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CHOLINERGIC RECEPTORS OF RAT LYMPHOCYTES DURING ADJUVANT POLYARTHRITIS

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Evidence for muscarinic receptors was provided by [^{3}H]quinuclidinylbenzilate binding to rat lymphocytes and the competitive inhibition of the radiolabelled ligand binding by a specific muscarinic antagonist, atropine. An increase in the amount of ligand binding to the adjuvant polyarthritis lymphocytes was observed in the protracted inflammatory phase of the disease, whereas competitive inhibition of the [^{3}H]quinuclidinylbenzilate binding by atropine remained unchanged. An increase in the number of muscarinic acetylcholine binding sites of adjuvant polyarthritis lymphocytes during the acute inflammatory phase is suggested.

Introduction

Cholinergic receptors associated with non-neural and non-innervated cells have been shown in erythrocytes [1], murine lymphocytes [2], human lymphocytes [3] and rat lymphocytes [4], as demonstrated by direct binding assay. Cholinergic receptors, stimulated selectively by muscarine and blocked by such agents as atropine, have been described as the 'muscarinic' type. A direct binding assay of muscarinic receptors has been provided by using [^{3}H]quinuclidinylbenzilate, a specific, potent and long-lasting muscarinic agent [5,6].

We have examined the specific binding of [^{3}H]quinuclidinylbenzilate to rat lymphocytes during the protracted inflammatory phase of experimentally induced adjuvant polyarthritis.

Materials and Methods

Animals

For all experimental and control groups, 5 3-month-old female Wistar strain rats, initially weighing 150–200 g were used. Adjuvant polyarthritis was induced by intradermal injection of 0.1 ml Freund's complete adjuvant suspended in paraffin oil (6.0 mg/ml) into the foot pad of the left hind paw. The arthritis score was used as an index of the severity of the adjuvant polyarthritis and was determined by observation of extent of swelling, lesions and redness of each joint and the tail, with scores of 0 to 4 being assigned to each [7]. The maximum score for one animal was 20.

Cells

Lymphocytes were collected by removing and fragmenting the lymph nodes draining the sites of the adjuvant injection, as previously described [8]. The submaxillary, axillary and inquinal lymph nodes were collected from the control animals. The cells were resuspended in Parker 199 medium (Lublin, Poland) supplemented with 5% calf serum (Lublin, Poland), and adjusted to $2 \cdot 10^8$ cells/ml at 4°C. Nucleated cells were counted in a haemocytometer and their viability checked by means of the 0.2% Trypan blue test.

Lymphocyte cultures

Stimulation of the lymphocytes during adjuvant

polyarthritis was measured by in vitro culturing of the cells in 0.2 ml microplates (Linbro). The cells were resuspended in Parker 199 supplemented with 20% calf serum, 0.3% glutamine (Reanal, Hungary) and canamycine (Sigma). The cells ($5 \cdot 10^5$ cells per well) were incubated for 24 h in an Assab incubator at 37°C, 5% CO_2, 100% humidity, with the addition of 0.2 μCi/well of [^{3}H]thymidine (UVVVR, Czechoslovakia, spec. act. 2 Ci/mol), 4 h before the end of the culturing. The incorporation of [^{3}H]thymidine into DNA was determined by counting the radioactivity of filters containing material insoluble in 10% trichloroacetic acid. The stimulation index was calculated as a ratio of the radioactivity of adjuvant polyarthritis cells and control cells. The data presented are the mean of quadruplicate samples ± standard deviation.

Binding experiments

Lymphocytes ($2 \cdot 10^7$ cells) were incubated in 200 μl of Parker 199 supplemented with 5% calf serum, at 37°C in an Assab incubator with 20 μl of atropine sulfate (Calbiochem) at a final concentration of 10^{-4} M or with 20 μl of Parker 199, added for 10 min. The radiolabelled ligand, [^{3}H]quinuclidinylbenzilate (Amersham, spec. act. 8.4 Ci/nmol), in 20 μl solution was added for 2 min at 37°C. 50-μl aliquots of the cell suspension were immediately centrifuged through 5 ml of Parker 199 layered on the top of a 1 ml mixture of Ficoll-uropoline (9% Ficoll, Pharmacia and 34% uropoline (Polfa, Poland) in a 2.4:1 ratio), 2-fold diluted with phosphate-buffered saline (Lublin, Poland) at 4°C. After this one-step centrifugation, the cells were dissolved in 0.25 ml of Nuclear Chicago Solubiliser (NCS, Amersham/Searle) and the radioactivity of the samples was measured in a toluene scintillator. All data presented are the mean of quadruplicate samples ± standard deviation.

