THE TOXIC PROPERTIES OF MASSIVE INOCULUMS OF NEWCASTLE DISEASE VIRUS AND INFLUENZA VIRUS (PR8) FOR CELL STRAINS DERIVED FROM NORMAL AND NEOPLASTIC TISSUE

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It has been reported that massive inoculums of Newcastle disease virus and influenza virus have a "toxic" action *in vivo* under certain conditions.[1-5] More recently it has been demonstrated that these viruses also exert a toxic action *in vitro* against certain cell strains of human origin.[6,7] This report concerns experiments which describe and compare the toxicity of these 2 viruses for 5 other cell types *in vitro*. These cell types are of human and murine origin and are derived from both normal and neoplastic tissue.

The results demonstrate that cytotoxicity is produced only by exposure of cells to an inoculum which represents a ratio of more than one infectious unit of virus per cell. The data for 3 of the cell types (L, $LLCM_1$ and U_{12}) further suggest that whereas the virus units are responsible for this cell damage, their effect cannot be explained by the usual processes of intracellular virus multiplication, since the toxic effect of inoculums cannot be serially passaged indefinitely.

MATERIAL AND METHODS

Virus

The California strain, #11914, of Newcastle disease virus (NDV) and the PR8 strain of influenza virus were used (both strains were obtained from the American Type Culture Collection, Washington, D.C.). Nine to 10-day-old fertile eggs were inoculated with 0.1 ml. of a 10^{-4} dilution (PR8) or 10^{-3} dilution (NDV) and incubated for 2 (PR8) or 3 (NDV) days at 37°C. The harvested infected allantoic fluid was

This work was supported by United States Public Health Service Grant #C-1735 and grants from the Cuyahoga Unit of the American Cancer Society and the Alice Keith Mather Fund of the Cleveland Foundation.

A portion of this work was done under the terms of a Damon Runyon Cancer Research Fellowship.

Received for publication, November 6, 1959.

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stored in glass ampules in a carbon dioxide box. The median infective dose (ID_{50}) of different harvests of NDV varied from $10^{-9.2}$ to $10^{-9.7}$ per 0.1 ml., and of PR8, from $10^{-8.7}$ to $10^{-9.3}$ per 0.1 ml. Hemagglutination (HA) end points of dilutions of these two viruses usually ranged from 1:256 to 1:1024 for NDV and 1:2,000 to 1:8,000 for PR8 per ml. of allantoic fluid.

Assays of Virus and Immune Serum

One-tenth ml. of 10-fold dilutions of allantoic fluid in a buffered (pH 7.6) balanced salt solution (Hanks's) were inoculated into the allantoic cavity of 8 to 11-day-old fertile eggs. After 3 days' incubation at 37°C., 0.5 ml. of undiluted allantoic fluid was mixed with 0.5 ml. of 0.5 per cent of chicken erythrocytes, and the sedimentation pattern was interpreted after 1 hour at 4°C. The 50 per cent infectivity end points (ID_{50}) were determined according to Reed and Muench.[8] To avoid falsely negative hemagglutination,[9] a 1:16 dilution of fluid was frequently tested concomitantly with undiluted allantoic fluid.

The viral hemagglutinin (HA) titer was determined using equal volumes (0.5 ml.) of serial 2-fold dilutions of infected allantoic fluid and 0.5 per cent thrice-washed chicken erythrocytes. After one hour at 4°C., the highest final dilution which produced maximum (4+) hemagglutination was regarded as the titration end point.

The preparation of the NDV-specific immune rabbit serum has been described previously[10] and PR8-immune serum was prepared in a similar manner. If 8 hemagglutinating units (HAU) of homologous virus were added to increasing dilutions of serum, hemagglutination inhibition usually occurred in a 1:1,024 dilution of NDV-immune serum and a 1:2,048 dilution of PR8-immune serum.

