Lymphocyte Membrane Enzymes.
II. Cyclic 3',5'-Adenosine Monophosphatase Located on Unstimulated Human Small Lymphocyte Nuclear Membranes

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This study was undertaken to localize the enzyme cyclic 3',5'-adenosine monophosphatase in unstimulated human small lymphocytes. Cyclic AMP may be of importance in mediating lymphocyte stimulation, hence localization of the enzyme that is thought to modulate its intracellular concentration, cyclic AMPase, may point to the site of action of cyclic AMP in the cell. The histochemical technique employed was that of Shanta et al.\(^6\) Cyclic AMP substrate is degraded by the phosphodiesterase, then hydrolyzed, resulting in a lead phosphate precipitate at the site of enzyme action. This is subsequently visualized as lead sulfide. The enzyme was found to be localized on the nuclear membranes, which indicates that this is the site where cyclic AMP is destroyed. It does not necessarily mean that the nuclear membrane is the site where cyclic AMP is effective; this may be at some other point in the lymphocyte stimulation pathway, including the cell membrane, and the nuclear membrane may only be the site where cyclic AMP is degraded.

Increasingly, attention is being concentrated on the enzymes associated with lymphocyte membranes, as they may be of importance in the early events, immediately related to antigen recognition, that lead to lymphocyte stimulation. This study is concerned with freshly separated human small lymphocytes to ascertain the nature of the enzyme systems they possess while in the prestimulation resting state. The value of such information is that it may be possible to ascertain from it and related data what biochemical events occur along the intracellular stimulation pathway that leads from the lymphocyte surface to the nucleus.

In a wide variety of intracellular stimulatory events, cyclic 3',5'-adenosine monophosphate (cyclic AMP) is known to serve as a second-echelon hormone, particularly in mediating the effects of the peptide hormones.\(^1,2\) Recent work\(^3,4\) indicates that cyclic AMP may be an intermediary in thymocyte and

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Submitted April 20, 1971; revised May 20, 1971; accepted May 22, 1971.

This study was carried out in the Department of Pathology, Guy's Hospital, London, England, and was supported by the University of London Laura de Saliceto Studentship, the Pech Medical Research Trust, and the British Medical Association Ernest Hart Award.

lymphocyte stimulation, hence it was of interest to attempt the localization of the phosphodiesterase, cyclic 3',5'-AMPase, that destroys cyclic AMP and thus, in this way, probably serves to modulate the effect of cyclic AMP within the cell.

The histochemical technique employed was based on that of Shanta et al. Smears of lymphocytes were incubated in a medium containing cyclic 3',5'-AMP, and this was degraded by the phosphodiesterase located within the cells to form 5'-AMP. The latter compound was then hydrolyzed by the exogenous 5'-AMPase supplied in the medium in the form of snake venom from Crotalus atrox. The liberated phosphate radical precipitated with lead also present in the medium, in the form of lead phosphate, which was converted to black lead sulfide at a later stage. The black staining could be visualized microscopically and marked the presumptive site of enzyme activity.

**Materials and Methods**

**Lymphocyte Separation**

Human small lymphocytes were separated from healthy donors using the technique described by Coulson, Corner, and Coombs. Defibrinated blood was mixed with 100 mg carbonyl iron particles (Fine Dyestuffs and Chemicals, Manchester, England) and with one third of its volume of 3% gelatin in phosphate-buffered saline (Batch 277, Glue and Gelatine Research Association Birmingham England). The blood-gelatin-iron mixture was rotated on a blood cell suspension mixer for 30 min at 37°C, then decanted and allowed to sediment for a further 30 min at 37°C. The supernatant suspension of lymphocytes was diluted with an equal volume of 199 medium (Wellcome, lot M3413) to prevent the gelatin from gelling, and centrifuged to produce the pellet of cells used to make the smears. The time that elapsed from the initial venipuncture to the fixing of the slides in acetone was 2 hr. The slides on which the smears were made, and which subsequently came in contact with the incubation media, were scrupulously cleaned.