Results

The binding of [^{3}H]quinuclidinylbenzilate to lymphocytes, stimulation index of polyarthritis cells and arthritis score were determined during the protracted inflammatory period of adjuvant polyarthritis. This period included the nonimmune inflammatory phase characterized by the rapid swelling of the injected feet but with no significant swelling in the uninjected feet. The onset of adjuvant polyarthritis was signified by the appearance of marked swelling in all extremities, occurring between 7 and 17 days after injection. The sustained phase of the disease begins at about day 18 of the disease. The recovery phase of the disease was not examined. The maximal stimulation index of adjuvant polyarthritis cells was observed on the seventh day after adjuvant injection, as determined by [^{3}H]thymidine incorporation (Fig. 1), whereas the maximal severity of the disease was observed on the seventeenth day after adjuvant injection, as determined by the arthritis score (Fig. 2).

During the nonimmune inflammatory period no significant change in the [^{3}H]quinuclidinylbenzilate binding to lymphocytes was observed, nor in the inhibition of the ligand binding by atropine. During the onset of the disease, binding of [^{3}H]quinuclidinylbenzilate to adjuvant polyarthritis cells was significantly higher ($P \leq 0.001$ in Stu-

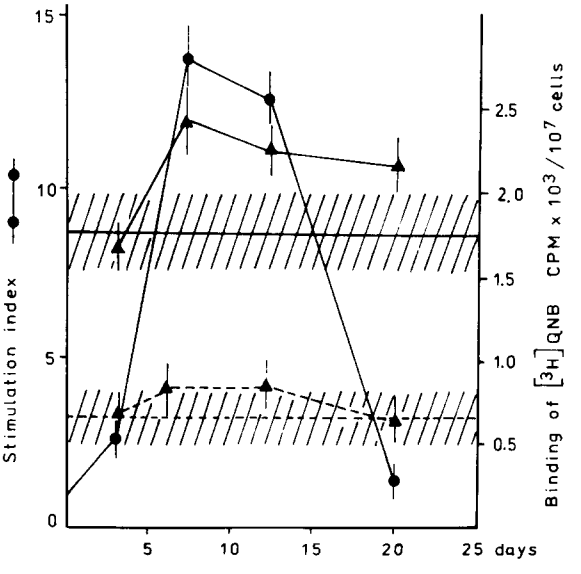

Fig. 1. Binding of [^{3}H]quinuclidinylbenzilate to lymphocytes during adjuvant polyarthritis: ———, control cells; — — — atropine-pretreated control cells; ▲———▲, adjuvant polyarthritis cells; ▲— — —▲, atropine pretreated adjuvant polyarthritis cells. The line: ●———● represents the stimulation index expressed as the ratio of the [^{3}H]thymidine radioactivity of adjuvant polyarthritis cells and control cells. Cross-hatching and the bars describe the standard deviation of the mean of four samples.

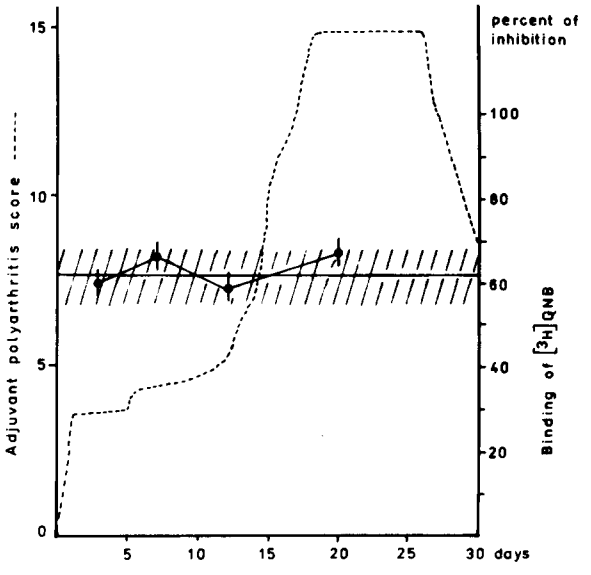

Fig. 2. Inhibition of the binding of [^{3}H]quinuclidinylbenzilate to control lymphocytes (———) and adjuvant polyarthritis cells (●———●) by atropine during adjuvant polyarthritis. The line: ------ describes the adjuvant polyarthritis score representing the severity of the disease. Results express percent of inhibition. Cross-hatching and the bars describe the standard deviation of the mean of four samples.

