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The Binding of Polypeptides by Neurophysins

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COMMUNICATIONS

A Requirement for Zinc Ions for Deoxyribonucleic Acid Synthesis by Lymphocytes Stimulated by Phytohaemagglutinin

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Alford (1970) reported that the transformation of human peripheral-blood lymphocytes by phytohaemagglutinin required a bivalent cation in addition to Ca^{2+} . His results suggested that this requirement was most efficiently met by Zn^{2+} .

Lymphocytes from pig blood obtained at slaughter were used for the present investigations. The incorporation of [^3H]thymidine into the DNA of the lymphocytes was inhibited by 80–90% by the addition to the medium of 0.6mM-EDTA or 0.6mM-diethylenetriamine-*NNN'N'N'*-penta-acetate but not by 0.6mM-nitrilotriacetic acid. The addition of Zn^{2+} as ZnSO_4 reversed the inhibition by EDTA or diethylenetriamine-*NNN'N'N'*-penta-acetate. With 0.6mM-EDTA complete reversal of the inhibition required 0.05mM- Zn^{2+} ; concentrations less than this caused only partial reversal, whereas higher concentrations again decreased the incorporation. The initial pattern of reactivation with increasing concentrations of Zn^{2+} suggested a competition between EDTA and a cellular chelator that, complexed with Zn^{2+} , was directly or indirectly involved in [^3H]thymidine incorporation. The competition between chelators for the metal ions present in the system was investigated by using a computer simulation based on the program described by Perrin & Sayce (1967). The simulation suggested that the cellular chelator had to have a stability constant of the order of 10^{12} to account for the observed competition with EDTA for the activating Zn^{2+} . The validity of the simulation was tested by using this value for the stability constant of the active site to calculate the proportion of the sites that had accepted Zn^{2+} over a range of added Zn^{2+} concentrations. These proportions were found to agree well over a range of suboptimal Zn^{2+} concentrations with the experimentally determined rates of thymidine incorporation.

The time-course of the requirement for Zn^{2+} was investigated by adding EDTA at various times after stimulation of the cells with phytohaemagglutinin.

It was further studied by adding EDTA before phytohaemagglutinin and subsequently attempting to reverse the inhibition by adding an optimal amount of Zn^{2+} . At least half the maximum rate of incorporation was still obtained provided EDTA was added not earlier than 12h before the incorporation was measured. If EDTA was added initially, Zn^{2+} had to be supplied at least 17h before the incorporation was measured if at least half the maximum activity was to be obtained.

The specificity of the reversal by Zn^{2+} of the inhibition caused by EDTA will be discussed together with the implications of an apparently high affinity constant for Zn^{2+} at the active site within the cells.

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Electrophoretic Slowing of Sensitized Lymphocyte–Macrophage Mixtures: a Cellular Technique in Immunochemistry

By E. A. CASPARY and E. J. FIELD (*Medical Research Council Demyelinating Diseases Unit, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE, U.K.*)

Immunochemical studies have generally been confined to the interaction of substances with specific antibody prepared against them. The study of specific cellular immunity, however, is more difficult and not readily made quantitative. A very specific change in immunologically sensitized cells may, however, be measured by using cell electrophoresis in the presence and in the absence of the sensitizing antigen, when the mobility of the cell will be decreased by the antigen, and a number of studies of this kind have been reported over the last 5 years (Sundaram *et al.*, 1967a,b; Hartveit *et al.*, 1968; Deingdoh & Turk, 1968). An initial study of guinea-pig peritoneal-macrophage exudates from sensitized animals showed that the antigen slowing response depended first on the presence of lymphocytes and secondly that it could be inhibited by puromycin or other inhibitors of protein synthesis (Caspary, 1971). It was further

shown that the lymphocytes produce a slowing factor that then acts on the macrophages to produce a slowing effect in electrophoresis. Indeed the system is analogous with the mechanism of macrophage-migration inhibition (David, 1966; Bloom & Bennett, 1966), though the messenger protein need not necessarily be the same. Standard electrophoretic methods are not easily applicable to human cells for technical reasons, and a system was therefore devised whereby guinea-pig macrophages could be used as an indicator system for the specific interaction of human lymphocytes with antigen. The mixed-cell interaction of human and the contaminating guinea-pig lymphocytes (about 10–20%) was overcome by irradiating the exudate with 100rd of γ -radiation, which proved adequate for short-term experiments. Our final assay system was set up as follows: 10×10^6 irradiated guinea-pig macrophages, 0.5×10^6 human lymphocytes and antigen at concentrations up to $33 \mu\text{g/ml}$ made up to a final volume of 3 ml with medium TC199. The mixture was allowed to incubate at room temperature for 90 min before the electrophoretic mobility of the macrophages was measured in a Zeiss cytopherometer at 23°C . This instrument is extremely temperamental, large cells such as macrophages tend to cause turbulence, a multiplicity of joints tend to produce micro-leaks and electrode polarization restricts prolonged use, though with care, patience and experience many of these troubles may be overcome. Macrophages are timed in both directions of current, and we have found empirically that measurements differing by more than 10% must be discarded. For immune assays the results are expressed as a percentage slowing when the mobility in the presence of specific antigen is compared with the control mobility, i.e. no antigen.

This method was first used in the study of thyroid disease (Field *et al.*, 1970), later in nervous diseases (Caspary & Field, 1970) and cancer (Field & Caspary, 1970; Caspary & Field, 1971). Tests using encephalitogenic basic protein in neurological disease showed that the immune reaction was to the active determinant (P. R. Carnegie, E. A. Caspary, F. Westall & E. J. Field, unpublished work), an undecapeptide, and that this reactivity could be altered by substitution or deletion of amino acids. The immune interaction with encephalitogenic basic protein could also be inhibited by serotonin (Field *et al.*, 1971), but not by adrenaline or acetylcholine. Some hallucinogenic drugs proved almost equally effective.

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Identification of a Tumour Antigen

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Field & Caspary (1970) showed that lymphocytes from patients with malignant carcinoma reacted with the encephalitogenic basic protein from human brain and produced a factor that altered the surface charge of macrophages. When a variety of tumours were extracted by the procedure used to isolate encephalitogenic basic protein a mixture of proteins was obtained that was a more effective antigen than the encephalitogenic basic protein in the cytopherometer assay with these lymphocytes (Caspary & Field, 1971). The present report describes the identification of the protein that can react with lymphocytes from patients with malignant carcinoma.

Tumours of the vulva, cervix and breast were defatted with chloroform-methanol (2:1, v/v) and the residues were extracted with HCl between pH 3 and 2.5. The acid extracts were fractionated by gel filtration on Sephadex G-75 and the eluates were examined by electrophoresis in polyacrylamide gel (Adams & Fox, 1969). Pooled fractions were tested with lymphocytes from patients with cancer in the cytopherometer assay. Similar results were obtained with extracts of each tumour. The peak of antigenic activity was eluted with a volume 1.63 times the exclusion volume of the column. The major protein in this region, and the only component whose elution pattern paralleled the antigenic activity, had an electrophoretic mobility 0.85 times that of cytochrome *c*. The approximate molecular weight for this protein was calculated to be 16000. It contained one tryptophan residue.

Fractionation of a benign tumour (fibroid of uterus) and normal human liver yielded similar proteins, but their antigenic activity in the cytopherometer assay was significantly less ($P < 0.001$) than those of the protein from malignant tumours and of encephalitogenic basic protein.

The antigenic determinant in encephalitogenic