STRUCTURAL CHANGES PRODUCED IN BROWN-PEARCE CARCINOMA CELLS BY MEANS OF A SPECIFIC ANTIBODY AND COMPLEMENT*

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PLATE 6

An antibody appears in the blood of certain rabbits implanted with Brown-Pearce carcinoma cells which fixes complement specifically in mixture with a sedimentable constituent of them, as previous studies have shown (1–3); furthermore, the antibody inhibits the growth of the tumor cells *in vivo* and *in vitro* under a variety of experimental conditions (4). It also brings about striking structural changes in the carcinoma cells when held briefly in contact with them in the presence of complement, as will now be described (5). The findings have interest for cytology and for immunology, the more so since the effects of antibodies and complement on mammalian tissue cells have not hitherto been extensively studied.

Materials and Methods

The techniques employed were in general those previously utilized (1, 4). Briefly, the Brown-Pearce carcinoma cells were suspended as individuals in a buffered Ringer's solution containing added glucose; the suspensions were next mixed with sera containing the specific antibody, and, for control purposes, with other sera devoid of it; the effects of the antibody were then studied by examining the cells for structural changes, both in fresh and in fixed and stained preparations, and by determining their viability following implantation in susceptible hosts.

Suspensions of Brown-Pearce carcinoma cells were made as follows: firm, pale pink intramuscular growths, 6 to 8 days old and largely devoid of necrotic portions, were diced and pressed through a 40 mesh monel metal sieve into a buffered glucose–Ringer's solution, hereinafter BGR. (The Ringer's solution was buffered at pH 7.4 with phosphate buffer in approximately 0.01 molar concentration, and 500 mg. of glucose was added to each cc. of the buffered Ringer's solution.) The suspensions were left in tall cylinders for 10 to 15 minutes at room temperature, thus allowing the tissue debris and cell clumps to settle to the bottom and

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leaving the bulk of the tumor cells suspended as individuals in the supernatant liquid above, as microscopic examinations proved. The supernatant liquids were pipetted off and diluted with BGR so as to contain approximately 10,000 cells per c.mm.

Sera containing the specific antibody were procured on the day of the experiment or on the preceding day from Havana or chocolate-Dutch rabbits that were carrying Brown-Pearce carcinomas, or in which such growths had recently regressed (2, 3). The sera were stored at 4°C. until ready for use. Sera to be used as controls were procured at the same time from normal rabbits of identical breed and were stored similarly. Tests for the specific antibody were regularly made on samples of the sera by means of the standardized complement fixation test previously described (1, 2); the complement fixation titer of each serum was recorded in terms of the highest dilution of it giving complete or almost complete fixation of 2 units of complement in mixture with a standard quantity of antigen comprised of a saline extract of frozen Brown-Pearce tumor tissue in a dilution of 1:40.

For the cytological and viability studies, equal parts of cell suspension and serum were mixed in 15 cc. conical tip centrifuge tubes and incubated in the waterbath at 37°C. The viability of the cells was tested by implanting 1 cc. of a given mixture into various muscle situations in each of several test rabbits and measuring the resulting growths by palpation at frequent intervals thereafter. The structural changes were studied in fresh cells utilizing ordinary and phase contrast microscopes, and in fixed and stained preparations by means of a special technique now to be described.

Four cc. of the mixture was spun at about 400 × gravity for 5 minutes, with result that a pellet approximately 0.3 cc. in volume was deposited in the conical tip of the tube. This contained virtually all the cells, now loosely packed together. The supernatant liquid, usually quite opalescent but devoid of cells, as the microscope showed, was decanted and replaced by the desired fixative, which was allowed to run gently down the side of the tube from a pipette. This procedure usually resulted in the spontaneous detachment of the pellet, though occasionally this had to be removed with the aid of a thin wire. Fixation was allowed to proceed as for a small piece of soft tissue 1 to 2 mm. thick, viz., during 3 to 6 hours when Zenker acetic fixative was employed. The fixed pellet was then imbedded in paraffin or in a mixture of celloidin and paraffin, sectioned, and stained as usual. 3 per cent Zenker acetic fixative was used as routine throughout the investigation, although Carnoy's, Bouin's, and Bensley's fixatives were also used at one time or other. Sections were cut at 4 μ for routine study, sometimes at 2 μ for special purposes. For comparison, the control and test preparations were often mounted and stained side by side on the same slide. Eosin-methylene-blue stain was used as routine, but hematoxylin-eosin, aniline fuchsin–methyl green, and Heidenhain's iron-hematoxylin were also used as indicated.

The bulk of the cells in the histological preparations made from control mixtures—i.e. those containing BGR or normal rabbit serum—and studied prior to incubation, were "normal" Brown-Pearce carcinoma cells, the cytological characteristics of which will be described in detail further on. Regularly, however, there were in each preparation some extraneous elements also—erythrocytes and, rarely, a leucocyte or connective tissue cell, and sometimes small bits of muscle. These extraneous elements were of course present also in the pellets made from the experimental mixtures containing immune rabbit serum. No further mention will be made of them. In addition a small proportion of the carcinoma cells in the control and experimental mixtures exhibited coagulated acidophilic cytoplasm and pyknotic or karyorrhexic nuclei, while others had varying amounts of pale staining cytoplasm together with nuclei having intact membranes, clear sap, and a very little stainable chromatin present in the form of extremely fine granules. The cells of both types differed greatly from the normal "healthy" Brown-Pearce carcinoma cells; they were identical with the dead or degenerating cells to be found in the more central and necrotic parts of nearly all good sized Brown-Pearce carcinomas in situations obviously devoid of adequate blood supply. These altered tumor cells will likewise be ignored in the descriptions that follow.

Character of the Structural Changes

In previous experiments Brown-Pearce carcinoma cells that had remained 2 hours or longer in contact with sera containing the specific antibody remained visible and unagglutinated, as examinations of fresh preparations with the ordinary microscope showed; indeed under such circumstances the cells during long periods of observation appeared essentially like those in the control mixtures, though their viability was promptly lost (4). Much the same proved true when the mixtures were spun lightly in the centrifuge with subsequent fixation, embedding, and staining by means of routine procedures of the pellets of sedimented cells. Under these conditions the cytoplasm of the cells was largely destroyed, owing to the methods employed, but the nuclei appeared essentially intact and unchanged in the mixtures containing normal sera and in those containing immune sera as well. More refined cytological methods, however, have recently made it plain that under suitable conditions fresh rabbit sera containing the specific antibody will regularly induce striking structural changes in the cytoplasm of Brown-Pearce carcinoma cells, while the sera of normal rabbits, tested concurrently, have no such effect.