Cell Strains and Maintenance

The cell strains used in these studies consisted of L, $LLCM_1$ and Sarcoma 180 of murine origin and U_{12} and KB[12] of human origin.* L, $LLCM_1$ and U_{12} cell strains were maintained as a stock supply of cells in 8 ounce prescription bottles (Kimble Neutraglass). A nutrient medium used for growth and maintenance of cells consisted of 20 per cent horse serum and 80 per cent synthetic medium #199, with the addition of 100 units of penicillin and 100 μg. of streptomycin per ml. Tube cultures of KB cells (human epidermoid carcinoma) and S180 cells (mouse sarcoma) were obtained from a commercial source. Upon receipt, their nutrient fluid contained 90 per cent Eagle's Basal Medium and 10 per

* The authors are indebted to Dr. R. N. Hull[11] for the supply of $LLCM_1$ cells and to Drs. H. E. Swim and R. F. Parker[12] for the U_{12} cells.

cent horse serum. In order to standardize the conditions of these experiments, the medium of commercial tube cultures was replaced by the same 20 per cent horse serum and 80 per cent 199 mixture used for stock culture cell types within 24 hours.

Preparation of Tube Cultures

Since cultures of KB and S180 cells were obtained in tubes, it was only necessary to supply them with the 20 per cent horse serum and 80 per cent 199 mixture used for other cell types to have adequate material for experiments. These were nourished until their growth was satisfactory for comparison with the tube cultures prepared from stock bottles of the other cell strains. To prepare cultures of L, $LLCM_1$ and U_{12} cells, 15 by 150 mm. test tubes were each inoculated with 1.5 ml. of a cell suspension containing 200,000 to 300,000 cells. After stationary incubation for 2 or 3 days, cultures were inspected for contamination and suitability for comparison in experiments. At the time of allantoic fluid inoculation, culture fluids of all cell strains were replaced with a fluid medium which contained the same concentrations of horse serum and mixture 199, except that the horse serum had been heated (56°C., 30 minutes) to reduce and standardize any antiviral properties. The dilution of any inoculum is reported as the final virus concentration in the culture medium (1.5 ml.). Sets of 3 or 4 tubes were inoculated with aliquots of the same dilution.

Serial Transfer of Supernatants from Infected Cultures

To determine if the cytotoxic effect of massive virus inoculums could be passed serially through sets of cultures, 12 tubes of cells received a 1:2 dilution of either NDV or PR8. After 2 days were allowed for maximum development of virus-induced changes, the fluid medium of each culture was aspirated, pooled with others of the same set, and centrifuged. One ml. aliquots of supernatants so obtained were substituted for the medium of healthy, previously uninoculated cultures of the same age. The total fluid volume in all tubes was brought to 1.5 ml. by an additional 0.5 ml. of fresh medium. Another separate portion of the supernatant was used for infectivity and hemagglutinin titration. Control sets of cultures which contained normal allantoic fluid in place of infected allantoic fluid were treated identically. In several instances a fraction of the cultures which were recipients of passaged medium were treated 30 minutes prior to use with specific immune serum or normal serum. Their appearance afforded an additional check on the specific effect of passaged virus.

RESULTS

Newcastle disease virus and influenza virus exhibited toxicity for each of the 5 cell strains used in this study; viz., strains L, $LLCM_1$, U_{12}, S180 and KB. This toxic damage was consistently demonstrable when a fluid consisting of 1:2 or 1:5 dilution of virus-infected allantoic fluid in maintenance solution was substituted for the growth medium. The toxic changes usually reached their maximum intensity by 24 hours following exposure to either virus. In most instances this was manifested by detachment and destruction of the entire cell layer which had covered the surface of the tube. The only evidence of the previous cell layers consisted of remnants of cells floating in the culture fluid and smaller suspended cell components which produced turbidity in the growth medium. The phenol red contained in the fluid became distinctly more alkaline than that of cultures in the control groups with non-infected allantoic fluid. Control cultures which received the same high concentrations of normal allantoic fluid or heat-inactivated (56°C., 30 minutes) infected allantoic fluid had no cell destruction or intracellular damage similar to that found in cultures which received either of the two viruses.

