REGULATION OF THE MOUSE B LYMPHOCYTE CELL CYCLE BY SUBSTANCES RAISING INTRACELLULAR cAMP and cGMP LEVELS

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Proliferation of lymphocytes is a very important element in the development of the normal immune response. According to modern theories of immunity, this process lies at the basis of the formation of an antigen-specific clone of lymphocytes and it determines the intensity of the immune response [2, 6]. It has been suggested that entry of the cells into the phase of DNA synthesis and its emergence from that phase are the limiting factors which are under the control of various regulatory factors, including T helper and suppressor lymphocytes [6, 13]. At the same time, the view is held that cyclic nucleotides play a central role in the regulatory effects of the various mediators of immunity [7, 8]. However, there is no direct evidence on the effect of cyclic nucleotides on passage of lymphocytes through the cell cycle.

The aim of this investigation was to study the effect of acetylcholine, which increases the cGMP concentration in cells [12], and of adrenalin, which increases the cAMP concentration in cells [5], on the passage of mouse B lymphocytes through the phases of DNA synthesis in the course of sensitization of animals with the protein antigen ovalbumin, proceeding from data showing that these substances have an immunoregulatory effect [11, 15].

EXPERIMENTAL METHOD

Experiments were carried out on 150 male C57BL/6 mice weighing 20-25 g. The animals were sensitized by intraperitoneal injection of 250 μg ovalbumin with 5 mg of aluminum hydroxide gel. The animals were used in the experiments on the 3rd day of sensitization — at the peak of the proliferative reaction [1]. Spleen cells were obtained by gentle homogenization of the spleen in medium 199 and filtration through nylon. The cells were then treated with anti-Thy-serum and complement to produce lysis of the T cells. A suspension of B lymphocytes was obtained by centrifugation in a Ficoll—Verografin density gradient [9]. Anti-Thy-serum was obtained by immunizing rabbits with mouse brain homogenate [10]. To obtain B-lymphocytes in different stages of the cell cycle, the method of spontaneous sedimentation of cells of different diameters was used [14]. The reaction of incorporation of [^{14}C]methionine was carried out with cells of the different fractions to estimate protein synthesis, and incorporation of [^{3}H]thymidine to estimate DNA synthesis, for which purpose 10^6-2 × 10^6 cells of the different fractions were incubated in medium 199 at 37°C with 1 μCi of [^{14}C]methionine and 1 μCi of [^{3}H]thymidine. After incubation for 1.5 h the cells were washed to remove unbound label twice with Hanks' solution and once with 10% TCA solution, the residue was dissolved in 0.2 ml of 1% Triton X-100, and radioactivity was counted by a double label counting program on a Merck-III scintillation Beta-Counter. When determining the effect of the neurotransmitters, acetylcholine and adrenalin, concentrations of 1 μM were incubated with lymphocyte fractions under analogous conditions and the results were expressed as percentages of action of the substance:

$$\frac{\text{Incorporation of } ^{3}\text{H-thymidine with substance}}{\text{Incorporation of } ^{3}\text{H-thymidine without substance}} \times 100\%.$$

To assess the effects of the neurotransmitters on mitogen-stimulated lymphocytes, spleen cells of intact mice were incubated for 36 h with 10 μg of lipopolysaccharide (LPS) — (B-mi-

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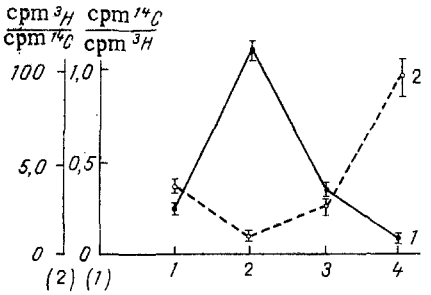

Fig. 1

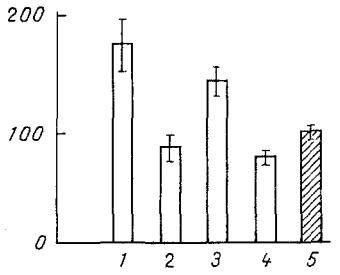

Fig. 2

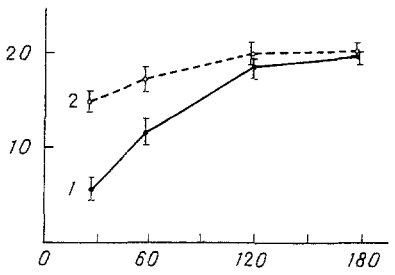

Fig. 3

Fig. 1. Effect of neurotransmitters on DNA synthesis in mouse B lymphocytes, separated by the spontaneous sedimentation method. Abscissa, nos. of fractions; ordinate, effect of substances (in %). 1) Acetylcholine, 2) adrenalin.