Figs. 1 and 2 illustrate the characteristic structural changes brought about by the antibody as they may be viewed with the ordinary microscope in fixed and stained preparations. These will now be described in detail.

The Brown-Pearce carcinoma cells of Fig. 1 had stood in contact with the fresh serum of a normal Havana rabbit during 2 hours at 37°C. before being prepared for microscopic examination as already described. The cells, it may be observed, remained structurally intact. In every instance their cytoplasm was deeply basophilic, and visible within it were numerous closely packed, clear, starch-like granules of approximately uniform size, each surrounded by a thin rim of basophilic material—the cytochondria of Opie (6). The contours of the untreated cells were regularly sharp, yet no distinct plasma membranes were visible at their margins. The nuclei of the unchanged cells were large, occupying at least half of the cell volume; as a rule they were spherical though sometimes flattened on one side or ovoid and occasionally irregularly serrated or indented; they were sharply demarcated from the surrounding cytoplasm by a very thin nuclear membrane, which sometimes remained visible in part during mitosis; the nuclear sap was transparent while the chromatin in the fixed and stained preparations seemed generally scanty, being distributed unevenly throughout the sap as small clumps, strands, and granules of darkly stained material; the nucleoli by contrast were regularly large and conspicuous, usually one in each nucleus, more or less centrally placed, and darkly stained in the methylene blue and eosin preparations.

Fig. 2 shows Brown-Pearce cells from the same suspension as that providing the cells of Fig. 1, though here the cells had been incubated 2 hours with fresh rabbit serum that contained much specific antibody (titer 1:256, as determined by complement fixation), with histological processing afterwards. It becomes obvious at a glance that the cells exposed to the antibody-containing serum (Fig. 2) were very different from those exposed concurrently to the normal serum (Fig. 1). The antibody-treated cells were readily identifiable as altered cells, however, and they were apparently as numerous as were their fellows that had been exposed under comparable conditions to the normal serum; furthermore they were comparably arranged as individuals and in loosely packed clumps in the microscopic fields, as careful examination of the entire histological preparations showed. The cytoplasm of the antibody-treated cells, however, was swollen, so that each cell seemed perhaps a third larger than normal—a

fact that will be referred to again further on; but other alterations were even more conspicuous. For now the cytoplasm had almost completely lost its ability to take the basophilic stain and it was in addition largely disorganized; indeed in every cell great irregular structureless spaces now occupied much of the cytoplasmic space, while the remains of the protoplasm, now obviously diminished in amount and greatly changed in character, lay irregularly distributed in the structureless spaces as larger conglomerates and smaller clumps of more or less eosinophilic and amorphous matter. Within the latter, however, the cytochondria could still be made out, some greatly swollen, and often these granular or swollen vesicular bodies were identifiable as discrete entities randomly arranged in the structureless spaces, and not infrequently outside the cells also, apparently floating free in the liquid medium. The plasma membranes—hardly visible, it will be recalled, in the cells treated with normal serum—appeared in the antibody-treated cells as rather thick irregular more or less acidophilic rims to which the particulate bodies often adhered; although usually intact, these were occasionally rent in the fixed and stained preparations. The nuclei of the antibody-treated cells were generally shrunken to about half or two-thirds the size of those of the unchanged cells, and the membranes about them appeared thickened, wrinkled, and darkly stained, perhaps owing in part at least to marginated chromatin; the nuclear sap, however, remained transparent. The most conspicuous change within the nuclei of the antibody-treated cells, however, was that exhibited by the nucleoli. For these elements, regularly large and conspicuous in the nuclei of the control cells, were generally small and inconspicuous in, or even absent from, the nuclei of the antibody-treated cells—a finding that will be referred to again further on.

Figs. 3 and 4 illustrate the fact that the structural changes began to appear almost immediately following contact between the carcinoma cells and sera containing the specific antibody, and that these progressed rapidly, so that within 15 minutes extreme alterations such as those described above were regularly manifest.

After only 5 minutes' contact with the antibody-containing serum the cytoplasm of the carcinoma cells was already swollen and irregularly vacuolated (Fig. 3) with vesicular bodies (swollen cytochondria) present here and there in it; furthermore the plasma membrane had by this time become visible while the ability of the cytoplasm to take the basic dye had already been largely lost. Within the cytoplasm the cytochondria were generally swollen, some of them enormously so, though the irregular structureless spaces were not yet manifest. The nuclei were now perhaps very slightly shrunken, and their membranes were more prominent than was the case in their normal counterparts; within many of them the nucleoli had become inconspicuous, while the chromatin was already aggregated and beginning to collect on or near the nuclear membrane, the sap meanwhile remaining quite transparent. The cytoplasmic changes in dividing cells after 5 minutes' contact with antibody-containing sera were identical with those just described, though the nuclear alterations were quite distinctive: the dividing chromosomes instead of remaining clumped in patterns and suspended in clear nuclear sap (see Fig. 1 for example), seemed to fuse together with the other contents of the dividing nucleus into a more or less homogeneous spherical mass which stained very deeply with the basic dyes and remained sharply demarcated from the changed cytoplasm without being obviously encompassed by a nuclear membrane, the changes as a whole giving the structure a "pyknotic" appearance.

After 15 minutes' contact, the antibody-treated cells displayed even more marked changes (Fig. 4). By this time the irregular structureless spaces were conspicuous in many of the cells, while the cytoplasmic and nuclear changes already described were also manifest. Again the nuclei of dividing cells exhibited the curious "pyknotic" change previously mentioned.

Longer periods of incubation did not bring about additional cytological changes. Thus after 4 and 24 hours' contact with antibody-containing sera the carcinoma cells in several preparations were quite similar in appearance to those seen after 2 hours' exposure (Fig. 2).

More refined histological preparations provided additional information about the cytoplasmic particles and nuclei of the antibody-treated cells.

