyted indicating that the inhibition of phagocytosis was reversed. × 1000.



(Carey *et al.*: Anticellular serum and HeLa cells)

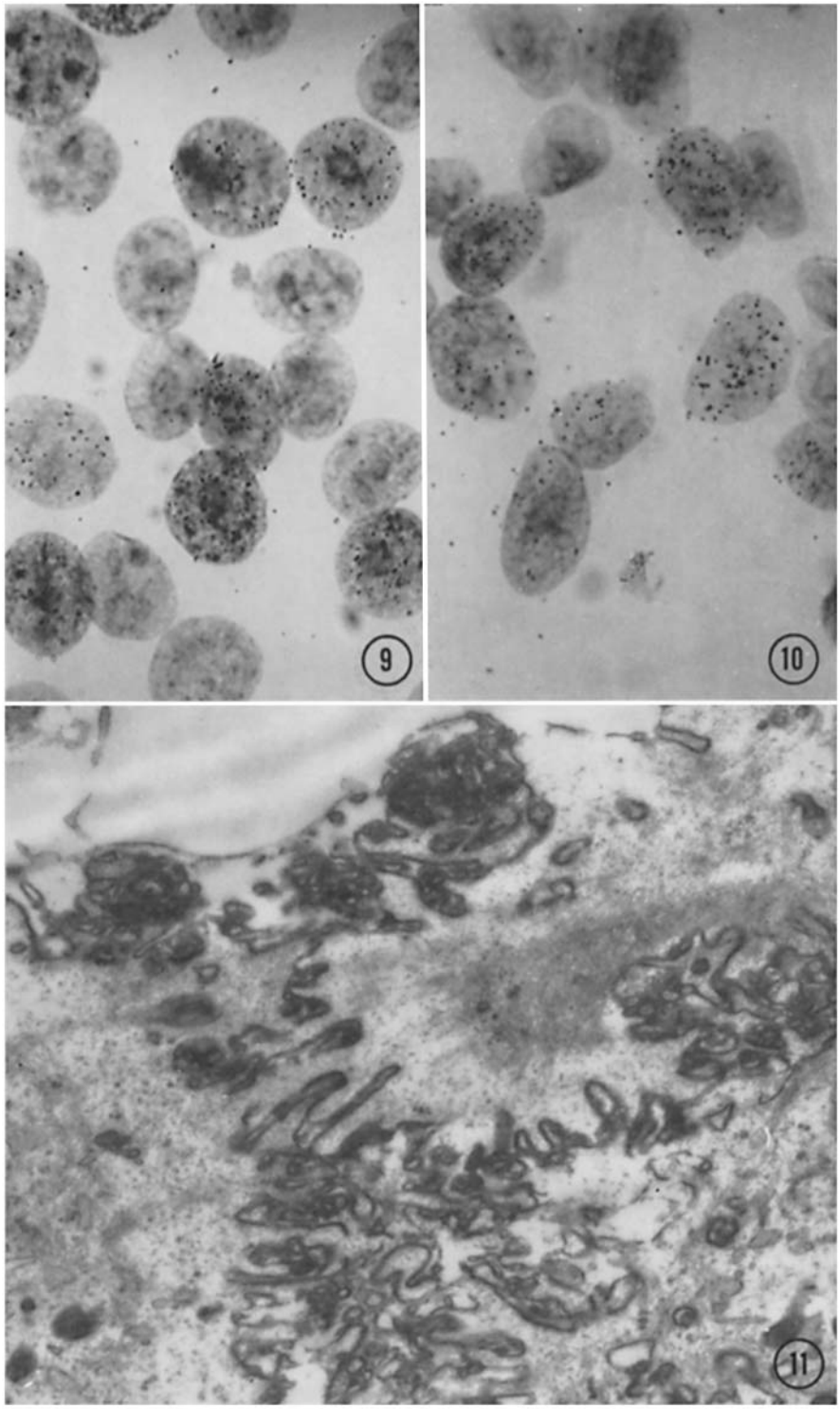
PLATE 80

FIGS. 9 and 10. HeLa cells were cultured on coverslips for 24 hours and then incubated with a 1:7 dilution of rabbit serum for 8 hours. Tritiated uridine was added in a concentration of 1 μc per ml. After 4 hours the cell monolayer was rinsed with growth media, fixed in methyl alcohol, stained with Feulgen reagent and coated with Ilford K5 emulsion. × 1000.

FIG. 9. Normal serum and tritiated uridine.

FIG. 10. Anticellular serum and tritiated uridine.

FIG. 11. Micrograph demonstrating the marked folding of membrane which occurred in HeLa cells incubated with anticellular serum. The cells were incubated with a 1:2 dilution of anticellular serum for 72 hours. Uranyl acetate. × 16,000.



(Carey *et al.*: Anticellular serum and HeLa cells)