Activation of Mouse Spleen Cells with Acetylated Concanavalin Coupled to Red Blood Cell Stroma (37093)

LEON S. KIND, HELEN WONG, AND SPENCER H. S. LEE

Department of Microbiology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia

There is currently much interest in how antigen must be presented to the lymphocyte surface to induce blast transformation, and in recent reviews both Moller (1) and Mitchison (2) stressed the importance of multipoint binding. Mitogens also are useful agents for studying events at the lymphocyte membrane: phytohemagglutinin (PHA) bound to red blood cells (RBC) potentiates lymphocyte stimulation (3), and mouse B cells can be stimulated by PHA bound to "Sepharose" particles (4) or concanavalin A (Con A) cross-linked to the bottom of tissue culture petri dishes (5) but not by soluble PHA or by soluble Con A (5). The following experiments show that the acetylation of concanavalin A eliminates its ability to stimulate murine splenic lymphocytes to undergo DNA synthesis. Activity was restored by coupling tritiated acetylated Con A ([^{3}H] AcConA) to RBC stroma.

Materials and Methods. Cell suspensions were made from spleens of SWR inbred mice raised in our laboratory from pairs obtained from Jackson Memorial Laboratory, Bar Harbor, ME. Pooled samples were from all males or all females. Cells were cultured by the method of Adler *et al.* (6). We prepared Con A by the procedure of Agrawal and Goldstein (7), and the New England Nuclear Corp., Boston, MA acetylated it with [^{3}H] acetic anhydride (8); specific activity of the [^{3}H]AcConA was 10 μCi/mg.

[^{3}H]AcConA was coupled to rabbit RBC stroma with chromic chloride (9). [^{3}H] AcConA (0.5 ml; 1 mg/ml) was mixed with 0.5 ml of 1% chromic chloride in 0.85% saline; 0.5 ml of packed rabbit RBC was added and the mixture was left at room temperature for 5 min. The RBC were lysed by three washes with distilled H_2O, and Con A per volume of stroma was estimated from the preparation's radioactivity. To rule out lymphocyte stimulation by rabbit RBC or

TABLE I. Activation of Spleen-Cell Cultures by Concanavalin Preparations.[a]

Preparation	Dose (μg/ml)	Response ([^{14}C]thymidine uptake, cpm)	
Tritiated acetylated Con A ([^{3}H]AcConA)	0.3	988 ± 16	(1250 ± 143)
	1.0	475 ± 65	(521 ± 165)
	1.6	922 ± 91	(1250 ± 143)
	4.0	2552 ± 61	(4045 ± 157)
Unmodified Con A	1.0	11,545 ± 850	(4045 ± 157)
Tritiated acetylated Con A coupled to RBC stroma ([^{3}H]AcConA–RBC)	0.3	2045 ± 50	(1250 ± 143)
	1.0	57,438 ± 1745	(521 ± 165)
	1.6	38,404 ± 5200	(1250 ± 143)
	4.0	26,457 ± 449	(4045 ± 157)
[^{3}H]AcConA–RBC with 0.06 *M* α-methyl mannoside	1.0	581 ± 14	(521 ± 165)

[a] Values in parentheses are background counts (duplicate tubes; no Con A added).

TABLE II. Binding of [^{3}H]AcConA to Spleen Cells.

Added			Bound	
Unmodified Con A	[^{3}H]AcConA		[^{3}H]AcConA	
	(μg)	(cpm)	(cpm)	(%)
0	8.0	16,854	542 ± 8	3.2
5.0	3.0	6849	157 ± 5	2.6

chromic chloride, ovalbumin coupled to RBC stroma with chromic chloride was added to lymphocyte cultures. No activation of lymphocytes occurred, and rabbit RBC suspensions alone, also, were inactive.

