GENE EXPRESSION OF ERYTHROCYTE RECEPTOR: REGULATION OF ERYTHROCYTE-RECEPTOR SYNTHESIS IN T-LYMPHOCYTES

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1. Introduction

Human T-lymphocytes bind sheep erythrocytes (SRBC) under stringent in vitro conditions to form rosette-shaped figures when viewed under a microscope [1–5]. Although the phenomenon has no known in vivo consequences it has been an extremely useful marker of human T-cells and an indicator of maturation. The binding appears to be the consequence of the presence of specific glycoproteins on the lymphocyte surface which have a selective affinity for a fetuin-like glycopeptide on the sheep erythrocyte membrane [6–9]. Peptide hormones synthesized by thymic epithelial cells have been shown to induce its biosynthesis [10,11], but very little is known regarding the molecular events underlying the induction process or the regulation of the biosynthetic process once it is initiated. These experiments are the first in a series designed to elucidate the regulatory mechanism of thymic hormones on expression of erythrocyte receptor. Using the experimental model in [12] we demonstrate that the biosynthesis of the erythrocyte binding protein is under a transcriptional control mechanism and once the genome of this protein is turned on during the maturation process, it continues to express without any further hormonal requirement.

2. Materials and methods

2.1. *Isolation of human lymphocytes*

Lymphocytes were obtained from normal laboratory personnel by centrifugation of fresh heparinized venous blood through Ficoll-Hypaque (LSM-Bionetics, Kensington, MD) [13].

2.2. *Sheep erythrocyte binding by T-lymphocytes (E-rosette formation)*

The quantitation of T-lymphocytes by their interaction with SRBC to form E-rosettes was performed by a modification of the method in [3]. Each procedure was performed in triplicate.

2.3. *Trypsinization of lymphocytes*

Lymphocyte trypsinization was done as in [14,15]. Fresh trypsin solution (Sigma, St Louis MO) 1 mg/ml was prepared daily by reconstitution in Dulbecco's phosphate-buffered saline (D-PBS) (Gibco, Grand Island NY) and pre-warmed at 37°C immediately prior to use. Mononuclear cells, separated as described, were washed twice in Hank's balanced salt solution (HBSS) (Gibco) with no added fetal calf serum (FCS) (Gibco) and resuspended in 10 ml pre-warmed trypsin solution. After incubation for 5 min in 37°C water bath, the cell suspension was placed in ice for an additional 5 min before washing twice with cold D-PBS. The trypsinized lymphocytes were resuspended in HBSS without FCS at 7.5×10^6/ml for determination of the proportion of lymphocytes retaining their SRBC receptors by standard E-rosette formation. Viability was assessed by trypan blue exclusion.

2.4. *Culture conditions*

Cells were incubated at RPMI 1640 (Flow Lab., McLean VA) without serum at 37°C in an atmosphere of 5% CO_2 and air on a rocking platform.

2.5. *Metabolic blocking agents*

The following agents were added to trypsinized lymphocytes for final concentration of: puromycin 5, 10 and 20 μg/ml (Boehringer Mannheim, Indianapolis IN); cycloheximide 10 and 20 μg/ml (Nutritional Bio-

chem, Cleveland OH); actinomycin-D, 0.1, 1, 5, 10 and 20 μg/ml (Sigma); cordycepin (Sigma) 50 μg/ml. Following incubation the cells were washed, their viability assessed by trypan blue exclusion, and prepared for the E-rosette assay.

3. Results

3.1. *Recovery of sheep erythrocytes binding capacity following trypsinization*

Trypsinization of normal human peripheral blood lymphocytes reduced the number of lymphocytes capable of forming E-rosettes, e.g., binding erythrocytes, from the pre-treatment levels of 55–65% down to 2–8%. The viability of the trypsinized cells remained >97%.

Fig.1 illustrates the recovery of erythrocyte binding capacity following trypsinization while maintained in vitro in RPMI 1640. The % of E-rosette forming cells returned to the pre-trypsin level after 9 h incubation. This regeneration curve is extremely reproducible.

3.2. *Effect of metabolic inhibitors on recovery of erythrocyte binding capacity*

Table 1 summarizes the effect of metabolic inhibitors on the regeneration of erythrocyte binding protein when assessed after 9 h incubation. These data show the results of 1 dose for each inhibitor, but com-

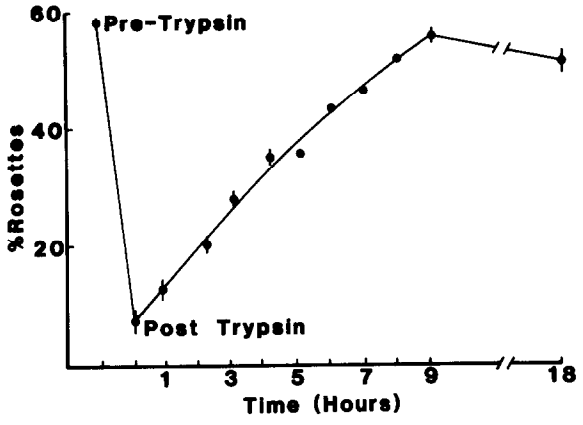

Fig.1. Regeneration of sheep erythrocyte binding protein following trypsinization. The values indicate observed % (±SD) of rosettes formed over untrypsinized control after different time intervals of incubation at 37°C in 5% CO_2 and air. The viability of these cells after 9 h incubation is ~97%. Details of the experiment are in section 2.

