USE OF LIPOSOMES TO STUDY *c*AMP TRANSPORT BY LYMPHOCYTE MEMBRANES

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Cyclic nucleotides are known principally as intracellular mediators, but there is some evidence which points to the presence of receptors for them on the outer cell membrane. In primitive multicellular organisms receptors of this type are responsible for the regulation of mobility, proliferation, and differentiation of cells [4], and the presence of receptors for cyclic nucleotides on thymocytes and on other types of cells has been demonstrated in mammals [5, 9], and active transport of *c*AMP through the cell membrane also has been shown to be possible. Since transport of cyclic nucleotides through the membrane inside the cell may be an important regulatory mechanism of lymphocyte activation, the study of such mechanisms is undoubtly of great theoretical and practical interest.

To study the mechanisms of *c*AMP transport into lymphoid cells, we used a population of regulatory lymphocytes activated in the course of isoproterenol-induced salivary gland hyperplasia in mice [1], for these cells can be obtained in a highly purified form from regional lymph nodes in the neck at a time when a sharp increase in the *c*AMP content is observed in salivary gland tissues in response to isoproterenol. This phenomenon suggests that *c*AMP is a specific activator for this cell population.

To abolish the effect of endogenously synthesized *c*AMP and of the powerful intracellular mechanisms of *c*AMP degradation, we used a model system in which lymphocyte membranes were built into an artificial cell-like formation, or liposome. This approach to the use of liposomes as a convenient experimental tool was first used in the USSR in the Laboratory of Molecular Mechanisms of Allergy, under the direction of Academician of the Academy of Medical Sciences of the USSR A. D. Ado to study some properties of bacterial allergens [2].

EXPERIMENTAL METHOD

Experiments were carried out on 150 female BALB/*c* mice. To obtain activated lymphocytes the animals were given an intraperitoneal injection of 5 mg isoproterenol, 30 min later they were sacrificed, and activated lymphocytes were isolated from their lymph nodes by centrifugation in a low-density Ficoll gradient, as described previously [1]. Ability to activate a hyperplastic response in salivary gland cells was confirmed by syngeneic transfer of $5 \cdot 10^6$ cells into intact animals, followed by recording the weight of the salivary glands 24 h after injection of the cells. The phenotype of the activated lymphocytes was judged by disappearance of the effect of anti-SC- and anti-Thyl-serum; the anti-Thyl-serum (against T lymphocytes) was obtained by immunizing rabbits with mouse brain homogenate, as described in the literature [6], with adsorption by erythrocytes and bone marrow cells, whereas the anti-SC-serum (against precursors of T cells) was obtained by adsorption of antibrain serum by thymocytes until a negative reaction was obtained in the cytotoxic test in a dilution of 1:4 [3].

To obtain lymphocyte membranes the cells were homogenized in 10 volumes of a solution containing 1 mM $NaHCO_3$ 5 mM Mg ions, and 3.2 mM $CaCl_2$, with isolation of the membrane fraction by ultracentrifugation in a sucrose gradient, as described in [7]. To incorporate the membranes into liposomes the method of dialysis in the cold of a 2% Triton solution of membrane fraction, lecithin, and cholesterol, against 0.15 M KCl, pH 6.0, was used, employing the technique in [8]. The lipo

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TABLE 1. ELISA of Binding of Anti-SC- and Anti-Thyl-Sera by Liposomes

Serum	Liposomes	Optical density units
—	Control 1 (without membranes)	0,021±0,004
—	Control 2 (with thymocyte membranes)	0,022±0,007
—	Experimental (with lymphocyte membranes from lymph nodes)	0,017±0,003
Normal rabbit serum	Control 1	0,043±0,007
	Control 2	0,041±0,008
	Experimental	0,045±0,014
Anti-SC-serum	Control 1	0,035±0,005
	Control 2	0,040±0,007
	Experimental	0,109±0,011*
Anti-Thyl-serum	Control 1	0,035±0,03
	Control 2	0,122±0,014*
	Experimental	0,040±0,019

*$p < 0.01$.

