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PHOTOBLEACHING RECOVERY STUDIES OF MEMBRANE EVENTS ACCOMPANYING LECTIN STIMULATION OF RABBIT LYMPHOCYTES

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SUMMARY

Fluorescence photobleaching recovery methods reveal marked changes in lateral mobilities of rabbit lymphocyte membrane components during the course of stimulation with succinyl concanavalin A (S Con A). The diffusion constant of S Con A receptors on T lymphocytes falls from 1.6×10^{-10} cm^2/sec to 6.5×10^{-11} cm^2/sec within 4 hr after stimulation, remains constant for 14 hr, and returns to its former value. The mobility of B cell receptors similarly falls from 1.4×10^{-10} cm^2/sec to 5.5×10^{-11} cm^2/sec but regains its unstimulated value much more slowly. In contrast, a fluorescent phospholipid analog shows constant mobilities of 1.9×10^{-8} cm^2/sec and 1.5×10^{-8} cm^2/sec in T and B cells, respectively, throughout the experiment.

INTRODUCTION

The initial event in lectin activation of lymphocytes is the physical attachment of the mitogen to receptors on the cell membrane. The mitogen need not be internalized (1,2) but must remain on the cell surface for an extended period (3). While binding of the lectin is necessary for stimulation, it is not sufficient since Con A binds equally well to both T and B lymphocytes (2,4), but stimulates only T cells. Activation must therefore be initiated by a more complicated process involving both the lectin and the membrane.

Efforts to observe via ESR (5,6) and fluorescence depolarization (7) lectin-induced changes in membrane viscosity during the

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Abbreviations: FPR, fluorescence photobleaching recovery; Con A, concanavalin A; S Con A, succinyl concanavalin A; diI, 3,3'-dioctadecylindocarbocyanine iodide; TMR, tetramethylrhodamine; ATS, antithymocyte serum; HBSS, Hanks balanced salt solution.

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course of cell activation have yielded inconclusive results. Changes in receptor mobility and distribution accompanying the activation process have been reported (4,8) and the essential nature of such changes proposed (4). Activation by the divalent S Con A is particularly interesting in this connection. While S Con A is fully as mitogenic as Con A (1,3), it does not induce receptor capping or patching. The mobility of receptor-bound S Con A should provide an indication of any modulation of receptor mobility essential to activation, this modulation being distinct from the gross receptor aggregation induced by Con A and most other lectins.

We report here the mobilities in T cells of Con A receptors and of the lipid analog diI during the course of activation by S Con A. We compare these receptor mobilities with those in B cells, to which S Con A binds without activating.

METHODS AND MATERIALS

Antithymocyte serum (ATS) was prepared from guinea pigs of the Hartley strain twice injected intraperitonically with approximately $4x10^8$ rabbit thymocytes. Two weeks after the second injection, serum was collected, absorbed five times with rabbit erythrocytes,and complement inactivated for 30 minutes at 56°C. S Con A was purchased from Vector Laboratories and used without further purification. 1.0mg samples were fluorescently labeled with tetramethylrhodamine isothiocyanate using the method of Rinderknecht (9). DiI was synthesized by the procedure of Sims et al. (10) and used as a working solution of 0.5mg/ml in absolute ethanol.

Lymphocytes were teased from adult New Zealand white rabbit spleens and T cells isolated by selective nylon wool absorption of B cells (11). B lymphocytes were purified by the addition of 5% ATS and complement in RPMI-1640 (Grand Island Biological Co.). Purities of fractionated populations were verified by [^{3}H]thymidine incorporation after 72 hours (table I).

Cultures of $1x10^6$ cells/ml were maintained in cultures of RPMI-1640 supplemented with 10% homologous serum under a 5% CO_2 atmosphere. Cell viability, measured by trypan blue exclusion, remained greater than 80% throughout the experiment. Multiple small samples of purified T or B cells were continuously incubated with 20µg/ml S Con A from the onset of the experiment. At specified times samples were labeled for 10 min at 37°C either with diI (5µg/ml) or with TMR-S Con A (20µg/ml) which exchanged with bound unlabeled S Con A. This latter procedure was used in preference to direct stimulation with TMR-S Con A to avoid possible internalization of fluorescent lectin. Cells were washed twice with fresh HBSS and read within 20 min after initial removal from the incubator.

Table I: [^{3}H]Thymidine Incorporation into Stimulated Lymphocytes[a]

	CELL TYPE		
Treatment[b]	T (cpm)	B (cpm)	Unfractionated (cpm)
+ S Con A	7600±560	395±120	3745±145
- S Con A	340±70	380±130	340±105

[a]2mCi[^{3}H]thymidine was added after 72 hrs of continuous incubation with S Con A. Cell samples were counted 12 hrs later.

[b]Fractionated cell samples were treated with 20μg/ml of S Con A to stimulate the responding lymphocytes. Purity of cell fractionations can be observed by comparison of T and B populations to unfractionated samples. Unfractionated rabbit spleen lymphocytes contain approximately equal numbers of T and B cells.

Diffusion constants were measured by fluorescence photobleaching recovery techniques introduced by Axelrod et al. (12). A detailed description of our experimental and data handling procedure is found elsewhere (13). Individual cells were examined under coverslip at 37°C on a thermal stage using a 50x water immersion objective. A Leitz Panphot microscope with an Ortholux Ploem fluorescence vertical illuminator was used with a custom photometer optically similar to the Zeiss MPM01. Flourescence was measured with an RCA 4832 photomultiplier tube coupled to a PAR 1108 photon counter. A Data General Nova 3/12 computer afforded on-line data analysis. A Coherent Radiation CR-2 laser was operated at 40mW output power at 488nm or 514nm. The laser output was separated by a beamsplitter into two parallel beams, one attenuated by neutral density filters and the other blocked by an electronic shutter. The beams were recombined with a second beamsplitter. The resultant 10μW interrogation beam was focused on the cell surface to a $1/e^2$ spot radius of 0.81 microns. Opening the shutter exposed this spot to the unattenuated beam. Diffusion constants were calculated from spot size and half-time of fluorescence recovery after photobleaching. Fluorescence recovery after bleaching averaged 90% for diI and 60% for TMR-S Con A.