The pellets from several experiments were fixed in Bensley's fixative and embedded in celloidin and paraffin (7) in order to provide optimal conditions for studying the cytoplasmic particles; sections cut at 2 μ were then stained with Altmann's aniline-fuchsin in mixture with methyl green, as utilized by Cowdry (8). The cells that had been incubated with normal serum displayed in their cytoplasm numerous spherical bodies each measuring about 0.5 to 1.0 μ in diameter and staining red with the aniline-fuchsin; they were usually discrete and crowded closely together, most densely next the nucleus and usually not in contact with the plasma membrane; thus they corresponded essentially with the cytoplasmic particles generally labelled mitochondria, though Opie's designation—cytochondria (6)—seems preferable and is here employed.

In the antibody-treated cells, the bulk of the cytoplasmic bodies rapidly became swollen and vesicular and they promptly lost the ability to take the red stain, though some of them retained this in part for a while, appearing briefly as vesicles having red-stained rims or crescents and not infrequently more densely stained polar bodies as well. After 15 to 30 minutes, however, most of the swollen vesicles failed to take the red stain at all. But this was not so in every instance. Indeed many of the antibody-treated cells still displayed a few to a dozen or more brilliantly stained red particles that were unchanged in form or only slightly swollen. Even after 4 to 24 hours the cells often exhibited red-stained particles that were entirely normal in form and staining affinity, and these were approximately as numerous as in cells exposed to the antibody and complement for only brief periods.

In the Bensley-fixed, Altmann-stained preparations, the fact was abundantly confirmed that the nuclei of Brown-Pearce carcinoma cells remained relatively unchanged during contact with fresh immune serum for periods up to 24 hours; for in these preparations the nuclei were exceptionally well shown. The nuclear membranes remained unrent and often unwrinkled in cells exposed to immune serum during 4 and 24 hours, while within them the sap stayed clear and the chromatin remained largely unchanged in form and staining properties though often displaying slight clumping and margination; the nucleoli, however, were either inconspicuous or absent, as already stated.

Samples of the mixtures supplying pellets for histological studies, as described above, were also studied as wet preparations under the phase contrast microscope. The findings confirmed many of the observations already recited and provided additional information as well.

The rapid swelling of the carcinoma cells in the mixtures containing fresh immune sera was especially conspicuous under the phase contrast instrument; the cells not only increased perhaps a third again in size after a few minutes' contact, but their shape changed, becoming spherical rather than polygonal. It was obvious too from scrtuiny of the wet preparations that the cells as individuals were neither lysed nor agglutinated in the immune serum mixtures, for even after prolonged periods of incubation—4 and 24 hours at 37°C., for example—the antibody-treated cells in each mixture were approximately as numerous as they had been at the beginning of the experiment. Furthermore, throughout the periods of observation up to 24 hours the plasma membranes remained readily visible as thin structures which were

unbroken but apparently stretched tight about the swollen cytoplasm. It was especially noteworthy that "blisters" did not form on the plasma membranes of the antibody-treated cells though they frequently did so on the surfaces of Brown-Pearce cells kept 30 minutes or longer in the mixtures with normal rabbit serum, the finding confirming observations previously made in this laboratory (9).

The cytoplasmic particles of Brown-Pearce carcinoma cells suspended in normal serum appeared under the phase contrast microscope as numerous minute spherical or rod-like bodies often displaying polar condensations and usually distributed more or less regularly throughout the cytoplasm; most of them were motionless, though a few, especially those near "blisters" (9), showed Brownian movement. In the carcinoma cells incubated 30 minutes or more in mixture with fresh immune sera, the cytoplasmic bodies were reduced in number, and many of those remaining were swollen and vesicular, sometimes with conspicuous polar condensations; these were scattered irregularly in the fluid that distended the cytoplasmic spaces, or were loosely adherent to the plasma membranes, and they all displayed more or less active Brownian movement. Not a few cytoplasmic particles, however, remained unchanged in form and size in the markedly altered cells; these were usually seen in close proximity to the nucleus, often in a small cluster, and they did not move.

The nuclei of the antibody-treated cells were regularly spherical and usually placed eccentrically. The nuclear membranes were more refractile than was the case in the cells suspended in normal serum, but the nuclear sap always remained transparent even after many hours of incubation. The chromatin was seen indistinctly in the nuclei of cells exposed to normal serum, appearing as a faint irregular meshwork or as small faint condensations; it was not notably different in the cells of immune serum mixtures, though perhaps to some extent clumped and marginated, with result in the increased refractivity of the nuclear membrane, ` as already mentioned. The nucleoli of the carcinoma cells in normal serum were conspicuous under the phase contrast microscope as large dense irregularly spherical condensations; those of the antibody-treated cells were one-half to one-third as large, but otherwise they appeared similar.

A total of 34 antibody-containing sera were employed in the work, and 28 normal rabbit sera. Every one of the antibody-containing sera brought about the structural changes in Brown-Pearce carcinoma cells when held in contact with them under the specified conditions, while none of the control sera did so. The implications of some of the observations will be considered in the discussion. Meanwhile attention will be given to several of the conditions necessary for development of the structural changes.

The Role of Complement

Since complement is required for the lysis of bacterial cells and erythrocytes by antibodies, it seemed essential to determine whether it likewise acts in concert with the specific antibody in bringing about the structural changes here observed. Hence the following experiments.

A suspension of Brown-Pearce carcinoma cells, prepared as already described, was mixed in equal parts with each of the following: (a) 2.0 cc. of fresh rabbit serum containing the specific antibody (complement fixation titer 1:256); (b) 2.0 cc. of the same serum specimen heated at 56°C. for 30 minutes and then cooled to room temperature; (c) 1.6 cc. of the inactivated serum employed in (b), to which 0.4 cc. of fresh guinea pig serum was added; (d) 1.6 cc. of BGR, to which 0.4 cc. of fresh guinea pig serum was added. The mixtures were incubated 2 hours at 37°C. and then prepared for microscopic study as already described.

Virtually all the cells of mixtures (*a*) and (*c*) exhibited structural changes similar to those illustrated in Fig. 2, while the cells of mixtures (*b*) and (*d*) remained unchanged and like those of Fig. 1.

Several experiments of similar sort gave identical results, while additional tests showed that sera that had stood for weeks or months in the refrigerator at 4°C., although containing the specific antibody in high titer, as complement fixation tests showed, regularly failed *per se* to induce the structural changes when incubated 2 hours at 37°C. with the Brown-Pearce carcinoma cells, though they always did so when fresh guinea pig or rabbit complement was added.