TABLE 2. Effect of Anti-SC-Serum on ^{3}H-*c*AMP Transport from Liposomes

Liposomes	Serum	Radioactivity of samples (cpm)
Control (without membranes)	—	13 897±818
	Normal rabbit serum	39 868±1407
	Anti-SC-serum	44 579±392
	Anti-Thyl-serum	41 434±415
Control (with thymocyte membranes)	—	11 827±820
	Normal rabbit serum	28 557±1275
	Anti-SC-serum	25 434±1122
	Anti-Thyl-serum	24 455±1257
Experimental (with lymphocyte membranes)	—	4 750±608
	Normal rabbit serum	21 362±704
	Anti-SC-serum	12 182±1340*
	Anti-Thyl-serum	20 127±988

*$p < 0.001$.

somes thus obtained were washed by centrifugation and were suspended in Hanks' solution, pH 7.0, in samples containing 1.5-3 mg of the preparation, were used.

The presence of SC-reactive protein in the composition of the liposomes was recorded by ELISA. For this purpose 5 mg of liposomes was incubated with anti-SC-serum in a dilution of 1:10 for 1 h at room temperature, and then washed with a solution containing 2 mg/liter of bovine serum albumin and 0.05% Tween-20, 5 times for 5 min each time at 3000 rpm, and finally with anti-immunoglobulin serum, labeled with horseradish peroxidase, under similar conditions. Activity of liposome-bound peroxidase was recorded in the reaction with *o*-PDA and H_2O_2 by photometry.

To record the *c*AMP inflow, up to 10^{-7} M ^{3}H-*c*AMP (from "Izotop," Leningrad) was added to the samples, which were incubated for 15 min and then washed quickly 3 times, for 3 min each time, at 5000 rpm. The outflow of ^{3}H-cAMP was recorded after incubation for 90 min by centrifuging the samples 3 times, with 10-min intervals, under the same conditions.

The radioactivity of the samples was counted on a "Rack-Beta" liquid scintillation counter.

The results were subjected to statistical analysis by Student's test.

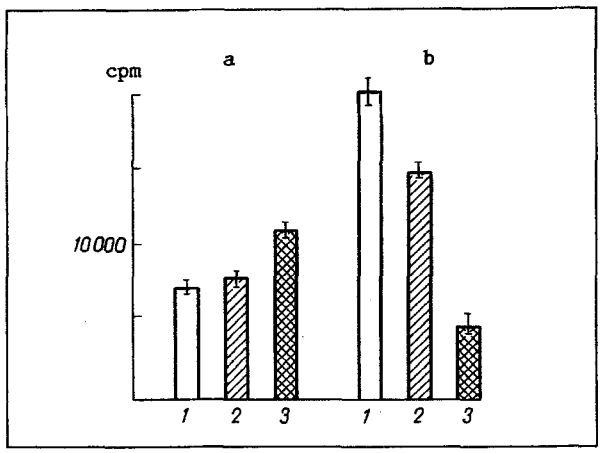

Fig. 1. Transport of ^{3}H-*c*AMP through wall of intact liposomes and liposomes modified by lymphocyte membranes. a) Study of inflow of ^{3}H-*c*AMP, b) study of outflow of ^{3}H-*c*AMP: 1) intact liposomes, 2) liposomes containing thymocyte membranes, 3) liposomes containing lymphocyte membranes from cervical lymph nodes (experimental). Abscissa, conditions of reaction; ordinate, radioactivity of samples (cpm).

