Some Factors Controlling Alkaline Phosphatase Isoenzymes in HeLa Cells

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(Received 17 December 1969)

Alkaline phosphatase (EC 3.1.3.1) is separated on electrophoresis into several isoenzymes, which contain different amounts of sialic acid (Sarawathi & Bachhawat, 1968; Moss, Eaton, Smith & Whitby, 1966). The removal of these sialic acid residues by neuraminidase produces an isoenzyme of decreased mobility (Butterworth & Moss, 1966). The alkaline phosphatase present in HeLa cells has four isoenzymes and each isoenzyme is sensitive to the action of neuraminidase (Spencer, 1967). In contrast the S₃ clone of HeLa cells contains a glucocorticoid-inducible alkaline phosphatase that consists of two isoenzymes (Cox & Griffen, 1967). This induction is believed to involve a change in conformation of the enzyme rather than an increase in the amount of enzyme (Griffen & Cox, 1966).

The purpose of the present paper is to show that the specific activity and the isoenzyme pattern of HeLa S₃ cells is dependent on the type of serum present in the medium and that the effect of prednisolone also depends on the serum.

Methods. HeLa S₃ cells were obtained from Dr R. Berry (Churchill Hospital, Oxford, U.K.) and from Grand Island Biological Co. (New York, N.Y., U.S.A.). The cells were grown as monolayer cultures in medium 199 (Morgan, Morton & Parker, 1950); 10% of calf serum or of foetal calf serum was added as required. Prednisolone (3 µM) or cysteine (1 mM) was added to 3-day cultures and the cells were harvested after a further 3 or 5 days' growth. A partially purified fetuin fraction of foetal calf serum was prepared by the method of Fisher, Puck & Sato (1958) and used at 0.5 mg/ml. The cells were harvested by trypsinization and homogenates prepared by Morton's (1954) butanol procedure.

Alkaline phosphatase activity was assayed spectrophotometrically at 400 nm with 2 mM *p*-nitrophenyl phosphate (Sigma 104; Sigma Chemical Co., St Louis, Mo., U.S.A.) as substrate in 0.2 M-2-amino-2-methylpropan-1-ol hydrochloride buffer, pH 9.8, at 37°C. Protein was measured with Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951). The isoenzymes were separated by polyacrylamide-gel electrophoresis (Spencer, 1967). The gels were stained in a solution of sodium β-naphthyl phosphate (1 mg/ml) and Fast Blue BB (1 mg/ml) dissolved in 0.2 M-tris hydrochloride buffer, pH 8.7.

Results. The specific activities and isoenzyme patterns of S₃ cells grown in medium containing foetal calf serum or calf serum are shown in Figs. 1(a) and 1(c). The enzyme has a higher specific activity

and the induction is markedly increased when the cells are grown in calf serum instead of foetal calf serum. Similar results were obtained for cells from both sources, but the 'Oxford' cells had a consistently higher specific activity. A different isoenzyme pattern is found for both cell lines when grown in foetal calf serum instead of calf serum. The effect of prednisolone on the cells is shown in Figs. 1(b) and 1(d). No change in isoenzyme pattern or specific activity was found in the presence of foetal calf serum; however, when the cells were grown in calf serum there was an increase in specific activity and a change in the isoenzyme pattern. These changes were shown to be independent of the nutrient composition of the medium, since identical results were obtained when medium 199 was replaced by Dulbecco's modified Eagle's medium (Dulbecco & Freeman, 1959) or by Waymouth's (1959) medium.

S₃ cells were also grown in medium 199 with serum that had been dialysed for 24 h against 5 mM-tris-HCl buffer, pH 7.5. The electrophoretograms gave the same patterns as shown in Figs. 1(a) and 1(c) for non-dialysed serum. This shows that the serum factor has a high molecular weight. The main component of foetal calf serum that is not present in calf serum is fetuin. Therefore S₃ cells were grown in medium 199 + 10% of calf serum with fetuin

Fig. 1. Alkaline phosphatase isoenzyme patterns on polyacrylamide-gel electrophoresis of HeLa S₃ cells grown in medium 199 with: (a) 10% of foetal calf serum; (b) 10% of foetal calf serum + 3 µM-prednisolone; (c) 10% of calf serum; (d) 10% of calf serum + 3 µM-prednisolone; (e) 10% of foetal calf serum and the extract incubated with neuraminidase for 30 min. The values below the gels are specific activities of the samples expressed as nmol of *p*-nitrophenol/min per mg of protein for cultures from the two sources.