dent's *t*-test), as compared with the ligand binding to control lymphocytes (Fig. 1). The binding of [^{3}H]quinuclidinylbenzilate to polyarthritis lymphocytes pretreated with atropine, however, was not significantly different from that seen with control cells. Furthermore, no significant difference of the atropine-induced inhibition of ligand binding by polyarthritis cells and by control cells was observed during the examined period of the disease (Fig. 2).

Discussion

Specific binding of the muscarinic ligand, quinuclidinylbenzilate, to the lymphocyte surface requires the presence of specific cholinergic receptors of the muscarinic type. Evidence for muscarinic receptors is provided by the [^{3}H]quinuclidinylbenzilate binding observed and its competitive inhibition by a specific muscarinic antagonist, atropine, as we have previously shown for rat lymphocytes [4]. Similar results were obtained for murine lymphocytes [2]. Our results for muscarinic ligand binding to adjuvant polyarthritis lymphocytes show the increase of [^{3}H]quinuclidinylbenzilate binding during the acute period of the disease, but not during the nonimmune inflammatory period (phase I, Fig. 1). This result suggests that the increase of [^{3}H]quinuclidinylbenzilate binding by adjuvant polyarthritis lymphocytes is specific to the immune stimulation of lymphocytes during the acute phase of the disease. Since we found that competitive inhibition of [^{3}H]quinuclidinylbenzilate binding by cells pretreated with atropine remained unchanged during the disease (Fig. 2), we conclude that the total number of binding sites of the muscarinic ligand per cell increased during the protracted phase of the adjuvant polyarthritis. However, another possibility exists. The population of adjuvant polyarthritis lymphocytes could have been enriched by the cells bearing the muscarinic receptor. This question is planned to be resolved by checking the difference in acetylcholine receptor distribution within the distinct subpopulations of lymphocytes.

There are some indirect data supporting an increased sensitivity of stimulated lymphocytes to acetylcholine. We have previously shown that the cholinergic stimulation in vitro of lymphocytes was obtained only when the cells had previously been subjected to suboptimal doses of phytohemagglutinin [9]. Both stimulators affecting lymphocyte responses, phytohemagglutinin and acetylcholine, interact. Their effect is synergistic when acetylcholine is added soon after stimulation of lymphocytes with mitogen [9]. In lymphocytes stimulated previously with phytohemagglutinin, acetylcholine produced changes in RNA synthesis [10].

Hadden et al. [11] found that an intensification of RNA and protein synthesis in normal lymphocytes and in phytohemagglutinin-stimulated lymphocytes was observed by an increase of cGMP acetylcholine. Atkinson et al. [12], however, did not observe either the changes in cGMP level in lymphocytes treated with acetylcholine and plant mitogens, or the effect of acetylcholine on the course of stimulation of lymphocytes by phytohemagglutinin, when mitogen and acetylcholine were applied simultaneously. This possibly altered the effect being studied [9]. Acetylcholine and/or the cholinergic agents have been shown to mod-

ulate the spontaneous and anti-Ig-stimulated motility of lymphocytes [13], enhance human mixed lymphocyte reaction [14] and initiate thymic lymphoblast DNA synthesis and proliferation [15]. Our finding of the direct relationship between lymphocyte immune stimulation and the changes in the level of the muscarinic receptors in cells during the acute phase of adjuvant polyarthritis further suggests the functional importance of cholinergic modulation of the lymphocyte response [9–11, 13–15]. It may be assumed that the relationship observed between muscarinic receptor level and lymphocyte stimulation during the acute phase of adjuvant polyarthritis as determined in vitro reflects the in vivo mechanisms of immunological cellular response.

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