The same cellular alterations were produced by only an hour of exposure to the high concentrations of infected allantoic fluid followed by aspiration of virus and its replacement by fresh maintenance solution. Although only a fraction of infectious virus remained after aspiration and subsequent dilution by the fresh nutrient fluid, the cytotoxic alterations were nonetheless severe and were manifest within the same period of time as that of the first method. Thus it was arbitrary whether to remove the virus seed after incubation with cultures for one hour or to leave the original inoculum for the full time of observation. The latter method was adopted for the majority of experiments involving titration of the toxic properties of material containing virus.

High concentrations of NDV or influenza-infected fluids induced similar visible degenerative cell changes as early as 14 to 16 hours. In most tube cultures, the earliest alterations consisted of increased cytoplasmic granularity and irregularity and refractility of the cell margins. Simultaneously, other cells in the same culture were more rounded than usual, and it appeared that cohesiveness of cells was increased, as evidenced by small clumps. Various degenerative changes in both the nucleus and cytoplasm appeared in rapid succession until dislodgment and lysis of cells occurred. In some cultures, however, occasional isolated cells were found which had remained intact and continued to maintain adhesion to the glass surface. They possessed irregular, highly refractile

cell margins and no intracellular detail; furthermore, their appearance did not change during observation for periods up to 6 days. In spite of their intact cell membranes and adhesiveness to the glass surface, they appeared to be irreversibly damaged or dead.

Higher magnifications of cells infected by NDV were examined on cover slip cultures and cultures in micro tissue culture plates. Cover slip cultures were prepared by placing a large drop of cell suspension on each of 8 22-mm. glass cover slips enclosed within a sealed 10 by 150 mm. Petri plate. The cells were allowed 30 minutes to settle on the glass surfaces and then submerged by 10 ml. of medium which contained dilutions of normal or infected allantoic fluid. Under these conditions, virus-induced changes in L cells were noticed as early as 4 to 8 hours after infection and consisted of a marked increase in granularity and rounding of the cells (Figs. 1 and 2). The cells had a definite tendency to clump, and there was also a distinct increase of the average diameter in many. These changes were followed in several hours by the gradual appearance of cytoplasmic vacuolation and irregular cell outlines (Fig. 3). As the toxic damage progressed, the observable manifestations were nuclear shrinking and pyknosis (Fig. 4). Cellular alterations in these preparations were usually complete within 24 to 36 hours (Fig. 5).

The cell changes described above were used to devise an arbitrary grading system for comparison of the cytopathogenic effect of various dilutions of either virus on cultures of each of the 5 cell strains. Toxic cell alterations were interpreted on a scale ranging from ± (slight cell changes, probably due to virus) to 4+ (total cell population damaged and dislodged from the growth surface). A value of 1+ represented the least degree of unequivocal virus damage. Table I contains the grade of cytopathogenicity produced by dilutions of NDV with cultures of each of the 5 cell strains. Similar values for each strain were obtained with the same dilutions of influenza-infected allantoic fluid. A direct relation is suggested between the grade of damage and the concentration of infectious virus placed with the cells. It is important to note that low concentrations of infectious seed virus, below a certain range, were unable to produce toxic changes in cells of each strain during the entire period of observation. An identical observation was made for low concentrations of influenza virus on the 5 cell strains used for these experiments.

During the period of observation, there were variations of the grades of toxicity recorded for tube cultures. If only a small proportion of the cell population showed minor toxic changes, it was not uncommon to find that the early evidence for slight damage disappeared upon con-

TABLE I

CYTOPATHOGENIC EFFECT OF NEWCASTLE DISEASE VIRUS ON CELL STRAINS L, U_{12} AND $LLCM_1$, SARCOMA 180 AND KB

Virus dilution	Time in days	Degree of cytopathogenicity: Cell strain L	U_{12}	S180	KB	$LLCM_1$
1:2	1	3	3	3	3	3
	3	4	3	4	4	3
	5	4	3	4	4	3
1:10	1	2	2	3	2	2
	3	2	2	3	3	3
	5	2	2	3	3	3
1:100	1	1	2	1	1	1
	3	1	2	1	1	2
	5	1	2	2	1	1
1:1000	1	1	1	0	0	1
	3	±	±	0	0	±
	5	±	0	0	0	1
1:10,000	1	0	0	0	0	0
	3	0	0	0	0	0
	5	0	0	0	0	0

tinued cultivation (Table I). Reproduction of unaffected cells and cell crowding were regarded as important factors which masked these slight toxic alterations.