RESULTS AND DISCUSSION

Con A receptors on both T and B lymphocytes undergo, within a 4 hr period after S Con A treatment, an almost 2-fold reduction in mobility (Fig. 1). Diffusion coefficients of 1.6×10^{-10} cm^2/sec and 1.4×10^{-10} cm^2/sec for T and B cells, respectively, start to decrease within the first hour after S Con A stimulation. By four hours, diffusion constants stabilize at 6.5×10^{-11} cm^2/sec and 5.5×10^{-11} cm^2/sec for T and B cells, respectively, which values remain stable for the following 12 hours. In contrast, receptors on unstimulated

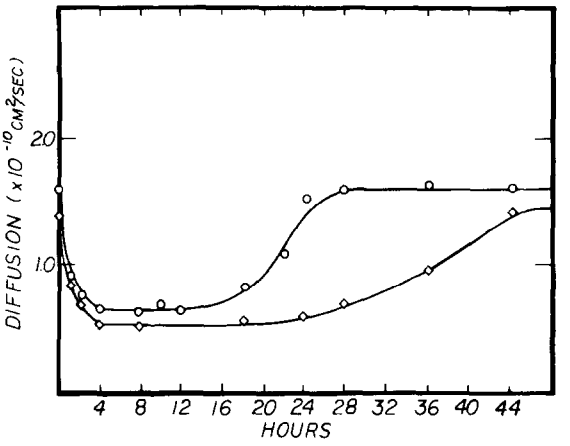

Figure 1: Diffusion coefficients of Con A receptors as a function of time after stimulation. Cell samples of both T and B rabbit lymphocytes were incubated with a stimulating dose of 20μg/ml of S Con A. At designated times, cells were labeled with 20μg/ml of TMR-S Con A. Each point is the average of measurements made on at least 20 individual cells at 37°C. Circles and diamonds indicate measurements on T and B cells, respectively.

cells labeled with TMR-S Con A at the times of measurements show no discernable changes in receptor diffusion throughout the 48-hr time course (table II). Moreover, the mobility of diI in the lipid matrix remains relatively constant at 1.9×10^{-8} cm^2/sec for T cells and 1.5×10^{-8} cm^2/sec for B cells (table III). Reduced mobilities of protein receptors therefore do not reflect gross changes in the surrounding lipid matrix. This general result supports Dodd's (5) and Barnett's (6) ESR results on lymphocyte stimulation by phytohemagglutin.

Cells treated for 30 min with .02 M azide of 1μM cyanide following 3 hrs of continuous stimulation regain the receptor mobilities of unstimulated cells. Cytochalasin B or colchicine treatment after brief stimulation produces a similar effect. These results suggest that Con A receptors undergo an energy-dependent restriction of mobility requiring between 3 and 4 hours to complete. Furthermore, they show that this lectin-induced process requires intact

Table II: Diffusion Constants of Con A Receptors on Unstimulated Lymphocytes[a]

	TIME IN CULTURE (in hours)					
	0	6	9	12	24	48
T Cells	1.92±.34	1.94±.43	1.83±.33	1.86±.33	1.90±.32	1.79±.29
B Cells	1.11±.25	1.08±.33	1.09±.26	1.06±.39	1.15±.31	1.32±.29

[a]Cells were fractionated and placed in culture but not stimulated. Samples were labeled with 20 g/ml TMR-S Con A immediately prior to measuring diffusion constants. Diffusion coefficients are given as 10^{-10} cm^2/sec. Standard errors given are standard errors of the mean.

Table III: Diffusion Constants of diI in Stimulated T and B Lymphocytes as a Function of Time after S Con A Addition[a]

	TIME (in hours)					
	0	6	9	12	24	48
T Cells	1.92±.26	1.96±.29	2.00±.19	2.00±.22	1.96±.19	1.89±.21
B Cells	1.43±.18	1.59±.19	1.67±.15	1.67±.18	1.65±.16	1.54±.21

[a] Cells were frationated and continuously incubated with 20µg/ml of S Con A. At designated times, the cells were labeled in 1 ml of HBSS with 1% of 0.5mg/ml diI in ethanol. Diffusion coefficients are given as 10^{-8} cm^2/sec. Standard errors given are standard errors of the mean.

cytoskeletal structures. These results are in general agreement with the photobleaching recovery results on myoblasts reported by Schlessinger et al. (8).

Between 18 and 24 hours after stimulation, T cells recover a receptor mobility characteristic of the unstimulated state, while B cells require a full 24 hours more. The significance of this particular period in the stimulation time course is two-fold. First, as shown by [^{3}H]thymidine incorporation, the onset of DNA synthesis is just then occurring. Second, after 24 hr washing the lectin from the surface no longer reverses the progression of cells toward blast transformation. In contrast, cells washed free of S Con A prior to this time show complete reversal (3).

In conclusion, we have demonstrated a direct membrane effect occurring upon lectin activation of T lymphocytes. This phenomenon can be reversed by metabolic inhibitors and cytoskeletal disruptors. If indeed commitment to proliferation occurs about 24 hours after stimulation, there may be a direct link between commitment and the apparently simultaneous release of receptor restriction.

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