**Fixation and Histochemistry**

Lymphocyte smears were air dried within a period of 5 sec and then plunged into 60% acetone in distilled water at 20°C for 120 ± 5 sec. The slides were washed gently for 3 min in tap water and then in two changes of glass distilled water for a total of 4 min. Test slides were incubated in a medium containing the following constituents: cyclic 3',5'-adenosine monophosphate (1.44 mM, Sigma, lot 60C-1620), Tris-maleate buffer (pH 7.6, 50 mM), magnesium chloride (10 mM), lead acetate (2 mM), and Crotalus atrox venom (1 mg in 10 ml, Sigma, lot 78B-1460). When the medium was made up in this way, the final pH was 7.30, and it had to be adjusted to 7.60 with Tris base. Incubation was continued for 90 ± 5 min at 37°C in disposable Petri dishes. At the end of incubation the pH of the medium was still 7.60. The molarity of the incubation medium was unavoidably reduced slightly by the residual dampness of the lymphocyte smear.

After incubation, the slides were washed carefully by gentle agitation in five changes of glass distilled water for a total of 25 min. Any significant curtailing of this washing led to nonspecific background staining on the slide. The next stage in the staining procedure was immersion of the slides in freshly prepared ammonium sulfide solution (1% weight/volume in distilled water), followed by three washes in distilled water totaling 9 min. Finally the slides were mounted in glycerol jelly.

In some preliminary experiments different methods of fixation were tried, including 100% methanol and 70% ethanol is distilled water.

**Control**

With every staining run the following controls were employed: (1) the substrate cyclic AMP was omitted; (2) exogenous 5'-nucleotidase was omitted and fluoride was added to
inhibit endogenous 5'-nucleotidase; (3) or aminophylline was added as an inhibitor of cyclic AMPase. In practical terms, when the cyclic AMP was omitted, 1 ml of distilled water was substituted for the cyclic AMP solution, so that the final concentration of the other ingredients of the medium was not affected. When the exogenous 5'-nucleotidase was omitted, sodium fluoride solution was substituted to produce a final concentration of 20 mM; in addition, the smears were preincubated in 100 mM sodium fluoride dissolved in 50 mM Tris buffer (pH 7.8) for 30 ± 3 min at 37°C. The aminophylline control studies involved preincubation of the slide in 100 mM aminophylline B.P. (British Pharmacopoeia) for 30 min at 37°C, followed by incubation in a medium identical to the normal test medium but with added aminophylline B.P. to a final concentration of 20 mM.

Further control studies were carried out on two additional donors, including heat inactivation of the enzyme, inhibition with formalin, and variation of the pH of the incubation medium. In practical terms, heat inactivation was achieved by warming the separated lymphocytes to 60°C for 120 ± 5 sec prior to centrifugation and fixation. Formalin treatment involved immersion of the slides for 10 min in formalin B.P. at room temperature between the acetone fixation stage and the wash in distilled water. Variation in the pH range for the final incubation media was achieved by making up the test medium to the following different pH values: 6.0, 6.5, 6.8, 7.0, 7.6, 7.8, and 8.0.

Results

Lymphocyte Separation, Fixation, and Morphology

The leukocytes in the supernatant from the final sedimentation procedure contained more than 99% lymphocytes, although red cells outnumbered the lymphocytes in the ratio roughly 6:1 in the smears. Care was taken to examine the lymphocytes just before the tail of the smear where, on the Leishman film, their morphology appeared classical. The head of the smear was avoided because the cells were crowded too closely together; conversely, the tail of the film showed excessive spreading of the lymphocytes.

Preliminary trials were done with lymphocyte smears, which were air-dried but not fixed in acetone prior to incubation with the substrate. The cells appeared swollen and considerably distorted after this treatment, and there was marked reduction in the density of positive staining. Methanol and 70% ethanol similarly yielded inferior final histochemical preparations compared to the acetone fixed slides.

Localization of the Cyclic AMPase

Positive staining was found to be localized on the nuclear membrane in the small lymphocytes of all 10 of the different donors employed. The final reaction product was sharply defined with no extension into the nucleus or cytoplasm, and no black deposits were seen elsewhere in the cell (Fig. 1). The cell membranes were closely examined and were consistently negative. The erythrocytes in the smear showed no staining.