In still other experiments, numerous samples of fresh and frozen guinea pig serum (pooled specimens from several animals) were used; all had high titers of complement, as subsidiary tests showed, and all failed to induce any structural change when the undiluted specimens were mixed in equal parts with suspensions of Brown-Pearce carcinoma cells and incubated therewith as usual. The same proved true of numerous specimens of fresh normal rabbit serum.

The findings showed plainly that the structural changes did not become manifest in Brown-Pearce carcinoma cells exposed to sera containing high titers of the specific antibody unless complement was also present, the latter alone being likewise ineffective.

Specificity of the Phenomenon

Will rabbit sera devoid of the specific Brown-Pearce antibody induce the structural changes in Brown-Pearce carcinoma cells?

Serum specimens were procured from 8 hybrid rabbits (English spotted, agouti, and chinchilla breeds) that were either carrying Brown-Pearce tumors or had recently overcome them; these all failed to manifest the specific Brown-Pearce antibody in complement fixation tests, the findings conforming to previous experience (1, 4). Without exception they also failed to induce the structural changes in Brown-Pearce carcinoma cells when held in contact with them under the conditions already described, while sera containing the specific antibody regularly brought about the characteristic changes in concurrent tests. The same proved true with serum specimens procured from 5 rabbits carrying the transplanted Vx2 carcinoma (10); these specimens likewise contained none of the specific Brown-Pearce antibody though they often contained high titers of antibodies with affinities for other sedimentable constituents of various normal and neoplastic cells (11, 12).

In the tests just referred to, rabbit sera devoid of the specific Brown-Pearce antibody—whether procured from rabbits that had overcome implanted Brown-Pearce carcinoma cells without developing the specific antibody, or from rabbits carrying the transplanted Vx2 carcinoma—all regularly failed to induce the characteristic structural changes in Brown-Pearce carcinoma cells when tested under the standard conditions. Several experiments were next made to learn whether rabbit sera containing the specific Brown-Pearce antibody will bring about structural changes in the cells of normal rabbit tissues, or in those of another rabbit neoplasm.

In 5 experiments, different specimens of fresh whole rabbit sera containing the specific antibody in high titer, and others procured from normal rabbits, were mixed in equal parts with suspensions of Vx2 carcinoma cells, normal rabbit lymphocytes, normal rabbit liver

cells, and normal rabbit kidney cells, and with Brown-Pearce carcinoma cells, all prepared as previously described. Following incubation at 37°C. for 2 hours, pellets were prepared in the usual way for cytological study.

The preparations comprised of Vx2 carcinoma, normal rabbit liver, and normal rabbit kidney cells were to some extent unsatisfactory for cytological purposes because they always contained a large proportion of clumped and otherwise altered cells, irrespective of whether the cells had been incubated in BGR, in normal rabbit serum, or in antibody-containing serum. Even so, scrutiny of the stained preparations showed plainly that the Vx2 carcinoma cells that had been exposed *in vitro* to sera containing the specific Brown-Pearce antibody were essentially like those that had been held in contact with the normal rabbit sera, and the same was true of the cells of normal rabbit liver, kidney, and lymph nodes; in particular, none of the cells mentioned displayed structural changes like those regularly exhibited by Brown-Pearce carcinoma cells following contact with the antibody-containing sera.

The question whether structural changes can be induced in normal or neoplastic rabbit cells by means of antibodies induced in other species by various rabbit tissues remains to be studied.

Other Factors Influencing Development of the Structural Changes

The Influence of Local Conditions.—As already stated, the structural changes described heretofore were regularly displayed by Brown-Pearce carcinoma cells suspended as individuals in mixtures containing the specific antibody and complement. In several experiments, however, small bits of the tumor tissue were included inadvertently in the mixtures; the bulk of the cells contained in these bits of tissue usually remained unchanged even following long contact with the specific antibody and complement and while the individually suspended cells in the same mixtures manifested the characteristic changes already described. To study this phenomenon further, the following experiment was done.

"Healthy" Brown-Pearce carcinoma tissue, freshly procured from a rabbit that had been implanted 9 days before, was cut into slices 1 to 2 mm. thick and with a surface area of about 2 × 4 mm. For control purposes, two of the slices were dropped into 3 per cent Zenker acetic fixative. Two others were put into freshly procured rabbit serum, and two into freshly procured serum containing the specific antibody (complement fixation titer 1:256). For further control purposes, a cell suspension was made from other parts of the same tumor, and 2 aliquots of this were mixed in equal parts with the normal and immune sera respectively. The four serum preparations were incubated 2 hours at 37°C., then prepared for histological study.

The slices that had been incubated with normal serum contained Brown-Pearce carcinoma cells that for the most part were entirely normal; a few of those at the periphery of the blocks, however, had become more or less detached, perhaps owing to the trauma of cutting, and some of these displayed a slight to moderate loss of cytoplasmic basophilism with or without early pyknotic changes of their nuclei. The slices that had been incubated in the antibody-containing serum were in general similar in histological appearance to those that had been incubated in the normal serum, though they differed in one significant respect. For while the carcinoma cells *in situ* within the bits of tissue were entirely normal in arrangement, structure, and staining properties, a few of those that had become more or less completely detached from the periphery displayed the swelling and vacuolation of the cytoplasm already

described as characterizing the effects of the antibody. These changes were likewise exhibited by practically all the cells in the pellets made from the mixture containing immune serum and individually suspended cells, though the pellets made from the normal serum-cell suspension mixture contained unchanged cells for the most part.

The findings made it plain that carcinoma cells *in situ* in excised tumor tissue were not acted upon by the antibody and complement, while those suspended as individuals in a liquid milieu containing the active immunological reagents were regularly and markedly changed. The precise meaning of this interesting fact remains undisclosed.

Refractory Cells.—The cytological changes were manifest in virtually every individually suspended cell in the suspensions suitably exposed to the antibody and complement in the experiments already given. In the pellets made from each of these suspensions, however, a few discrete tumor cells were regularly seen, scattered at random amidst the great number of altered cells, which were structurally intact and indistinguishable from those that had been concurrently suspended in normal serum or saline. The proportion of these unchanged cells in various tests was regularly approximately 0.5 per cent; furthermore, the refractory cells were often seen, and in approximately the same proportions, in antibody-containing mixtures that had been held 24 hours or longer at 37°C. The essential finding will now be described in detail; its implications will be mentioned in the discussion.