EXPERIMENTAL RESULTS

Injection of 5 mg isoproterenol intraperitoneally into the mice was followed, in agreement with data in the literature, by hyperplasia of the submandibular salivary glands by 1.45-1.62 times 24 h after injection of the drug. The number of lymphocytes with a density of 1.07-1.08, among which 27-32% reacted with anti-SC-serum and 40-45% with anti-Thyl-serum in the complement-dependent cytotoxic test, increased by 2.5-3 times in the regional lymph nodes in the neck 30 min after injection of the isoproterenol (the time of the peak *c*AMP level in the gland tissues). After intravenous injection of the cells into intact animals, the mass of salivary glands in them increased by 23-32% after 24 h ($p < 0.01$), which was not observed after injection of lymph-node and spleen cells or thymocytes. The reaction was completely abolished by treating the cells with anti-SC-serum and complement, and depressed by 27-35% by treatment with anti-Thyl-serum and complement.

The use of ELISA showed that liposomes containing SC membranes plus lymphocytes, but not thymocyte membranes (control) bind anti-SC-serum (Table 1).

Addition of ^{3}H-*c*AMP to the liposome samples was accompanied by accumulation of the nucleotide in the vesicles, a phenomenon which ought to reflect both the physicochemical properties of the process of diffusion of the low-molecular-weight substance through the lipid membrane, and also processes of active membrane transport, if such exists. During quick washing and brief incubation (15 min) the liposome preparations containing membranes of SC-lymphocytes became much more able to accumulate ^{3}H-*c*AMP than control liposomes or liposomes with thymocyte membranes, evidence of the existence of active membrane transport into liposomes (Fig. 1).

During incubation for 90 min and prolonged washing with 10-min intervals between washings, creating conditions favorable for the outflow of stored *c*AMP, the ^{3}H-*c*AMP content was, on the other hand, smaller for SC-containing liposomes, i.e., active transport by membranes incorporated into a liposome takes place in both directions (Fig. 1).

To study the effect of anti-SC-serum on transport of ^{3}H-*c*AMP into liposomes, this serum or the control serum of intact animals was added to the samples together with ^{3}H-*c*AMP (Table 2). Under these conditions, and when outflow of ^{3}H-*c*AMP from liposomes was being recorded, the normal and anti-SC-serum increased accumulation of ^{3}H-*c*AMP equally by liposomes not containing membranes, evidently because of stabilization of the liposomes. For membrane-containing liposomes the anti-SC-serum differed significantly in its action from the control, evidence that SC-structures are involved in *c*AMP transport into the liposome.

The effect of anti-SC-serum on liposomes containing membranes of lymphocytes with the SC-antigen could not be explained by an antigen-antibody reaction on the surface of the liposomes, for anti-Thyl-serum did not induce such an effect for liposomes containing thymocyte membranes carrying the Thyl-antigen (Table 2), although ELISA confirmed the presence of this antigen on the liposome membrane (Table 1).

Thus membranes of SC-positive lymphocyte can effect *c*AMP transport through the artificial liposomal membrane. They may also possess a similar function when in the composition of cells. Since we know that *c*AMP is a regulator of differentiation of SC-cells [3], it can be tentatively suggested that exogenous *c*AMP can exert a marked influence on the activity of these cells, which exhibit the properties of cells regulating proliferation in various experimental systems. It can also be postulated that the SC-antigen found on lymphocyte precursor cells may participate directly in *c*AMP transport through the cell membrane of these cells.

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Na,K-ATP-ASE ACTIVITY OF INDIVIDUAL STRUCTURAL COMPONENTS OF THE GUINEA PIG VISUAL SYSTEM DURING HYPOXIA

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The study of the ATPase systems in various organs and organelles during hypoxia has attracted great attention [2, 4, 7-9, 12]. The increased interest in this problem in recent years is connected with the fact that hypoxia leads to ATP deficiency and to disturbance of ionic gradients, which occupy a special place in pathogenetic injury to biological membranes [5]. Nevertheless, there are no data as yet on changes in ATPase activity in individual structural components of the visual system during hypoxia. These data are essential for our understanding of the physicochemical mechanism of cell damage in the visual system during exposure to hypoxia, for depression of the components of the electroretinogram (ERG) and of the visual cortical evoked potential in various types of hypoxia has been established [3, 10, 13, 14].

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