Controls

In all the controls where cyclic AMP was omitted or where aminophylline was added, the lymphocyte nuclear membranes were unstained. In two out of 18 control experiments where exogenous 5'-nucleotidase was omitted and fluoride added, the nuclear membrane appeared slightly gray and the cytoplasm very slightly gray. On the other 16 occasions and during the prelimi-
Fig. 1.—Freshly separated small lymphocytes from different donors showing localization of cyclic 3',5'-AMPase on nuclear membranes. Smears of lymphocytes were incubated in a medium containing cyclic AMP, exogenous 5'-nucleotidase and lead ions; lead phosphate deposited at the site of enzyme activity is converted to black lead sulfide by ammonium sulfide treatment. \( \times 1750 \). In Fig. 1A the two small black granules to the right of the lymphocyte and the two small circles below it are artifacts. The eight erythrocytes discernible in the picture are unstained.

In binary trials, the nuclear membrane was unstained.

Treatment with formalin and heat inactivation resulted in complete inhibition of the enzyme activity. When the pH of the incubation medium was either 8.0 or 7.0, the density of the staining was considerably reduced; at pH 6.8 and below there was no nuclear membrane staining.

**Discussion**

The inhibitory effect of formaldehyde and aminophylline and the susceptibility of the staining procedure to both prior heating of the cells and to departures from the optimal pH of the incubation medium strongly support the view that an enzymatic process initiates the degradation of the cyclic 3',5'-AMP. Pearse has criticized the histochemical technique employed because it is a multistep procedure, and he has questioned the meaningfulness of the localization of the final product. However, in the case of the cells investigated in these experiments, the final reaction product was persistently and sharply localized at the nuclear membrane. This lack of diffuseness of the final lead sulfide deposit argues against the second stage of the degradation of the cyclic 3',5'-AMP substrate occurring at a site distant from the first stage. Confusion between the lead sulfide reaction product and traces of residual carbonyl iron left from the separation procedure did not occur, because with the high magnifications employed, the characteristic shape of any particles was clearly recognizable. A related enzyme, 5'-nucleotidase, has previously been demonstrated in the perinuclear zone of NK/Ly lymphoma cells.

As Sutherland has pointed out, cyclic AMP is known to play the role
of a secondary intracellular hormone-like substance in a wide variety of situations. The intracellular content of cyclic AMP at any moment appears to be the result of a balance between the enzyme adenyl cyclase that effects the synthesis of cyclic AMP, and the modulator enzyme cyclic 3',5'-AMPase that degrades it.\textsuperscript{11} Localization of cyclic AMPase to the nuclear membrane does not necessarily point to the latter structure as the site where cyclic AMP is effective within the lymphocyte. It is possible that the site where cyclic AMP is effective is at the cell membrane or some other site between the cell membrane and the nucleus and that the nuclear membrane is merely the site of degradation. The presently available evidence for cyclic AMP playing any part in small lymphocyte activation can best be described as suggestive. Various investigators\textsuperscript{2-5} have found that cyclic 3',5'-AMP will suppress phytohemagglutinin-stimulated lymphocyte transformation, but in addition that some concentrations of cyclic AMP, in the absence of phytohemagglutinin, will stimulate lymphocyte transformation. Studies with rat thymic lymphocytes indicate that cyclic AMP appears to mediate the mitogenic effect of parathyroid hormone, bradykinin, and growth hormone.\textsuperscript{12-14}

Small lymphocytes are noted for the paucity of their enzyme content,\textsuperscript{15} however, the small amount of enzyme present, particularly that associated with membranes, may play an important role in early triggering events following antigen recognition,\textsuperscript{16} and thus be of immunologic significance. In this context an earlier observation\textsuperscript{17} on the increase in lymphocyte membrane-associated nucleoside diphosphatase during transformation may be correlated retrospectively with the synthesis and release of lymphokines.\textsuperscript{18,19} However, in the case of cyclic AMP and its modulator enzyme cyclic AMPase, it is still too early to ascribe to them a definite place in the lymphocyte stimulation pathway.

References

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