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EFFECT OF CYCLIC AMP-DEPENDENT PHOSPHORYLATION OF SHEEP'S RED BLOOD CELL MEMBRANES ON THEIR ABILITY TO INDUCE THE HUMORAL IMMUNE RESPONSE

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Cyclic AMP is known to participate in the regulation of antibody formation in vitro, and in proliferation and differentiation of lymphocytes stimulated by a mitogen or antigen [1]. The function of cyclic AMP as secondary mediator in the transmission of signals is that of activation of a series of protein kinases (PK) phosphorylating different protein substrates [7, 13-15]. Cyclic AMP-dependent phosphorylation of cell membranes of many tissues has been found to be at a low level [14, 15]. The high sensitivity of thymocytes and spleen cells, stimulated by mitogen or antigen, to concentration of exogenous or endogenous cyclic AMP at the beginning of culture [1] suggests that membrane proteins which are antigenic receptors may undergo cyclic AMP-dependent phosphorylation and that cyclic AMP-dependent PK may be located in the immediate vicinity of receptors for the antigen or mitogen. The present writers have also suggested that cyclic AMP-dependent phosphorylation may also influence activity of the antigenic determinants of various cellular antigens on account of changes in the charge and/or conformational changes in their membranes.

The object of the present investigation was to study the possible effect of phosphorylation and of activity of cyclic AMP-dependent and cyclic AMP-independent membrane PK of sheep's red blood cells (SRBC) on the antigenic activity of those membranes in the induction of the humoral immune response in mice.

EXPERIMENTAL METHOD

Experiments were carried out on female (CBA × C57BL/6)F_1 mice obtained from the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR.

ATP and (γ-^{32}P)ATP, with a specific activity of 60 Ci/mmole, were obtained from the All-Union Research Institute of Molecular Biology, Head Office of the Microbiological Industry, Academy of Sciences of the USSR. Mice were immunized intravenously with 0.5 ml of a 5% suspension of intact (ISRBC) or phosphorylated SRBC (PSRBC). On the 4th, 5th, and 6th days after immunization the animals were killed and the number of antibody-forming cells (AFC) estimated from the number of plaque-forming cells (PFC) in the spleen by the modified method of local hemolysis in semiliquid medium [3]. The tests for determining the number of PFC were carried out after addition of ISRBC or PSRBC to the reaction medium. The significance of differences was assessed by Student's t-test.

Phosphorylation of SRBC was carried out by the method described in [12], with induction of synthesis by cyclic AMP. The SRBC were washed with medium No. 199 and then with an incubation medium containing 0.03M Tris-HCl buffer, pH 7.5, 0.15M NaCl, 10 mM $MgCl_2$, 10 mM CH_3COONa, 0.3 mM EDTA, 2 mM theophylline, 5 mM NaF, 0.1 mM Na_3PO_4, and 2 μM ATP. The cell residue was resuspended in this same medium to a concentration of 1×10^8 cells/ml. Incubation was carried out at 37°C for 30 min, after which the cells were cooled to 4°C, washed in medium No. 199, and injected as a 5% suspension into mice.

Phosphorylation of SRBC was verified from the incorporation of ^{32}P into cellular material insoluble in TCA [12].

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TABLE 1. Phosphotransferase Activity of Extract of ISRBC and PSRBC Membranes (in nmoles ^{32}P transferred by 1 mg of enzymes to 1 mg substrate per minute)

Substrate	Cyclic AMP (5 μM)	Activity of membrane PK (M ± m)	
		ISRBC	PSRBC
Calf thymus histone H3	—	0,8±0,05	0,2±0,01
	+	0,9±0,06	0,3±0,01
Bovine serum albumin	—	0,5±0,02	0,2±0,01
	+	0,5±0,01	0,2±0,01
Structural proteins of SRBC membranes	—	7,5±0,6	2,8±0,1
	+	7,0±0,4	2,8±0,1

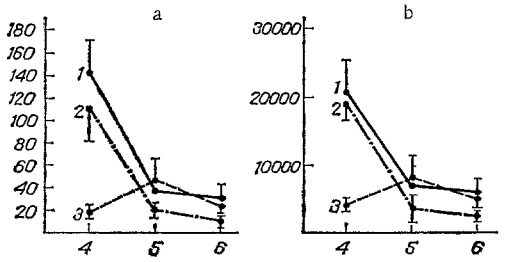

Fig. 1. Number of AFC in spleen of (CBA × C57BL/6)F_1 mice immunized with ISRBC or PSRBC. Abscissa, days after immunization; ordinate, number of AFC. a) Number of AFC per 10^6 cells; b) number of AFC per spleen. 1) Number of AFC in mice immunized with ISRBC; 2) number of AFC in mice immunized with SRBC, incubated in medium No. 199 at 37°C for 30 min; 3) number of AFC in mice immunized with PSRBC. Data obtained on 4th day after immunization are mean results of eight experiments, whereas data for 5th and 6th days are mean results of two experiments (on five mice in each group).

