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Variation of Alkaline Phosphatase Activity Among Cells of Inducible and Constitutive Strains of Human Fibroblasts.* (29287)

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Investigators concerned with enzyme induction in microorganisms commonly assume that all of the cells in a genetically homogeneous population behave similarly. Evidence is presented here that this may not be true for an instance of enzyme induction in cultures of diploid human fibroblasts.

Cox and Pontecorvo(1) reported differences among established strains of human fibroblasts with respect to the activity of alkaline phosphatase of lysates of the mass cultures. Under the conditions of their experiments, most strains had no measurable alkaline phosphatase activity unless permitted to grow in the presence of small amounts of a substrate of that enzyme. Two more unusual types were noted: "constitutive" strains which demonstrated relatively high enzyme activity and "non-inducible" strains, which had no measurable enzyme activity even after treatment with substrate.

Methods. Histochemical stains for alkaline phosphatase were originally performed (on coverslip monolayers) for the purpose of conveniently classifying strains with respect to their enzyme activity when grown with or without an inducing substrate (1 mM phenyl phosphate). The diploid strains, of typical "fibroblast" morphology, had been established for periods of 2-10 months by explant or trypsinization techniques and were grown in Waymouth's medium with 10% calf serum. Cultures for pleuropneumonia-like organisms were negative. A modification of the method of Fredricsson(2) was employed for the alkaline phosphatase stains. Fredricsson discovered that diffusion of enzyme could be greatly diminished when the Gomori technique was performed in an incubation medium of 40% acetone; only slight inhibition of enzyme activity resulted. Our experience confirmed this; aqueous incubations yielded a more diffuse precipitation of calcium phosphate, with less sharp localization and considerably more nuclear staining, generally accepted as an artifact(3). Fixation in formalin (at 4°C, pH 7.0 for 15 min) did not prevent this diffusion, which was quite evident when aqueous incubations were carried out in the presence of di Na p-NO_2 phenyl PO_4; even after the coverslip was removed from the incubation medium, enzymic hydrolysis continued at a rapid linear rate (as measured by the optical density of p-NO_2 phenol at 410 mμ). Markedly improved preparations resulted even with final concentrations of acetone of only 16%; the higher concentration recommended by Fredricsson was less preferable because a variable degree of precipitation of substrate was observed. A second modification was a doubling of the calcium concentration, designed to favor the "capture reaction" (precipitation of the enzymically liberated phosphate as calcium phosphate). Finally, in order to prevent resolubilization of the precipitated phosphate, all rinses subsequent to the enzyme incubation were in 40% acetone buffered with 10 mM 2 amino-2-methyl 1-propanol-HCl, pH 9-10. Controls

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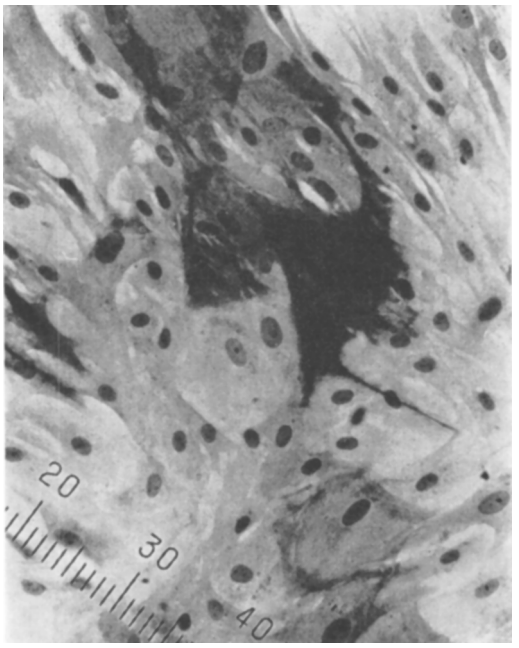

FIG. 1. Alkaline phosphatase stain of monolayer of human fibroblasts grown in medium containing 2 mM phenyl PO_4 for 6 days. Fixed in neutral buffered formalin at 4°C for 5 min. Incubated in 2.5 ml 2% Na β-glycerophosphate, 2.5 ml 2% Na veronal, 0.5 ml 4% $Ca(NO_3)_2$, 0.5 ml 0.8% $MgCl_2$ and 4 ml of 40% acetone for 5 hr at 37°C. Calcium phosphate precipitate converted to CoS by treatment with 2% $Co(NO_3)_2$ and 0.2% $(NH_4)_2S$, both in 40% acetone. Nuclear staining is due to light counterstain with safranin. Scale = 0.01 mm per division.

incubated without substrate and treated with enzyme inactivating agents (heat and methyl alcohol) failed to show intracellular deposits of cobalt sulfide.