In two experiments Brown-Pearce carcinoma cells were mixed in separate tubes with freshly procured normal and antibody-containing serum, and the mixtures were incubated at 37°C. for 1 and 2 days respectively, while in a third experiment similar mixtures were left at room temperature for 6 days, an aseptic technique being utilized throughout so as to avoid bacterial contamination. Pellets of each mixture were prepared in the usual way and studied histologically. In the immune serum preparation kept 24 hours at 37°C., the unchanged cells were found in the proportion stated above; furthermore there was no decrease in the proportion of the refractory cells following 48 hours' incubation. Indeed, after 6 days' contact at room temperature there were about as many refractory cells as in pellets of the same mixture prepared on the 1st day, though these cells had undergone autolytic changes which were identical in nature with those manifested by the carcinoma cells autolyzing in the normal serum control mixtures, as will be brought out in the next section.

Attempts to Reproduce the Structural Changes by Other Means

A number of experiments were made to learn whether the exposure of Brown-Pearce carcinoma cells *in vitro* to other agents (bacterial toxins, various enzymes, surface active agents, hypotonic solutions, for example) would induce in them structural changes like those brought about by means of the antibody and complement. The results were interesting and they will here be briefly described, though it may be stated categorically that in no instance were changes induced which resembled those effected by the antibody and complement.

Perfringens toxin, procured from the research division of the Lederle Laboratories and known to contain both lecithinase and proteolytic enzymes, after 30 to 60 minutes' contact with initially living, individually suspended Brown-Pearce carcinoma cells brought about in them a diminution of cytoplasmic basophilism together with some irregular cytoplasmic vacuolation; furthermore, the cytoplasmic bodies of such cells failed to stain with aniline fuchsin or with Heidenhain's iron-hematoxylin, though their nuclei displayed clear sap and unaltered chromatin network, and indeed seemed unchanged except for the nucleoli, which were swollen, sharply contoured, and darkly stained, and sometimes surrounded by a transparent "halo." After 2 to 4 hours' exposure to the perfringens toxin at 37°C., however, the carcinoma cells uniformly presented the appearance of coagulation necrosis, with swollen, granular, brightly pink cytoplasm and homogeneous slightly shrunken and darkly stained "pyknotic" nuclei, which occasionally displayed beginning karyorrhexis—their appearance as a whole being quite similar to that of Brown-Pearce carcinoma cells in suspensions that had been heated at 56° for a few minutes *in vitro,* while these in turn were similar to the cells which had died in the center of large Brown-Pearce tumors *in vivo* as result of insufficient blood supply.

The effects with *ribonuclease* were also interesting. A crystalline preparation, procured from the Schwarz Laboratories Inc., New York City, was used in a concentration of 200 μg./cc. After 15 minutes' contact at 25°C. the carcinoma cells did not differ from those in the control BGR suspension. After 2 hours at 37°C., however, about half of the cells had lost their cytoplasmic and nucleolar basophilism, the rest being wholly unchanged; following 5 hours' contact with the enzymes still more of the carcinoma cells displayed the loss of cytoplasmic and nucleolar basophilism, though 10 to 20 per cent nevertheless remained unchanged—the findings suggesting that the cells remaining viable during the prolonged incubation were unaffected by the enzyme while those that had been damaged during handling or had succumbed during the long sojourn at 37°C. were acted upon by it in characteristic fashion.

In similar studies, *lysozyme* and *hyaluronidase* (in final concentrations of 500 μg./cc.), and salt-free *trypsin* and crystalline *chymotrypsin* (in final concentrations of 20 mg./cc.) failed to induce visible changes in the bulk of the carcinoma cells exposed to the respective agents following incubation with them for 2 hours at 37°C.

Likewise, the *surface active agents* zephiran, sodium desoxycholate, tween 80, triton A-20, and tyrocidin, in concentrations up to 500 μg./cc., did not bring about any structural alterations in the majority of the Brown-Pearce carcinoma cells with which they were held in contact for periods of 2 hours at 37°C.

Brown-Pearce carcinoma cells kept long in buffered glucose-Ringer's solution, and those placed in hypotonic saline solutions—for example when a suspension of the carcinoma cells in BGR was mixed in equal parts with distilled water—exhibited changes such as those previously described by others (6, 9); in general these were characterized by a vesicular swelling of the cytoplasm together with the formation of numerous blebs or "blisters" on the plasma membranes (9) and a loss of cytoplasmic basophilism except for that at the rims of the cytochondria; it was obvious that the changes in no way resembled those induced by means of the antibody and complement.

Relationship of Structural Changes to Viability

In previous work, Brown-Pearce carcinoma cells that had been incubated in mixture with sera containing the specific antibody for 2 hours at 37°C. regularly failed to grow upon implantation in the muscles of susceptible test rabbits, whereas carcinoma cells from the same suspensions which had been incubated with normal rabbit serum grew readily in the same hosts (4). It was further shown in the previous work that the specific antibody exerts its antiprolifer-

ative effect on tumor cells exposed to it *in vitro* in the absence of complement, though, as formerly suggested, complement may have been supplied by the host (4). Viability studies carried out during the course of the present investigation made it clear that the carcinoma cells incubated with the specific antibody and complement regularly lost their viability within a few minutes and quite as promptly as they began to manifest structural changes.

In three experiments, three samples of fresh sera, procured from Havana rabbits in which Brown-Pearce carcinomas had recently regressed and containing the specific antibody in high titer, were held in contact with individually suspended Brown-Pearce carcinoma cells; in each experiment 2 aliquots of the cell suspension were used, one being kept 15 minutes at room temperature, the other 2 hours at 37°C. For control purposes samples of the same tumor cell suspensions were concurrently incubated with freshly procured normal rabbit serum, and with BGR. The cells of the various mixtures were then studied for structural changes as already described, and for viability by injecting 1 cc. of each mixture intramuscularly into three test rabbits in each experiment, with palpation of the injection sites at regular intervals thereafter.

