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Cytotoxicity and Cell Cycle Studied with a Combined Tetrazolium-Feulgen Reaction.* (31286)

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The response of mammalian cells to irritants such as chemicals, X-irradiation, virus infection, change in temperature, etc., has been found to vary in relation to certain phases of the cellular life cycle(1). When studying the effect of cytotoxic antibodies on cultivated mammalian cells we noticed that within a non-synchronized population paired daughter cells regularly showed the same degree of cytotoxicity whereas marked variability was observed between different pairs. This observation seemed to indicate that the effect of cytotoxic antibodies was likewise influenced by the cellular life cycle. Experiments to substantiate this assumption are described here.

As a more precise tool for morphological evaluation and quantitation of cytotoxicity, we developed a combined tetrazolium-Feulgen stain. This procedure makes it possible to observe simultaneously in the same cell specific nuclear as well as cytoplasmic changes. It is assumed that this staining procedure might have wider applicability. Therefore, a description of the technique is included in this paper.

Materials and methods. Cultured mammalian cells. Chang's human conjunctiva cells were used in these studies. The strain has been continuously maintained in our laboratory for the past 4 years on Eagle's medium without antibiotics supplemented with 17% newborn calf serum. Periodic examinations assured absence of contaminants, particularly mycoplasma.

Synchronization of cell cultures to a degree of 80% was achieved by employing the technique of Terasima and Tolmach(2) with minor modifications.

Preparation of cytotoxic antiserum. Monolayer cultures of Chang's conjunctiva cells were kept on serum-free medium for 24 hours prior to harvest. The monolayers were washed with balanced salt solution (BSS) and scraped from the culture vessel with a rubber scraper. The cell sediment was washed again repeatedly and homogenized in an ordinary culture tube by means of a high speed teflon pestle in the presence of alundum (60 mesh). The cell homogenate, an equivalent of three to five million cells per dose, was repeatedly injected intraperitoneally, subcutaneously and intramuscularly (with Freund's complete adjuvant) into guinea pigs over a period of up to 10 weeks. Sera were collected intermittently by heart puncture and inactivated at 56°C for 30 minutes before use.

Cytotoxicity tests. Coverslip cultures were prepared from synchronized and asynchronous cells. Synchronized cultures were sub-

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jected to cytotoxicity tests at hourly time intervals up to 30 hours after initially seeding mitotic cells onto the coverslips. Thus, a complete cellular life cycle with an additional 8-hour period was covered in these experiments.

The coverslip cultures were gently rinsed in BSS and overlaid with a 1:1:3 mixture of test (or control) serum, fresh guinea pig complement, and Eagle's medium. Controls included (a) guinea pig pre-immunization serum, (b) 20% newborn calf serum (without guinea pig complement). Incubation was carried out at 37°C in a moist chamber with a 5% CO_2-atmosphere. The optimum incubation time under the conditions of these experiments had been determined to be 90 minutes. Additional control coverslips were taken at the beginning of each cytotoxicity incubation period and immediately stained with May-Gruenwald-Giemsa. The cytotoxicity test coverslips were stained with May-Gruenwald-Giemsa and with the combined tetrazolium-Feulgen procedure.

Combined tetrazolium-Feulgen staining procedure. Rinse coverslip culture in warm BSS at pH 7.2. Incubate the unfixed living culture for 15 minutes at 37°C in BSS, pH 7.2, containing 0.05% Nitro-BT dye. Wash for about one minute in Carnoy's fluid; transfer to fresh Carnoy's fluid for at least 30 minutes. Rinse in absolute ethanol for 5 minutes. Hydrate through 95% and 70% ethanol. Rinse in distilled water. Incubate in 1 N HCl at 60°C for 8-10 minutes (glass coverslips) or for 10-12 minutes (plastic coverslips). Rinse in distilled water. Stain for 12 minutes in Schiff's reagent. Rinse for 2 minutes each in 3 changes of sulfite wash water (1% sodium metabisulfate in 0.1 N HCl). Wash in running water for 15 minutes and rinse in 70% ethanol. Counterstain with 0.01% fast green FCF in 95% ethanol for 1-3 minutes. Dehydrate in absolute ethanol and clear in xylene. Mount in a synthetic resin or Canada balsam.