Results. It was surprising to find that all preparations examined (over 100 coverslip monolayers from 12 strains) showed marked variation in enzyme activity from cell to cell. The heterogeneity could not be solely attributable to the histochemical "all or none" artifact(4). The appearance varied from no discernible precipitate of cobalt sulfide to jet black deposits covering the entire cytoplasm, with all gradations in between. A typical field is shown in Fig. 1, which was from a monolayer which had grown for 6 days in the presence of 2 mM phenyl PO_4 (without change of the medium). As is suggested in the illustration, clusters of cells (? small clones) with high enzyme activity could occasionally be noted, but more commonly, the distribution of "active" cells appeared to be random. Fields identical to that of Fig. 1 could be found in a "constitutive" strain classified by the method of Cox(1) and in a strain tentatively classified as constitutive by a method developed by the author(5). With long incubation times (up to 16 hours), definite enzyme activity could be demonstrated in one non-inducible and in all non-induced strains having low specific activities; here again, one could find all gradations of activity from cell to cell.

By far the greatest enzyme activity was observed in monolayers which had been grown for periods of up to 3-4 weeks without change of medium. This "self-induction" phenomenon(1) is now thought to be related to the degradation of cystine and cysteine, which repress the synthesis or activation of alkaline phosphatase in human fibroblast cultures(6). Degree of enzyme activity varied markedly from cell to cell. There was an apparently artifactual deposition of cobalt sulfide along the loose margins of partially detached cells, but enzyme activity was not otherwise related to any discernible morphologic differences. Enzyme activity was not related to the degree of cytoplasmic degenerative change as evidenced by fine and large droplet fatty alteration (stainable with neutral fat stains such as Fettrot 7B and Oil Red O) or accumulations of lipochrome pigments. Apparently phagocytized material was occasionally noted, but could not obviously be related to the degree of enzyme activity. The degree of confluence of various groups of cells within a given monolayer was definitely not correlated with enzyme activity. Enzyme activity did not appear to be correlated with the number of nucleoli or with the size of cells, nuclei and nucleoli. The distribution of enzyme activity within monolayers grown on 22 mm square cover slips vertically arrayed in Columbia staining jars containing 10 ml of medium could not be differentiated from monolayers grown in the conventional horizontal position; therefore, oxygen and other concentration gradients did not appear to influence enzyme activity.

Using a quantitative microfluorometric assay developed by Rotman(7), based upon

the micro-droplet spray technique of Collins (8), we have successfully measured the enzyme activity of single human fibroblasts grown in culture. The results substantiate the histochemical observations of heterogeneity; up to 9-fold differences in enzyme activity have been observed among cells derived from the same monolayer.†

Discussion. Synchronization and cloning experiments are in progress to evaluate further the cellular heterogeneity of alkaline phosphatase activity. Preliminary results using the dilution plating method of cloning (9) have been inconclusive, since in our hands "clones" of diploid human fibroblasts derived with this technique usually can be shown to arise from small clumps of cells rather than from single cells. Attempts to establish clones from isolated single cells (using micro-pipetting techniques, microcapillaries and feeder layers) have so far been unsuccessful. We hope that others more skilled in cloning techniques will also pursue this line of investigation.

Our knowledge of the frequency of mitotic recombinational events in organisms such as *Aspergillus nidulans*(10) suggests that such mechanisms are not at all likely entirely to account for the marked degree of heterogeneity observed. While there is still no information concerning the occurrence of mitotic crossing-over in mammalian cell cultures, mitotic non-disjunction is not likely to be a sufficiently frequent event in diploid human fibroblast cultures in view of the reports of prolonged karyotype stability from several laboratories(11). Should the variation in enzyme activity and/or synthesis prove to be "physiologic," these observations would still be significant, especially in view of the availability of quantitative techniques for assay of enzyme activity in single cells. We are profoundly ignorant of the detailed biochemical events which occur during the mitotic cycle of a cell, especially under conditions of enzyme adaptation. It would be of great importance to establish the stage of the cycle during which alkaline phosphomonoesterase activity and/or synthesis is enhanced and/or repressed and to discover the responsible mechanisms. Such investigations could clarify the ultimate function(s) of this group of enzymes.