In every instance the cells exposed to the immune sera and complement displayed the marked and characteristic structural changes, while those held in contact with the normal sera and BGR remained unaltered. Furthermore, the viability studies showed that the cells held in contact with the immune sera and complement had rapidly lost their viability, while the cells kept in BGR and in the normal serum-complement mixture had retained this. For example, the six mixtures containing cells exposed to normal serum and complement (three kept at room temperature for 15 minutes and three kept at 37°C. for 2 hours) were each implanted in one situation in each of three test rabbits; palpable tumors measuring 2.0 to 4.0 cm. across were present at every implantation site 9 days later, and these had all enlarged to measure 4.0 to 7.0 cm. across on the 16th day, there being no difference in the growths resulting from the mixtures kept under the two conditions. Palpable growths of comparable sizes likewise appeared at all the sites where cells exposed to BGR were implanted. By contrast the 18 sites (in nine test rabbits) implanted with the six mixtures containing cells plus the immune sera and complement all remained negative with one exception; in this instance a mixture of cells, immune serum, and complement that had been kept 15 minutes at room temperature gave rise to a tumor 1.0 cm. across which appeared on the 16th day in the most susceptible of the three test rabbits into which it had been implanted. It is interesting to speculate whether the exceptional tumor may have originated from the chance implantation in some favorable local situation in a specially susceptible host of a small fragment of tumor tissue in which the cells were unchanged by the antibody and complement, as already mentioned, or from the similar implantation of a number of individually suspended cells that were refractory to the effects of antibody and complement, or from some factor as yet undiscerned.

DISCUSSION

The structural changes here described were regularly induced in Brown-Pearce rabbit carcinoma cells by means of a specific antibody acting in conjunction with complement. While their nature and significance cannot be precisely defined at present the findings perhaps deserve some analysis and comment.

It seems noteworthy initially that the antibody employed is highly specific. It develops regularly in certain rabbits of suitable breeds following implanta-

tion with the Brown-Pearce rabbit carcinoma cells, and also following the injection into them of sedimentable particles extractable from the carcinoma cells (1–3). Thus implantations or injections of rabbit tissues into animals of alien species are not required for its production, while furthermore the antibody does not appear in the blood of rabbits implanted with rabbit tumor cells of other sorts, and it is not stimulated by injecting into rabbits sedimentable particles derived from other rabbit tissues. Moreover, the antibody, while regularly fixing complement in high titer in mixture with saline extracts containing cytoplasmic particles of Brown-Pearce carcinoma cells, does not react at all with extracts of other rabbit tissues, either normal or neoplastic, as much work has shown (1, 2, 4).

The structural changes seem to be unique. Mention has already been made of the fact that they were not duplicated by any of a number of means tried experimentally in the present work. Furthermore, they differ from the cellular changes manifested by Brown-Pearce carcinoma cells as these die from anoxia *in vivo*, many observations having shown that under such circumstances the cells display either a coagulation necrosis of ordinary sort, with granular acidophilic cytoplasm and pyknotic nuclei or a slowly progressive loss of cytoplasmic basophilism together with nuclear chromatolysis; and they differ also from the changes displayed by these carcinoma cells as they are overcome in resistant hosts, as has become clear from observations made recently in this laboratory and soon to be reported in another paper.

Many studies have been made of the effects on bacteria and erythrocytes of specific antibodies acting in conjunction with complement, while the effects of these immunological reagents upon certain protozoa and upon chick embryos have also been studied, though not extensively (13–15). Antibodies and complement have not been utilized in cytological studies of normal mammalian cells, though a number of workers have concerned themselves with the effects of antibodies on mammalian tumor cells (16); Lumsden *et al.* excepted, however, no one has heretofore looked for structural changes in tumor cells exposed to immunological reagents. The work of Lumsden and his associates was primarily concerned with the "cytotoxic" effects of heterologous "anti-cancer" antibodies acting in conjunction with other factors upon the cells of various transplantable neoplasms (17); in a concluding paper, however, Lumsden and his coworkers demonstrated that the serum of rats hyperimmunized with the cells of the Jensen rat sarcoma contained antibodies which, like those in the "heterologous anti-cancer serums," killed the cells of the Jensen rat sarcoma in tissue cultures *in vitro* within 10 to 20 minutes. Their description of the cellular changes induced under these conditions is brief and hence may be quoted in full: "The dying cells appear rounded and ragged in outline, and their fatty and protoplasmic granules become agglutinated. Their nuclei, previously invisible in unstained preparations, shrink and become sharply defined. Careful focussing not infrequently reveals the extremely thin cell wall distended by endosmosis away from the coarsely granular cell contents. If stained after 4 to 8 hours with Ehrlich's acid haematoxylin the nuclei are found to be pyknotic, while if 24 hours are allowed to elapse before staining complete chro-

matolysis is seen to have taken place" (17). Even from this brief description, it becomes obvious that the structural changes observed by Lumsden *et al.* differ notably from those encountered in the present work.

The structural alterations developed rapidly, indeed almost explosively, when Brown-Pearce carcinoma cells were mixed with the specific antibody in the presence of complement, and loss of cellular viability promptly became manifest also. Yet several distinct events can be discerned in the process, and upon analysis these provide interesting implications both for cytology and immunology.

Perhaps the earliest change displayed by the Brown-Pearce carcinoma cell exposed to the specific antibody and complement was an alteration in its contour from polygonal to spherical owing to the rapid swelling of its cytoplasm. The entrance of considerable quantities of fluid could well account for this alteration, and also for the vesiculation or "ballooning" of the bulk of the cytoplasmic particles, and for the appearance soon thereafter of structureless spaces in the swollen cytoplasm. The state of the plasma membrane is noteworthy in this relation. For this structure was obviously altered in the antibody-treated cell: thus it became visible in the fixed and stained preparations of cells exposed to fresh immune sera as it was not in those concurrently exposed to fresh normal sera, though this may have resulted more from alterations within the cytoplasm of the treated cells than from changes within their plasma membranes; what was more surely indicative of an alteration in the membrane, however, was the fact that "blisters" did not form upon it as they frequently did on the plasma membranes of viable Brown-Pearce cells sojourning in control fluids, the findings confirming and extending observations on the nature of the plasma membrane previously made in this laboratory by means of the phase contrast microscope (9). Yet the plasma membranes of the treated cells were not lysed, as scrutiny of the wet preparations showed; instead they remained visible and unruptured during many hours' contact with the antibody and complement, appearing almost from the beginning as membranes stretched thin about the greatly swollen cytoplasmic masses. The fact that the treated cells did not become agglutinated also has implications for the state of their plasma membranes (27).