Fig. 2. Effect of acetylcholine on DNA synthesis in mouse B lymphocytes under different conditions. Ordinate, effect of acetylcholine (in % of control). 1) Medium 199; 2) in presence of EDTA (1 mM) without acetycholine; 3) in presence of EDTA (1 mM) with 1 μM acetylcholine; 4) in presence of trifluoroperazine without acetylcholine; 5) in presence of trifluoroperazine (1 μM with 1 μM acetylcholine. Mice sensitized with 5 μg ovalbumin, 3rd day after beginning of sensitization.

Fig. 3. Effect of acetylcholine on frequency of mitoses of splenic B lymphocytes of sensitized mice. Abscissa, time (in min); ordinate, mitotic index (in %). 1) Background values; 2) 1 μM acetylcholine.

togen), after which the cells were treated with anti-Thy-serum and complement and the effect of acetylcholine was determined as described above.

To determine the effect of acetylcholine on emergence of the cells from the state of DNA synthesis, the number of cells in a state of mitosis was determined in the usual way by staining with azure-eosin and incubating the cells for 1-4 h in the presence of colchicine [4].

The results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

As was shown previously sensitization of the mice with ovalbumin was accompanied by an increase in proliferation of splenic B lymphocytes of the mice with peak incorporation of radioactive thymidine on the 3rd day of sensitization [1].

Investigation of incorporation of labeled precursors into the different fractions of B lymphocytes separated by the spontaneous sedimentation method at this time showed that the slowly sedimenting fraction (No. 2) incorporated mainly amino acids, and was thus in the G_1 phase of the cell cycle, whereas the quickly sedimenting cell fraction incorporated mainly thymidine, and was thus in the S phase of the cell cycle. Acetylcholine caused a marked increase in incorporation of [^{3}H]thymidine into the acid-insoluble pool of the slowly sedimenting lymphocyte fraction from sensitized animals, but inhibited statistically significant incorporation into the quickly sedimenting fraction (Fig. 1). Adrenalin, in the presence of the α-blocker phentolamine, had the opposite action (Fig. 1).

Acetylcholine and adrenalin did not affect [^{3}H]thymidine incorporation into B lymphocytes of intact mice. Sensitivity to acetylcholine, determined relative to effect on [^{3}H] thymidine incorporation, appeared in the B lymphocytes after culture of the spleen cells for 36 h with LPS (B mitogen) (Table 1): An increase in protein synthesis, but not DNA synthesis, was observed at this time.

The effect of acetylcholine was reduced only negligibly by binding of calcium ions in the medium with EDTA, but it was completely abolished when trifluoroperazine, a calmodulin blocker, was used (Fig. 2).

Determination of the number of mitoses in B lymphocytes in sensitized animals showed an increase in their frequency during incubation for 3 h in the presence of colchicine, preventing emergence of the cells from the phase of mitosis (Fig. 3). Acetylcholine increased the frequency of mitoses, but only during the first hour. After incubation for 2 h the effect of acetylcholine disappeared. Adrenalin reduced the frequency of mitoses at all times.

TABLE 1. Action of Acetylcholine on Mouse B Lymphocytes Activated for 36 h with LPS

Cells	Incorporation of, cpm	
	[^{3}H]thymidine	[^{14}C]methionine
Intact	3038±288	15 887±258
Intact + acetylcholine	3015±101	18 872±578
Activated by LPS	3434±672	22 134±1 708*
Activated by LPS + acetylcholine	7289±704**	21 520±1 801

Legend. *P < 0.05; **P < 0.001.

Acetylcholine thus stimulated passage of B lymphocytes through the cell cycle as a whole: It stimulated the beginning of DNA synthesis in cells in the G_1-phase of the cycle, which had not yet started DNA synthesis (stimulated the G_1/S transition) and stimulated emergence of the cells from the S-phase into mitosis. Abolition of the effect by trifluoroperazine indicates that stimulation of proliferation took place by calcium mechanisms, but not by extracellular calcium, for its binding by EDTA had little effect on the action of acetylcholine. It is likely that cAMP can raise the Ca^{++} level in the cytosol by redistributing intracellular calcium, as is the case for cAMP and thymocytes and certain other types of cells [7, 15].

Since the effect of acetylcholine was most marked during the first hours of its action, and later tended to disappear, elevation of the cGMP level by acetylcholine evidently facilitates more rapid G_1/S transition for cells in which this transition has already been triggered. Acetylcholine thus does not recruit new cells, but stimulates transition of B lymphocytes in the G_1 phase of the cell cycle into the phase of proliferation. The view is expressed in the literature that the G_1-cycle may be divided into two periods: the so-called G_{1C}+, dependent on the presence of serum and other agents stimulating proliferation in the medium, and the G_{1C}- period, independent of these agents. Cells in the G_{1C}- period can enter the phase of DNA synthesis spontaneously, because the G_1/S transition is already triggered in them [3].

The duration of the G_{1C}- period is about 2 h, which is in good agreement with the duration of the acetylcholine effect in the present investigation.

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