(0.5mg/ml). After 6 days in this medium the electrophoretogram showed the 'calf-serum' pattern but with a very weak slow band. Similarly S_3 cells previously grown in calf-serum medium and then transferred to foetal-calf-serum medium showed the 'foetal-calf-serum' pattern after 4 days.

S_3 cell cultures were grown in medium 199 + 10% of calf serum with 1mM-L-cysteine to investigate whether or not the two isoenzymes are regulated by a common pathway. L-Cysteine is an inhibitor of alkaline phosphatase activity (Martland & Robison, 1927), and in addition cysteine controls the amount of the enzyme in fibroblast cells (Cox & Pontecorvo, 1961) and in kidney cells (Griffen & Cox, 1967); these are two distinct processes. After 5 days in the presence of L-cysteine the specific activity had fallen to 12% of the starting value, but there was no change in the electrophoretic pattern.

To determine the relationship between the isoenzymes present with the S_3 cells grown in calf serum, use was made of the action of neuraminidase on alkaline phosphatase. An extract of S_3 cells grown in medium with 10% of foetal calf serum was incubated with 50 units of neuraminidase for 30 min in tris-acetate buffer, pH 5.5, and the sample was immediately subjected to electrophoresis in the usual manner. The pattern obtained (Fig. 1e) shows that incubation with neuraminidase produced new isoenzymes of lower mobilities, one of which corresponds to the slower isoenzyme found in cells grown in calf-serum medium.

Discussion. These results show that the specific activity, the effect of prednisolone and the electrophoretic pattern of alkaline phosphatase in HeLa S_3 cells depend on the type of serum present in the medium. The effect of prednisolone on these cells supports the concept that the 'induction' of alkaline phosphatase involves the formation of an enzyme with a modified conformation rather than an increase in the amount of enzyme synthesized (Griffen & Cox, 1966). This modified enzyme has a different mobility on polyacrylamide-gel electrophoresis from that of the normal enzyme. The action of cysteine on the control of alkaline phosphatase is uncertain; cysteine acts either as an inhibitor of synthesis of the enzyme or it increases the rate of degradation (Griffen & Cox, 1967). The present results show that the amount of both isoenzymes in S_3 cells is controlled by cysteine, so that if this control acts on the synthesis of the enzyme it takes place before the addition of the sialic acid groups to the enzyme.

The relationship between the two isoenzymes found in the presence of calf serum is shown by incubation of the enzyme from foetal-calf-serum grown cells with neuraminidase before electrophoresis. The removal of sialic acid residues from alkaline phosphatase by neuraminidase has been described

previously (Spencer, 1967; Butterworth & Moss, 1966). The products of the reaction migrate more slowly owing to removal of the anionic groups. With the short incubation period described here a series of isoenzymes was produced. The second band (numbering from the anode) correlates with the slower isoenzyme found in S_3 cells grown in calf serum.

The effect of serum on the isoenzyme pattern can be explained as a difference in sialic acid content between the two isoenzymes. Thus foetal calf serum inhibits the formation of the slower isoenzyme, and experiments described here show that fetuin is involved in this inhibition. Fetuin is also a glycoprotein, and its electrophoretic mobility is affected by incubation with neuraminidase (Oshiro & Eylar, 1968). The site within the cell where fetuin interferes with the addition of sialic acid is uncertain, but enzymes for such reactions have been located in cell membranes (Hagopian, Bosmann & Eylar, 1968), and alkaline phosphatase is present in the smooth and plasma membranes of HeLa cells (Bosmann, Hagopian & Eylar, 1968). This role for fetuin may also explain why the effect of prednisolone is less in the presence of foetal calf serum than in the presence of calf serum.

This work was started at the Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K., with support from the British Empire Cancer Campaign. I thank Miss J. Austin for her technical assistance.

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