The findings just recited speak in favor of the possibility that large quantities of fluid promptly entered the cells exposed to antibody and complement. Yet there was obviously more to the process. For in control experiments Brown-Pearce carcinoma cells placed in water and in hypotonic saline solutions, while manifesting after a time swelling of the cytoplasm and particularly of its cytochondria, together with loss of cytoplasmic basophilism similar to that described by Opie and presumably due to the imbibition of water (6), did not exhibit structural changes like those displayed by the cells exposed to the specific antibody and complement. The changes in the latter were much more explosive, and much more extensive also; furthermore they appeared to differ in kind even more than in degree. For they were characterized not only by swelling but also by disruption and disorganization of the bulk of the formed elements of the cytoplasm, with obvious dissolution of many of them, so that what had

been in the normal cell intact cytoplasm packed with numerous and more or less regularly distributed starch-like granules, became after a brief interval in the treated cell a swollen mass, largely comprised of fluid, in which could be seen irregular clumps of acidophilic granular debris—for the most part, the remains, one would say, of disrupted and irregularly conglutinated cytochondria—while about these masses discrete and often greatly swollen and vesicular cytoplasmic bodies lay free in the excess of cytoplasmic fluid while others adhered to the plasma membrane and still others to the nuclear membrane.

Another of the structural changes displayed by the carcinoma cells almost immediately after contact with the specific antibody and complement was a rapid and virtually complete loss of cytoplasmic basophilism. This was reminiscent of the change brought about by autolytic enzymes or by ribonuclease acting upon killed pneumococci and other bacteria, with loss of ribose nucleic acid and loss of ability to take the Gram stain in consequence (18, 19), though the two phenomena are by no means necessarily comparable. It is noteworthy in this relation, however, that in the present work *living* Brown-Pearce carcinoma cells were exposed to the action of the specific antibody and complement, which promptly induced in them the loss of cytoplasmic basophilism mentioned; in control tests, however, living Brown-Pearce cells exposed *in vitro* to ribonuclease in the concentrations customarily employed were wholly unaffected by it, only those cells presumably damaged or succumbing during handling or incubation displaying loss of cytoplasmic basophilism and this of a quite different sort. Still the finding suggests that one or another of the immunological reagents, or the two in concert, may rapidly alter the conditions within the cytoplasm so that ribose nucleic acid is promptly liberated from the structures with which it is normally associated. The finding becomes even more suggestive when the fact is recalled that the antibody reacts specifically *in vitro* with those components of Brown-Pearce carcinoma cells having a particle size and weight in the range of that of the microsomes (1, 4, 12); for these cytoplasmic particles are known to be comprised largely of ribose nucleoprotein (20, 21), and also to possess enzymes capable of assimilating amino acids (22).

When the structural sequences are viewed in the large it becomes quite plain that the nuclei of the antibody-treated cells were in general much less affected than was their cytoplasm. Indeed, the nuclei of the antibody-treated cells often remained essentially unchanged for long periods after the cytoplasm had become greatly swollen and its constituents largely disrupted; they did not take in fluid or display pyknotic changes, and their sap generally remained transparent for long periods of time while their chromatin persisted unaltered in form and staining properties except for some clumping and margination which was by no means regular or conspicuous. The fact that the nuclei of the treated cells remained relatively unaltered while their cytoplasm was conspicuously changed is not only striking but unusual, for the structural changes brought about *in vivo* and *in vitro* in Brown-Pearce carcinoma cells by means of a variety of injuries of other sorts (*e.g.*, anoxia, heat, bacterial toxins, etc.) almost regularly involved the nucleus as well as cytoplasm, while furthermore the alterations were generally stereotyped and comprised of an acidophilic coagulation of cytoplasm together with pyknosis or karyorrhexis of the nuclei. One interesting change was regu-

larly seen, however, in the nuclei of the antibody-treated cell: the nucleolus, which in the untreated cell is almost always large and conspicuous and darkly stained, in the treated cell usually shrank notably within a few minutes after the cell had been brought into contact with the immunological reagents. It seems not improbable that this rapid alteration in the nucleoli, which are known to contain ribose nucleoprotein (21), may be related to the rapid disappearance of the basophilic material from the cytoplasm of the treated cells, as already mentioned.

Although, as already stated, the meaning of the findings as a whole cannot be precisely defined at present, it is obvious that the structural changes have implications for cytology, and notably for those structures responsible for maintaining fluid balance in the cell and also for the integrity and structural relationships of the ground substance and the cytoplasmic particles. Furthermore they have implications for immunology, though again these cannot be precisely defined. Certain it is that the specific antibody and complement acted together or in sequence to bring about the structural changes, neither doing so alone. The manner in which the cells exposed to both antibody and complement almost instantaneously took in fluid and lost their cytoplasmic basophilism suggests strongly that enzymic action may have been involved, the observation recalling a possibility frequently considered hertofore on the basis of other data, namely that complement, or antibodies, or the two together, may function as enzymes (23, 24). The data at hand, however, do not allow one to decide upon this point, or upon the respective roles of the two immunological reagents in the present phenomenon. In this relation it should be stated, though, that while complement is involved in the *in vitro* reaction whereby the specific antibody is recognized (1, 2), other experiments made recently in this laboratory have shown that the antibody is readily absorbed *in vitro* by washed intact Brown-Pearce carcinoma cells, this reaction taking place in the absence of complement and without the development of visible alterations in the carcinoma cells (25). Could it be that the specific antibody alters the Brown-Pearce carcinoma cell in such a way that complement gains access to its cytoplasm and brings about the distinctive structural changes through enzymic action? Future work must tell.

Two observations made incidentally in the present work deserve mention as exemplifying the widely held principle that structural and functional units which seem to be identical in nature often differ in more or less subtle fashion from one another. These were that a small proportion of the cells seemed refractory to the action of the specific antibody and complement, and that a considerable proportion of the cytoplasmic particles also remained unchanged in form and staining properties following prolonged contact with the immunological reagents. It remains to be learned whether these differences have a relationship to one another, and also whether they are related to the recent observations of Opie on differences in the structure and staining properties of cytoplasmic particles (6), or to the interesting fact recently discovered

by Craigie, namely that a small proportion of the cells of certain transplantable growths differ from their fellows in structural characters, as defined by means of the phase contrast microscope, and also in their ability to withstand freezing and dessication under experimental conditions (26).