The first part in this procedure is the endogenous tetrazolium reaction. The colorless water-soluble dye is reduced to dark insoluble "formazan" deposits by cytoplasmic oxidative enzymes which are predominantly confined to particles resembling mitochondria in size, shape, number, and distribution. Finer granules seem to indicate sites of extramitochondrial oxidative activity possibly in association with the endoplasmic reticulum. The Feulgen reaction facilitates the study of the nuclear morphology including mitotic stages and aberrations.

Results. Morphological and staining characteristics of asynchronous and synchronized Chang conjunctiva control cultures. In fully developed monolayer cultures no differences were seen between individual cells with regard to endogenous enzymatic activity. However, in young cultures with small isolated colonies or single cells such differences were pronounced between, although not within, colonies. This seemed to reflect a certain degree of cellular heterogenicity which corresponded well with other morphological characteristics. At least 2 cell types were distinguishable in Chang's conjunctiva cultures, the one type growing in more tightly packed colonies with stronger enzymatic activity than the other. When different colony types expanded towards each other and began to merge very soon the same level of high enzymatic activity was reached in every cell of the colony complex. (This phenomenon was particularly pronounced when we mixed Chang's conjunctiva cells with mouse L-cells. The latter are almost completely void of endogenous enzymatic activity when grown alone under the cultural conditions of this experiment. But in mixture with conjunctiva cells they readily change to high activity as soon as contact is made between single cells or colonies.) The explanation probably lies in the fact that each colony creates an individual nutritional microenvironment in the rather stagnant layer of medium covering the cells. Colony contact then seems to mediate interchange of nutritional substrates within the fusing microenvironment.

These observations and studies helped to adjust cell density and medium conditions so that acceptable cultural uniformity was obtained for the following experiments.

Endogenous enzymatic activity in various phases of the cell cycle. This question was investigated on asynchronous and synchronized cultures. The results did not indicate