The results of many experiments involving titration of the cytotoxicity of both NDV and PR8 for these cell types suggested that no significant difference in sensitivity to virus toxicity existed among the cell strains. Thus the variation of cytotoxicity induced by NDV, as shown in Table I, only illustrates the variability obtained in experiments employing different batches of stock virus and cultures prepared on different occasions. Since cultures with low concentrations of either virus were not destroyed after 5 days, it was assumed that multiplication of virus did not account for the cell destruction produced by the higher concentrations of either virus. However, it was conceivable that an agent was produced by the cells in response to high virus concentrations which was responsible for cell damage. This was investigated by experiments to determine if the toxicity could be serially transferred through cultures of healthy cells. Tube cultures of the 3 stock cell strains, L, $LLCM_1$ and U_{12}, were used for these experiments, and aliquots of the cultures' supernatants were obtained at each passage interval for a determination of the amount of infectious and hemagglutinating virus which remained. Centrifuged culture supernatants pooled from sets of tube cultures destroyed by a 1:5 dilution of NDV or PR8 infected al-

lantoic fluid were serially passaged into fresh tube cultures at the end of successive 2 day periods. Table II contains values from one such

TABLE II

THE RELATIONSHIP OF SERIAL PASSAGE OF NDV AND PR8 ON L AND U_{12} CELLS TO THE CYTOTOXICITY, EGG INFECTIVITY AND HEMAGGLUTINATION

Virus culture passage		Cell strain							
		U_{12}				L			
		T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4
NDV	CP	4	2	1	0	3	2	1	0
	EID/0.1 ml. 9.7	8.7	6.5	4.5	0.5	8.3	6.0	<3.5	<0.5
	HA/ml. 512	256	256	128	64	256	256	128	32
PR8	CP	2	1	0	0	3	1	±	0
	EID/0.1 ml. 9.3	7.5	5.5	<1.0 *	—	8.0	4.0	<0.5	—
	HA/ml. 2000	>500	256	64	16	256	64	16	16

* 1 of 4 eggs infected.

experiment involving a total of 4 serial passages of NDV and PR8, each through L and U_{12} cell cultures. The almost complete cell destruction of the first set produced by the seed inoculum of either virus into cultures of the cell strains was found to possess diminishing cytotoxicity in the fresh cultures of each successive transfer. Moderate toxic changes were sometimes produced in culture sets of the third serial passage, and, in one experiment with NDV and L cells, slight but definite evidence of virus toxicity was recognizable in cultures of the fifth passage. Viral cytotoxicity diminished with each serial passage, and a concomitant, gradual reduction of both egg-infectivity and viral hemagglutinin was demonstrated in culture fluids. The correlation observed between decreasing virus concentration and decreasing cytopathogenicity in successive passages suggested that the toxicity observed in these experiments depended on a certain concentration of "active" seed virus similar to the correlation between toxicity and high concentrations of infectious virus in allantoic fluids (Table I). It was evident from these experiments that the cytotoxic effect of either virus could not be transmitted through series of cultures, and thus the toxic properties were associated with the infected allantoic fluid of the original inoculum.

Data suggesting that the toxic alterations produced in the cultures receiving passaged culture supernatant were related to both viruses were obtained by the use of specific immune serum in such experiments as those just described. If NDV or PR8-specific immune serum were added

to healthy cultures ½ hour before they received toxic, serially passaged supernatant, no cytotoxic changes developed in this group in contrast to untreated cultures. The degree of protection afforded this portion of experimental sets also provided a sharp contrast to the destructive alterations appearing in control groups to which normal instead of immune serum was added. The results of these experiments imply that the toxic factor in the passaged, toxic supernatants and the virus units of NDV or influenza virus are one and the same.

Additional, confirming evidence that NDV and influenza virus acted as cytotoxic agents and were responsible for the cell changes was provided by examining the toxicity of mixtures containing either virus and an equal volume of specific immune serum. For control purposes, both pooled normal and pooled nonspecific immune rabbit serum were used. Data from 2 representative experiments appears in Tables III and IV.