SUMMARY

Structural changes were regularly observed in Brown-Pearce rabbit carcinoma cells that had been brought into contact, in the presence of complement, with rabbit serum containing an antibody that reacts specifically with a distinctive sedimentable constituent of the carcinoma cells. The cellular changes appeared rapidly and were accompanied by an equally rapid loss of viability.

The structural changes, as disclosed by histological and cytological methods, including phase contrast microscopy, were described in detail and illustrated. Essentially they were characterized by the entrance of considerable quantities of fluid into the cell, together with swelling and disorganization of its cytoplasm, vesiculation and rupture of the bulk of its cytoplasmic particles, and rapid and virtually complete loss of cytoplasmic basophilism. The plasma membranes, though stretched thin about the swollen cytoplasm of the altered cells, and otherwise changed also, remained unruptured during many hours' observation. The nuclei of the altered cells, however, remained relatively unchanged, their membranes persisting unruptured and their sap remaining wholly transparent and undiminished in amount for long periods, while within it the chromatin retained its staining properties, though sometimes becoming clumped and marginated; by contrast, the nucleoli of the treated cells promptly shrank and lost much of their affinity for the basic dyes.

The experimental conditions under which the structural changes became manifest were given in detail, together with an analysis of the findings and brief mention of certain of their implications for cytology and immunology.

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EXPLANATION OF PLATE 6

The histological preparations were made by Miss Elizabeth Delano and the photographs by Mr. Julius Mesiar.

For a detailed description of the cytological altertions to be seen in the figures, see the text.

FIG. 1. Brown-Pearce carcinoma cells that had been suspended as individuals in buffered glucose-Ringer's solution and incubated 2 hours at 37°C. in mixture with fresh normal rabbit serum. The mixture was then centrifuged lightly, with fixation of the cell pellets in acid-Zenker fixative, followed by paraffin embedding, cutting, and staining with methylene blue and eosin. The arrow points to a cell in mitosis; so too in the figures that follow. ×1260.

FIG. 2. Brown-Pearce carcinoma cells, from the same suspension providing those of Fig. 1, here incubated 2 hours at 37°C. in mixture with fresh rabbit serum containing a high titer of the specific antibody. Histological preparation as above.

FIGS. 3 and 4. Brown-Pearce carcinoma cells and histological preparation as above. In Fig. 3 cells are shown which had been incubated 5 minutes at 37°C. with the immune serum. The cells of Fig. 4 had been held in contact with the immune serum for 15 minutes prior to fixation and histological preparation.
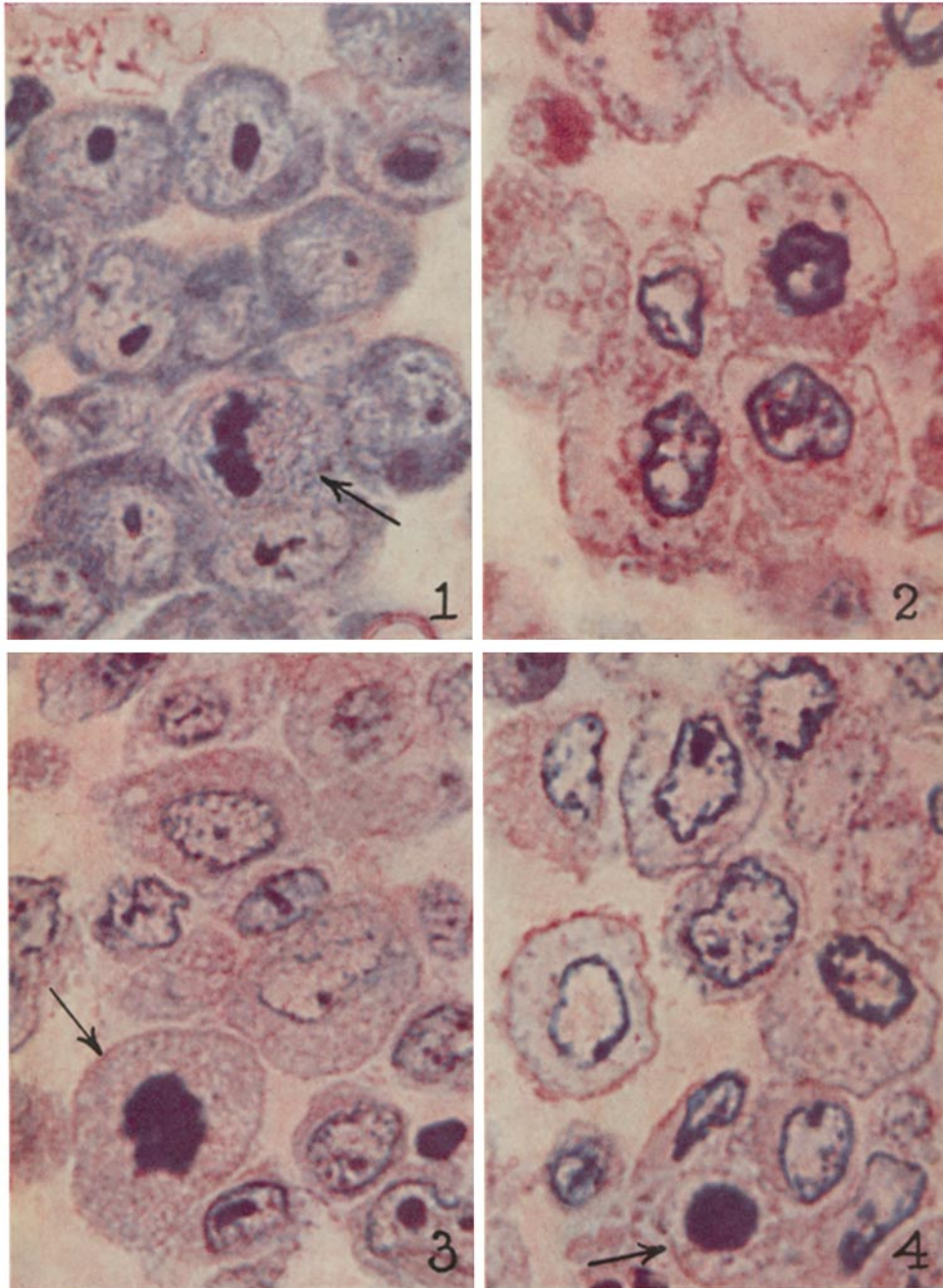
(Kalfayan and Kidd: Structural changes in carcinoma cells)