Binding of [^{3}H]AcConA to spleen cells was measured by a procedure described by Stobo, Rosenthal and Paul (10). [^{3}H]AcConA was added to 5×10^6 cells at 4° for 30 min, with and without unmodified Con A. Cells were suspended in 0.5 ml RPMI-1640 containing 0.1% sodium azide and 1% bovine serum albumin (BSA). After 30 min, binding was determined: the cells were washed with RPMI-1640 containing 5% BSA, and lysed with 0.5 ml of 1 *N* NaOH, and radioactivity was measured in a mixture of Liquifluor (New England Nuclear Corp.) and BBS-2 (Beckman).

Results. Table I shows that [^{3}H]AcConA failed to stimulate splenic lymphocytes at concentrations of 0.3 to 4.0 μg/ml. (In additional experiments, even in doses up to toxic concentration—50 μg/ml—it was non-stimulatory.) By contrast, 1 μg of unmodified Con A/ml induced an uptake of 11,545 cpm, and the same concentration of [^{3}H]AcConA coupled to RBC resulted in the incorporation of 57,438 cpm. Lymphocyte activation by [^{3}H]AcConA–Rbc was completely inhibited by 0.06 *M* α-methyl mannoside, a sugar that competitively inhibits the binding of Con A to its receptor sites on cells.

The failure of [^{3}H]AcConA to stimulate is not likely due to insufficient binding to lymphocytes, since its binding was similar to that of unmodified Con A (Table II): The addition of 8 μg of [^{3}H]AcConA to spleen cells resulted in 3.2% binding, whereas a mixture of 3 μg [^{3}H]AcConA with 5 μg of unmodified Con A resulted in 2.6% binding of the labeled substance.

The superiority of insoluble [^{3}H]AcConA–RBC over soluble unmodified Con A in inducing DNA synthesis in spleen-cell cultures is evident in Table III. Conditions for optimal stimulation were used. The optimal dose of Con A was 1 μg/ml in all experiments but the optimal dose of [^{3}H]AcConA–RBC was somewhat batch-specific, probably because of different spacing of the Con A molecules on the RBC stroma.

Discussion. The inability of [^{3}H]AcConA to stimulate lymphocytes cannot be attributed to toxic properties, since a count of viable cells 64 hr after incubation of cultures with the labeled acetylated Con A showed cell death no greater than with unmodified Con A. In addition [^{14}C] thymidine uptake was stimulated equally by Con A alone and by a mixture of [^{3}H]AcConA and unmodified Con A. [^{3}H]AcConA binds well to spleen cells (Table II); however, binding is a necessary but not a sufficient condition for stimulation. Thus, Con A binds as well to murine bone-marrow cells as to thymus and spleen cells, but B cells are poorly stimulated compared with thymus and spleen lymphocytes (10). Acetylation of Con A probably prevents the multipoint attachment of Con A necessary for stimulation, this deficiency being overcome when acetylated Con A is coupled to rabbit RBC stroma. It may be significant that acetylated flagellin has an enhanced ability to induce tolerance in rats, a property thought to be due to this agent's reduced

TABLE III. Enhanced Response of Murine Splenic Lymphocytes to Insoluble [^{3}H]AcConA–RBC[a] and Soluble Unmodified Con A.

Expt no.	Preparation	Dose (μg/ml)	Response ([^{14}C]thymidine uptake, cpm)
1	[^{3}H]AcConA–RBC	10	22,412 ± 449
	Con A	1.0	7500 ± 850
2	[^{3}H]AcConA–RBC	10	38,964 ± 1484
	Con A	1.0	15,294 ± 589
3	[^{3}H]AcConA–RBC	10	57,531 ± 4316
	Con A	1.0	16,787 ± 814

[a] Optimal dose was somewhat batch-specific, but the results shown here were obtained with three batches.

affinity for lymphocyte receptors (11, 12). Acetylated antibodies bind to antigen although they no longer precipitate with them (13, 14).

The experiments of Andersson *et al.* (5) perhaps offer the best explanation of our results. They found that locally concentrated Con A (chemically cross-linked to the bottom of petri dishes) stimulated synthesis in B cells whereas soluble Con A could not do so. Coupling of [^{3}H]AcConA to RBC likely results in similar local concentrations of this mitogen.

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