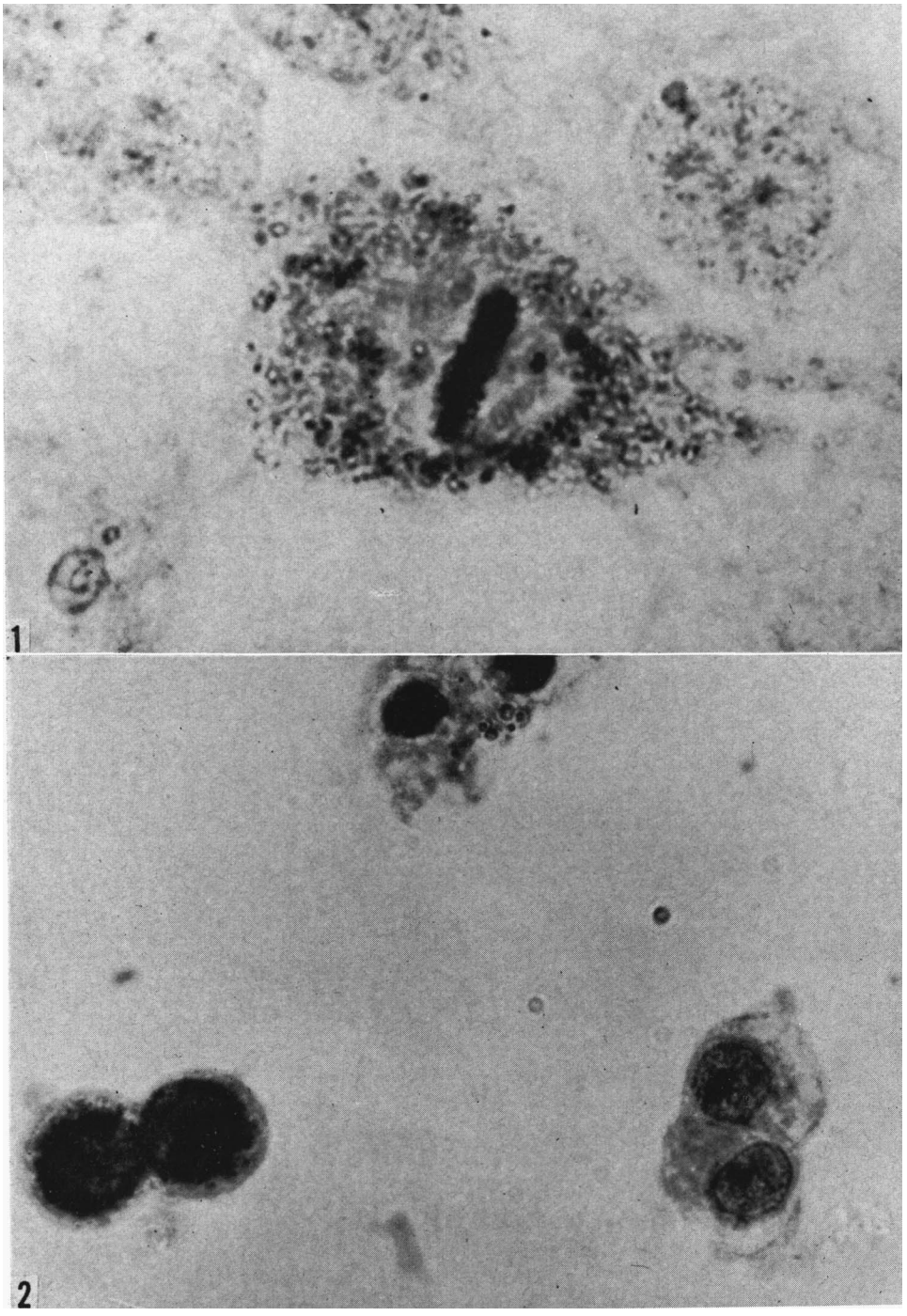

FIG. 1. Chang conjunctiva cell in metaphase, stained with combined tetrazolium-Feulgen reaction. Spindle apparatus free of formazan granules. (2500 ×.)

FIG. 2. Effect of cytotoxic antibodies on synchronized Chang conjunctiva cells during cytoplasmic cell division. Stained with combined tetrazolium-Feulgen reaction. (1200 ×.)

any fluctuation of oxidative enzyme activity in relation to certain phases of the cellular life cycle. Size, density, and distribution of formazan particles appeared to be uniform throughout interphase as well as the mitotic stages. In metaphase cells, however, the formazan particles were found pushed aside into the periphery by the spindle apparatus which never contained them, and, therefore, sharply contrasted with the cytoplasm proper (Fig. 1).

Effect of cytotoxic antibodies during various phases of the cell cycle. Cellular alterations induced by cytotoxic antibodies followed a consistent pattern of morphological events when the combined tetrazolium-Feulgen reaction was applied (Fig. 2). First, the cells begin to detach from the glass and from adjacent cells. The cells round up and the formazan particles condense around the nucleus. Gradually, all enzymatic activity is lost. The nuclei appear smaller and are stained darker. The nuclear membrane shows indentations. Finally, a halo separates the highly pycnotic nuclei and the disintegrating cytoplasm.

When cytotoxicity studies were performed on asynchronous cell populations, all stages of cytotoxic effect were recorded on the same coverslip ranging from entirely normal to complete destruction. However, it was striking to note that two neighboring cells of a pair were almost regularly affected to exactly the same degree. Different pairs, on the other hand, covered the whole range of cytotoxic effects. This observation (Fig. 2) suggested that these paired cells might have been killed by cytotoxic antibodies during or shortly after cell division.

This was confirmed in studies with synchronized cell populations. It was found that throughout interphase the cells are relatively resistant to the action of cytotoxic antibodies. Even the development of such early signs of cytotoxicity as detachment of cells and dislocation of formazan particles required longer incubation periods. Also, the phase of mitosis appeared to be little affected. Cells in prophase, anaphase, or metaphase were almost indistinguishable from those on the control coverslips incubated with plain newborn calf serum medium or with pre-immunization guinea pig serum. However, during the short period of cytoplasmic cell division the cells were seen to be killed almost at an instant. They did not move away from each other but stuck in pairs. Within about an hour the cells reached the endpoint of destruction (Fig. 2).