Isolation of the membranes from SRBC, extraction of PK from the SRBC membranes, and determination of their activity were carried out by the methods described in [4, 15].

Application of aliquots to the filters and their subsequent processing were carried out as in [13]. Nonenzymic structural membrane proteins were isolated by the method in [5] by extraction from the SRBC membranes with buffer containing 5 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 3 M urea. Calf thymus histone H3 was obtained as in [9] and purified as in [8]. The protein concentration in the extracts was determined as in [10], using bovine serum albumin as the standard.

EXPERIMENTAL RESULTS

Incubation of SRBC in medium containing 2 mM ATP and agents inducing cyclic AMP synthesis led to incorporation of 1.3 pmoles ^{32}P into material of 10^8 cells insoluble in TCA. These results confirmed those of investigations by other workers who found a low level of cyclic AMP-dependent phosphorylation of endogenous membrane proteins [7]. Both ISRBC and PSRBC underwent lysis and activity of cyclic AMP-dependent and cyclic AMP-independent isolated and purified membranes was determined. The results showed that the extracted PK from the membranes carried out cyclic AMP-dependent phosphorylation of the nonspecific substrate histone H3 and cyclic AMP-independent phosphorylation of bovine serum albumin and of structural membrane proteins (Table 1). Phosphorylation of SRBC in the presence of physiological concentrations of ATP

and induction of synthesis by cyclic AMP led to inhibition of both cyclic AMP-independent PK (by more than half) and of cyclic AMP-dependent PK (by two-thirds). Considering data in the literature [14] indicating the sharply increased ability of phosphorylated cyclic AMP-dependent PK to reassociate (and, consequently, to be inactivated), the preliminary assumption can be made that phosphorylation of SRBC in the presence of physiological concentrations of ATP led to autophosphorylation of the cyclic AMP-dependent PK of the SRBC membranes, as other workers also have suggested [2].

Investigations into the effect of phosphorylation of SRBC on their ability to induce the accumulation of AFC in the mouse spleen gave the following results (Fig. 1). The relative and absolute numbers of PFC in the spleen of animals immunized with PSRBC were more than two-thirds lower than the values for control animals immunized with ISRBC. Moreover, the peak of AFC during immunization of the mice with PSRBC was shifted to the 5th day, whereas in the control animals it was observed on the 4th day. It was also found that on addition of ISRBC to mouse spleen cells immunized with ISRBC the number of PFC in the chamber was 1.5 times greater than when PSRBC were added to the incubation medium (the number of PFC was 225.4 ± 25 and 149 ± 14 respectively). The local hemolysis test with spleen cells of animals immunized with PSRBC did not reveal any such differences (the number of PFC was 34.8 ± 7 when ISRBC were used in the tests and 28.1 ± 6 when PSRBC were used).

The results suggest that the depressed immune response to PSRBC may be the result of inactivation of a certain percentage of antigenic determinants located on SRBC, which participate in induction of the accumulation of AFC in the spleen. Inactivation of the antigenic determinants of PSRBC is also possible because of changes in the charge and/or of conformational changes in the phosphorylated SRBC cell membrane. For the same reason the possibility of changes in the resistance of the PSRBC membranes to the action of macrophagal enzymes and/or to the antibody–complement complex cannot be ruled out. To some degree this is confirmed by the results showing depressed cyclic AMP-independent phosphorylation of structural membrane proteins of PSRBC and the consequently altered permeability of those membranes. Changes in permeability of the PSRBC membranes may also have a definite influence on activity of the protein antigenic determinants of SRBC through a delegated chain of events on the membrane. However, this physiological process is readily reversible and can bring about only a very small shift of the peak of the immune response, the height of which is not less than that of the peak in the control animals. It can accordingly be tentatively suggested that the main cause of the lowering of the immune response to PSRBC was evidently inactivation of a certain percentage of the antigenic determinants of PSRBC due to changes in the charge on the membranes of these cells and/or conformational changes in them in the course of phosphorylation. Reduced activity of the cyclic AMP-dependent phosphotransferase reactions of the PSRBC membranes led to inhibition of the reverse process of dephosphorylation, not excluded after addition of PSRBC. Accordingly the immune response to PSRBC was enhanced only very slightly on the 5th day and it fell sharply again by the 6th day after immunization.

It can be concluded from these results that cyclic AMP-dependence of immune processes may be based both on the cyclic AMP-dependent state of receptors for antigens and on the state of the antigenic determinants of the antigen itself, and that activity of the latter may be largely determined by their phosphorylation–dephosphorylation. Research in this direction is continuing.

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