Table 1
Effect of metabolic inhibitors on regeneration of E-rosettes

Reagent	% E-rosettes	
	Non-trypsinized	Trypsinized + 9 h incubation
None	58.3 ± 4.1	55.0 ± 2.3
Puromycin 10 μg/ml	57.1 ± 3.8	6.0 ± 2.1
Cycloheximide 100 μg/ml	55.3 ± 0.7	4.1 ± 0.9
Actinomycin-D 1 μg/ml	57.3 ± 0.9	4.0 ± 2.1

Effect of transcriptional and translational inhibitors on recovery of erythrocyte binding protein following trypsinization. The concentration of inhibitors used in the experiment are at optimum level. The data represent observed % (±SD) rosettes over untreated controls

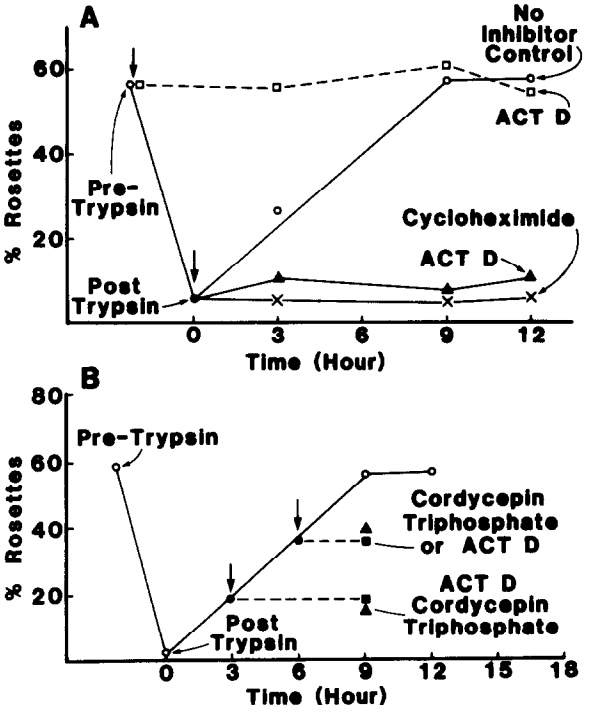

Fig.2. (A,B) Effect of metabolic inhibitors on regeneration of sheep erythrocyte binding protein following trypsinization. The concentration of the inhibitors used are at optimum level. The inhibitors are added at different times after incubation as indicated by the arrow and the cells were further incubated for a total of 9 h. Values indicate the observed % (±SD) rosettes. Plotted values with no error bars indicate an SD <1.

parable results were obtained with all doses utilized. The viability of these cells in the presence of inhibitors remains high, generally >95%. The results indicate the recovery of erythrocyte binding capacity is dependent on both DNA-dependent mRNA synthesis and protein synthesis.

Fig.2A illustrates the time course of recovery in the presence or absence of either actinomycin-D or cycloheximide. Recovery again was complete by 9 h in the untreated cultures. The metabolic blockers completely abrogated the development of erythrocyte binding capacity when added to trypsinized cells. Actinomycin-D had no effect on the binding capacity of non-trypsinized cells over the 12 h duration of this experiment.

Fig.2B illustrates the effect of actinomycin-D (1 μg/ml) or cordycepin triphosphate (50 μg/ml) when added to cultures after 3 or 6 h incubation. No additional lymphocytes acquire rosetting capacity once these inhibitors are added.

4. Discussion

Normal human T-lymphocytes, deprived of their erythrocyte binding capacity by trypsinization, will regain that capacity if permitted to synthesize mRNA and protein. No signal has to be received from outside the cell for this to be accomplished. Protein synthesis in most eukaryotic cells is regulated at the translational level using pre-synthesized long-lived mRNA [16,17]. In present experiments the recovery of erythrocyte binding proteins are accomplished by DNA-dependent mRNA synthesis without any exogenous hormonal influence. These results suggest that thymic hormones are important only in the initial induction step and once the cells have undergone differentiation, the genome for erythrocyte binding protein appears derepressed permanently.

The use of 5% human serum in the incubation medium in [12] makes it impossible to rule out a possible role for thymic hormones in the recovery process, but does not negate their conclusions regarding the need for nascent mRNA synthesis.

When actinomycin-D is added at the period (fig.3) that allows the time normally required for mRNA to be synthesized and transported into the cytoplasm, the regeneration of erythrocyte binding protein is still inhibited. In [18] were reported two classes of poly(A) mRNA in resting human lymphocytes, a stable class, which has half-life ~20 h and a labile class with half-life of <1 h. 70% of the total mRNA in resting human lymphocytes is of short-lived type (>1 h). Studies of the turnover of poly(A) mRNA for specific surface proteins such as erythrocyte binding protein have not been performed. A direct interference of actinomycin-D on the binding of sheep erythrocytes is ruled out since it had no effect on non-trypsinized cells cultured in its presence (fig.2). It is possible that erythrocyte binding protein continuously sheds and regenerates as a normal process. These data indicate that no shedding of the erythrocyte binding protein occurs in actinomycin-D blocked cells. Whether shedding occurs in non-blocked cells cannot be ascertained from this data, but it will be of interest to determine whether removal of the protein leads to new synthesis and, if so, whether the process is regulated at the transcriptional or translational level.

The use of normal peripheral blood T-cells precludes conclusions regarding the half-life of the mRNA and hence of the level of regulatory control. Many subsets of T-cells, all binding sheep erythrocytes, are present in this population and they may well have different biological time clocks when it comes to the biosynthesis of surface membrane proteins. Furthermore, these polyclonal cells are not synchronized. Consequently the observations (fig.3) that actinomycin-D or cordycepin arrest the recovery of further rosetting capacity by these cell populations is subject to alternative interpretations. An ongoing study of this matter in our laboratory utilizing a thymic hormone-inducible monoclonal leukemic cell line should facilitate the elucidation of these regulatory processes.

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