TABLE III

NULLIFICATION OF CYTOPATHOGENICITY OF NDV-INFECTED ALLANTOIC FLUID FOR U_{12} CELLS BY SPECIFIC IMMUNE RABBIT SERUM

Inoculum	Cytopathogenic effect *								
	Days of incubation								
	1			3			5		
NDV + NDV immune serum	0	0	0	0	0	0	0	0	0
NDV + PR8 immune serum	3	3	3	3	3	3	3	3	3
NDV + nonimmune serum	3	3	3	3	3	3	3	3	3
NAF † + nonimmune serum	0	0	0	0	0	0	0	0	0

* Results expressed for individual tube.
† NAF: Noninfected allantoic fluid.

TABLE IV

NULLIFICATION OF CYTOPATHOGENICITY OF INFLUENZA (PR8)-INFECTED ALLANTOIC FLUID FOR L CELLS BY SPECIFIC IMMUNE RABBIT SERUM

Inoculum	Cytopathogenic effect *								
	Days of incubation								
	1			3			5		
PR8 + PR8 immune serum	0	0	0	0	0	0	0	0	0
PR8 + NDV immune serum	2	2	3	2	3	3	2	3	3
PR8 + nonimmune serum	3	4	3	3	4	3	3	4	3
NAF † + nonimmune serum	0	0	0	0	0	0	0	0	0

* Results expressed for individual tube.
† NAF: Noninfected allantoic fluid.

The data in Table III indicate that NDV-immune serum is able to protect cells completely from the cytopathogenic effect of NDV-infected allantoic fluid. On the other hand, neither "normal" rabbit serum nor

PR8-immune serum prevented cytotoxic action by NDV-infected fluids. Using the same combinations of serums with influenza virus, only the PR8-specific immune serum prevented the virus-induced toxicity of PR8-infected fluids (Table IV). The high concentrations of rabbit serum used in this experiment produced slight and nonspecific cellular changes which, however, did not interfere with interpretation of results. The high specificity of the protection against cytotoxic effects offered by immune serum gave additional support to the conclusion that the toxic factor in infected allantoic fluids consisted either of active Newcastle disease or influenza virus.

Discussion

The evidence obtained from these investigations indicates that high concentrations of Newcastle disease virus and the PR8 strain of influenza virus can exert a cytotoxic effect on L, $LLCM_1$, U_{12}, KB and S180 cell strains. Similar concentrations of heat-inactivated, infected allantoic fluid and normal allantoic fluid had no such effect on cultures of these cells. The cellular alterations appeared as early as 4 to 8 hours after inoculation (NDV with L cells) and steadily increased until cell degeneration was usually complete. This frequently occurred before 24 hours with either virus under the conditions used in this study. The nature and rate of development of the cell changes did not possess characteristics diagnostic for either virus and presented a panorama of steadily increasing cytopathologic effects. No important differences were observed in this process among the 5 cell strains investigated.

The average inoculum of either seed virus required to induce these changes contained a high concentration of infectious virus particles, and if dilution of toxic allantoic fluids exceeded 1:1,000 they were frequently without effect. It was necessary for this virus concentration, which may be expressed in terms of egg-infectious doses (EID) per ml. of infected allantoic fluid seed, to be approximately $10^{6.5}$ EID in tube cultures containing $10^{5.5}$ cells for visible destruction of any one of the 5 cell types by either virus. Thus, this concentration in the higher dilutions of toxic allantoic fluid represented a virus:cell ratio in the order of 10:1. For maximum cytologic changes, however, virus concentrations which gave virus:cell ratios of 1,000 to 10,000 EID of NDV or PR8 per cell were required. In general, the severity of toxic cell alterations had a consistent, direct relation to the virus content of the inoculum.