It should be emphasized that even under apparently optimal conditions for induction of alkaline phosphatase *in vitro* ("self-induction"), substantially longer incubation times (60-120 minutes) were required to obtain results comparable to those which are regularly observed (using similar or identical techniques) with certain tissues (renal tubular and intestinal epithelia, healing wounds) from humans and other mammals, in which incubation times of 5-15 minutes suffice to demonstrate marked enzyme activity. If we assume that the enzyme activity which we observe histochemically is related to enzyme concentration and in turn, to rates of enzyme synthesis, this suggests that even when "induced," the genome of the cultured fibroblast is still in a state of relative repression with respect to the synthesis of alkaline phosphatase(s). Two regulatory loci and one structural locus have been described for the alkaline phosphatase of *E. coli*(12). Several genetic loci are also known to control the synthesis of the enzyme(s) in *Aspergillus nidulans* (13). The situation in man is likely to be even more complex, especially in view of the apparent heterogeneity at the cellular level.

Summary. Histochemical studies of 12 strains of diploid human fibroblasts derived from newborn foreskins revealed marked variation of alkaline phosphatase activity within all populations, whether classified as constitutive for the enzyme or inducible by substrate. Variation was also apparent in non-induced populations having low activity and in "self-induced" populations having high activity.

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† Unpublished data of J. Tierney and G. M. Martin.

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Antibody Response in Certain Domestic Animals to Influenza Viruses. (29288)

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Myxovirus immune sera are commonly produced in such laboratory animal hosts as chicken, ferret, guinea pig and rabbit. Increased epidemiological and diagnostic studies necessitate larger supplies of quality reference and diagnostic sera. An answer to this problem may be utilization of suitable larger animals where volume production with economy of manipulation and material can be achieved. Anti-influenza sera to human strains of virus have been produced in the horse(1). Recently, production of typing antisera in lambs inoculated by the intratracheal route with 3 type A influenza viruses has been reported(2). The present report summarizes a study undertaken to follow antibody response in goat, sheep and burro sera after immunization with influenza viruses.

Materials and methods. Viruses. The following influenza strains were employed as immunizing antigens: A2/Japan/305/56 ($E_4F_1M_3E_{23}$), B/Great Lakes/1739/54 ($E_4M_{10}E_{43}$), A/PR/8/34 ($F_8M_{593}E_{169}$), A1/FM/1/47 ($M_{37}E_{14}M_3E_{50}$), A/Swine/1976/31 ($M_{38}E_{45}$), B/Lee/40 ($F_8M_{137}E_{178}$) and C/Taylor/1233/47 (XE_{15}).* All strains were used for immunization of goats. The A2/Japan/305/57 and B/Great Lakes/1739/54 strains were used for sheep while only the A2/Japan/305/57 strain was used for immunization of the burro.

Immunizing antigens. These consisted of infected allantoic fluids treated by 2 cycles of adsorption and elution from chicken erythrocytes(3). Influenza type C antigen was untreated infected amniotic fluid. The materials were adjusted by dilution or by concentration (4) to contain approximately 200 hemagglutinating units.

Immunization and bleeding. Goats (Alpine cross, Nubian, Saanen and Toggenburg types) weighed between 55 and 125 lb, sheep (Suffolk and Rambouillet-Hampshire types) weighed 49 and 96 lb and a Mexican burro weighed 285 lb. All animals were inoculated intravenously at 0 time and again on day 7 and 35. The goat immunized with B/Lee/40 virus was also inoculated on days 56 and 77. Each inoculation consisted of 20 ml of virus material for goats and sheep and 40 ml of virus material for the burro. Bleedings were taken prior to immunization and at least 10 samples were collected from each animal at

* The letters E, F and M and the numerals designated in subscripts indicate passage history in the egg, ferret and mouse respectively. The complete history for C/Taylor/1233/47 virus is unknown except for a record of 15 egg passages.