Discussion. The extremely high degree of sensitivity to cytotoxic antibodies during cellular cytoplasmic division is compatible with other data. Cytotoxic antibodies are assumed to affect mainly the cell membrane where "functional holes" in the size range of ribosomes have been demonstrated(3). This leads to rapid loss of cations, macromolecules, and inactivation of certain oxidative enzymes(4). During cytoplasmic division the cell membrane is strongly involved, as has been illustrated by time lapse microcinematography. Approximately 30% newly synthesized material has to be incorporated into the membranes of the daughter cells(5). Moreover, in monolayer cultures it can be observed (Fig. 1) that mitotic cells round up and loosen their contact to the glass and to adjacent cells(2). In this way, a larger part of the membrane surface is exposed to attachment of antibodies in contrast to interphase cells which adhere flatly and firmly to the glass. Matsumoto(6) published photographs which support our experimental findings. However, he did not relate his observations to the cellular life cycle.

The use of the combined tetrazolium-Feulgen reaction in our studies allowed to correlate nuclear and cytoplasmic changes resulting in better evaluation and quantitation of cytotoxic effects. Presumably, this method has wider applicability. It can be refined further for the demonstration of specific oxidative enzymes by adding homologous substrates and co-factors. Mainly for control purposes, we have used the technique in these experiments also in an attempt to relate nuclear events and the endogenous activity of oxidative enzymes during various stages of the cell cycle. Furthermore, results were obtained concerning interaction within heterogeneous or mixed cell cultures. Recently, the technique was successfully employed for morphological studies on premalignant cells in plastic film-induced tumorigenesis(7).

Summary. On asynchronous and synchronized cultures of Chang's conjunctiva cells it was shown that cytotoxic antibodies (in the presence of complement) were most effective during the stage of cytoplasmic cell division, *i.e.,* between mitosis and G_1-phase. All other stages of the cellular life cycle were relatively resistant in that longer incubation periods were required for cell destruction. A newly developed combined tetrazolium-Feulgen reaction was used in these studies with advantage.

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Subgroups of γA Immune Globulins.* (31287)

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Recently it has been demonstrated that at least 4 heavy chain subgroups exist for γG globulin(1,2). These differ in a variety of properties in addition to the antigenic properties utilized in their classification(3). Perhaps most striking is the independence of genetic factors for each subgroup(4). The possibility that γA and γM, the other 2 major classes of immune globulins, possess analogous subgroups has been considered by a number of investigators. Some evidence for such a possibility has been obtained for the γM macroglobulins(5,6). However, no findings of a similar nature have been reported for the γA type. The present report indicates that the γA globulins can be classified into 2 distinct types based on marked differences in the heavy chains.

Material and methods. Thirty-two myeloma proteins of the γA type were utilized in this study. Most of these came from sera sent to this laboratory for diagnostic typing. In addition, 10 of the proteins came from patients studied at the Clinic and Hospital of The Rockefeller University.

Six different antisera were utilized. Two were made in cynomologous monkeys (Mc and Ms) and 4 were made in rabbits (R_2, R_c, R_4, R_5). Antisera Mc and Rc were made against the same γA protein (c). This came from an individual who showed a sharp γA band over a period of 10 years with no evidence of myeloma. The remaining animals were immunized with separate γA myeloma proteins. In addition, 4 rabbits were immunized with isolated γA proteins of the minor subgroup, 2 with Ha and 2 with Os. The γA antisera were usually absorbed with serum Ro which was previously shown to be entirely devoid of γA protein(7).

The techniques utilized for protein isolation, separation of the heavy and light chains and agar gel analysis were similar to those described previously from this laboratory(1).

Results. Subdivision of γA proteins into 2 types by antigenic analysis. In the course of classification of a myeloma protein from a new patient under study, it was noted that this protein was clearly γA in type with one γA antiserum but difficult to type with another γA antiserum. Further studies indicated that several γA antisera showed this protein to be deficient antigenically. A survey was then instituted of 32 γA myeloma proteins and two others were found which behaved identically to the initial protein.

Fig. 1 illustrates the lines formed by agar plate analysis for 6 isolated γA proteins with

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