A relationship between influenza virus concentration and toxicity for HeLa cells *in vitro* has been described[6]; however, virus multiplication has accompanied the destructive changes in cultures of various other cell types inoculated with NDV or influenza virus. These cell types

could, therefore, support the serial passage of the destructive viral agent.[10,14–16] This is distinctly different from the viral cytopathogenic changes described in this report, since serial passage of the toxic effect of NDV or PR8-infected allantoic fluid was unsuccessful in cultures of L, U_{12}, and $LLCM_1$ cells. Furthermore, the results suggested that the diminishing cytotoxic changes which appeared in cultures of succeeding passages were correlated to the gradually diminishing concentration of egg-infectious virus in the passaged fluid. Such experimental results implied that toxicity of NDV and PR8-infected allantoic fluids could be estimated in terms of the infectious virus concentration in seed inoculums, and also that no virus multiplication, at least in the usual sense, took place in these cell types.

Finally, support to indicate the role of both viruses in the cytolytic process was obtained by abolishing the alterations with specific immune serum. Moreover, this was demonstrated by two methods: adding this material directly to cultures before addition of virus; and testing the cytotoxicity of mixtures of virus and specific immune serum. The unequivocal results of these experiments made it evident that the virus contained in these fluids could be considered the etiologic agents of cell destruction.

Evidence would indicate that both "toxic" cell damage and that resulting from virus infection and multiplication of NDV or influenza virus are associated with a high concentration of infectious virus in the extracellular environment. It is immaterial, perhaps, whether this is supplied as inoculum, as in the experiments described here, or produced and elaborated by intracellular virus multiplication during the usual infectious process. For example, severe cytopathogenic changes in cultures of chick embryo tissues infected by NDV began to occur only after extensive virus multiplication and elaboration of virus into the extracellular environment had taken place (in the range of $10^{6.7}$ to 10^{7} EID per ml.[10]). *In vivo* experiments with influenza virus have also shown that lung lesions in mice become marked only after maximum virus titers are produced by the tissue and not during the stage of rapid virus multiplication within the cells.[2] The fact that each of the 5 cell types was susceptible to the toxic activity of both viruses would indicate that a broad spectrum of cell types can be damaged by appropriate concentrations of these agents. This conclusion is supported by other studies in which these viruses have been used.[6,7,17,18]

Summary

It was demonstrated that high concentrations of both Newcastle disease virus and influenza virus (PR8), in the form of infected allantoic

fluid, are cytotoxic *in vitro* for 5 cell strains derived from both normal and neoplastic human and murine sources. Cellular alterations were produced within 24 hours only by concentrations of infectious virus in the seed inoculum which represented virus:cell ratios in the order of 10:1 or greater; lower ratios were without effect on the cells. Both viruses exerted approximately the same degree of cytopathogenicity for each of the cell strains. There was lack of evidence for a relation between virus replication and destruction of L, $LLCM_1$ and U_{12} cells, since serial transfer of the destructive changes through cultures was impossible. The relation between the concentration of infectious viruses and allantoic fluid inoculums and the findings that only specific immune serum could nullify the toxicity of either virus indicated that the rapid cell changes were dependent on the virus content of this material.

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LEGENDS FOR FIGURES

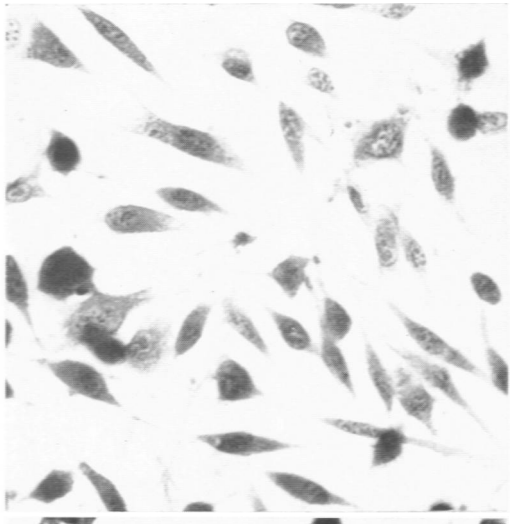
FIG. 1. L cells growing on a cover slip for 24 hours. Control culture; Papanicolaou stain. × 440.

FIG. 2. L cells 8 hours after NDV infection. Note the granularity of the cytoplasm. Grown on micro tissue culture plate; unstained. × 1068.

FIG. 3. L cells 12 hours after NDV infection. Note the rounding, granularity and clumping of cells, and irregularities of the nuclei and cytoplasm. Cover slip culture; Papanicolaou stain. × 360.

FIG. 4. L cells 16 hours after NDV infection. The cells are degenerated, and the nuclei are pyknotic. Cover slip culture; Papanicalaou stain. × 495.

FIG. 5. L cells 24 hours after NDV infection. Cell debris and a few damaged cells remain. Micro tissue culture plate; unstained. × 440.



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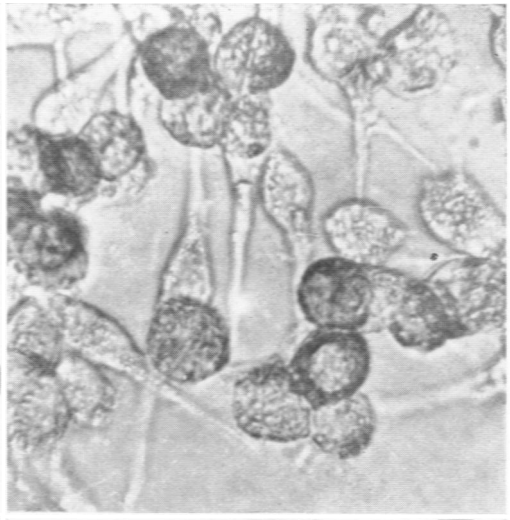

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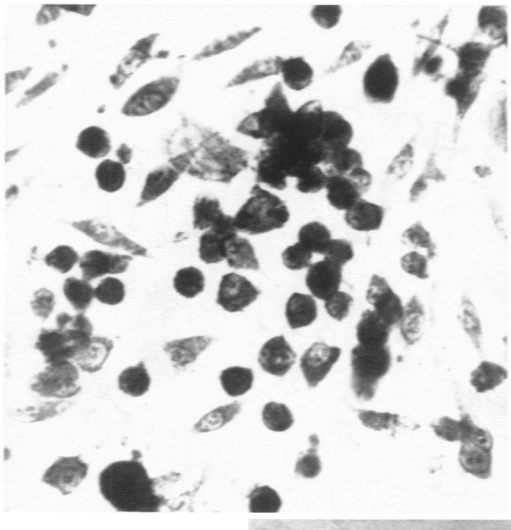

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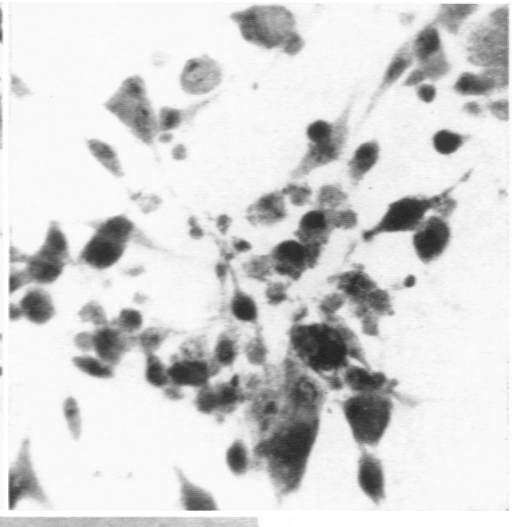

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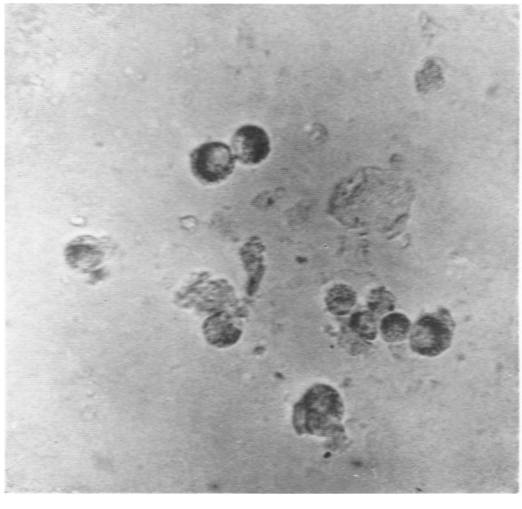

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