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In Vitro Stimulation of Hamster Lymphocytes with Concanavalin A

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The in vitro response of hamster lymphocytes to concanavalin A (Con A) was investigated by using the ^{3}H-thymidine incorporation assay, and the results obtained were compared to those with phytohemagglutinin (PHA-P). The optimum culture conditions for stimulation were obtained when one million lymph node cells were cultivated for 72 hr in 2 ml of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum in the presence of either 2 μg of Con A or 0.05 ml of PHA-P. The average ratios of stimulation using lymph node cells with Con A and PHA-P were 227 and 14, respectively. Bone marrow and thymus cells responded very poorly or not at all. Methyl α-D-glucopyranoside, when added to the culture medium at the time of addition of Con A, completely inhibited stimulation. The lymphocyte stimulation could be reversed by addition of the sugar as late as 18 hr after the addition of Con A. The lymphocyte response from hamsters bearing tumors induced by simian virus 40 tumor cells and from hamsters immune to tumor transplants was comparable to the response of lymphocytes from healthy donors.

The Syrian hamster is being used as an animal of choice for testing oncogenic potential of viruses. Simian virus 40 (SV40), a papovavirus which is not oncogenic in mice or rats (6), produces tumors readily in newborn hamsters (7, 10). Similarly, cells transformed in vitro by SV40 become readily transplantable in adult hamsters (3). The resistance to tumor development by viruses or by transformed cells is mediated by immune lymphoid cells rather than humoral antibody (15, 18, 19). Various in vitro tests have been described to measure cell-mediated immunity to tumor-specific transplantation antigens in spontaneous and induced tumors (15). The in vitro tests are based on the ability of immune lymphocytes to react either with intact target cells or with soluble antigen. With increasing interest and the need to understand host-tumor relationship in hamsters, it becomes important to study hamster lymphocyte functions.

In vitro activation of lymphocytes by phytohemagglutinins has been used to study lymphocyte functions in animals and man (1, 4, 14, 17, 18, 20, 22, 23). We have attempted to investigate the responsiveness of hamster lymphocytes to plant mitogen concanavalin A (Con A) derived from Jack Bean. Con A was chosen as mitogen because the nature of Con A combining receptors has been elucidated (11–13). Also, the binding of Con A to lymphocytes can be reversed at any time during blastogenesis simply by adding sugars which preferentially bind Con A. In the present investigation, attempts are also made to compare the response of hamster lymphocytes from normal and tumor-bearing hosts to Con A and phytohemagglutinin (PHA-P).

MATERIALS AND METHODS

Hamster lymphocytes. Eight- to 12-week-old inbred hamsters (MHA/ss LAK) were used as lymphocyte donors. The animals were first anesthetized by intraperitoneal inoculation of 0.1 ml of pentobarbital sodium. The animals were then bled by cardiac puncture. The inguinal, brachial, axillary, cervical, and mesenteric lymph nodes were removed aseptically and placed in RPMI-1640 medium without serum. The lymph nodes, having been cleaned from adhering fatty tissues, were placed on a 60-mesh stainless-steel wire screen and gently pressed through the wire gauge while being washed down with RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS), obtained from Denver Serum Company, Denver, Pa. The resulting cell suspension was pipetted to break cell clumps and centrifuged at 1,000 rev/min for 10 min at 4 C. The cells were washed again with RPMI medium, and viable counts were made in a hemocytometer using 0.1% trypan blue as a diluent. On the average, one hamster yielded 1.5×10^8 viable cells. When this method was used, the cell viability was 90 to 95%.

Spleen, thymus, and bone marrow cells were similarly prepared. The cells were cultured in 2-ml portions in Falcon plastic culture tubes (16 by 125 mm) and incubated in an upright position at 37 C in an atmosphere of 5% CO_2 in air and 90% humidity.

Lymphocytes were also tested from hamsters bearing a tumor transplant (3 by 3 cm) of SV40 tumor cells (kindly provided by Hilleman). These cells contain tumor-specific transplantation antigen at the cell surface and tumor antigen in the nucleus. No viral antigen has been detected in these cells (Tevethia, *unpublished data*). The tumors were produced by injecting 10^6 tumor cells subcutaneously.

Lymphocytes from hamsters vaccinated with live SV40 that had rejected a challenge of tumor cells were also tested along with lymphocytes from tumor-bearing and normal animals of the same age.

Mitogens. Con A (Calbiochem, no. 10229, grade A) was diluted in physiological saline to a concentration of 2 mg/ml and frozen at −20 C in 1-ml amounts. Con A was diluted to desired concentration just before each experiment from this stock solution.

Methyl-α-D glucopyranoside (MAG) was used to inhibit Con A activity.

PHA-P (Difco, control 547703) was reconstituted in 5 ml of distilled water. It was used in 0.05-ml amounts per culture tube.

Transformation assay. Appropriate mitogens (Con A, PHA-P) were added to culture tubes in desired concentration, and the tubes were allowed to incubate for 72 hr. The cells were pulsed with 1 μCi of thymidine-*methyl*-^3H (New England Nuclear Corp.; specific activity, 20.2 Ci/mmole) for the final 12 hr of incubation. At the end of the labeling time, the cultures were centrifuged at 1,500 rev/min for 10 min in a PR-6 refrigerated centrifuge. The cell pellets were washed with 5 ml of cold normal saline and then treated with 5 ml of 5% cold trichloroacetic acid for 30 min. The precipitate was washed once with 5% trichloroacetic acid and twice with absolute methanol. The precipitates were then transferred to glass tubes and digested at 45 C in 0.3 ml of Nuclear-Chicago solubilizer (NCS) (Amersham/Searle) for 2 hr. Ten milliliters of scintillation fluid was added [2,5-diphenyloxazole, 4 g; 1,4-*bis*-2-(5-phenyloxazolyl) benzene, 0.4 g; toluene, 1 liter] to each tube, and the contents were transferred to a plastic scintillation vial. The vial was shaken vigorously on a Vortex mixture, and the counts/min were determined by using a Beckman LS-250 liquid scintillation spectrometer.

RESULTS

Optimum culture conditions. To establish optimum culture conditions, hamster lymphocytes were cultured in RPMI-1640 medium supplemented with 5, 10, and 20% FCS. The viability of lymphocytes was checked at various time intervals. Results presented in Fig. 1 show that approximately 60% of lymph node cells survived after 72 hr of incubation in medium containing 10% heat-inactivated FCS. Survival of spleen cells showed a similar pattern. Unheated FCS was found to be toxic to hamster lymphocytes. All experiments were then performed in RPMI medium supplemented with 10% heat-inactivated FCS.

Stimulation of hamster lymphocytes with Con A. To determine the optimum period of stimulation,

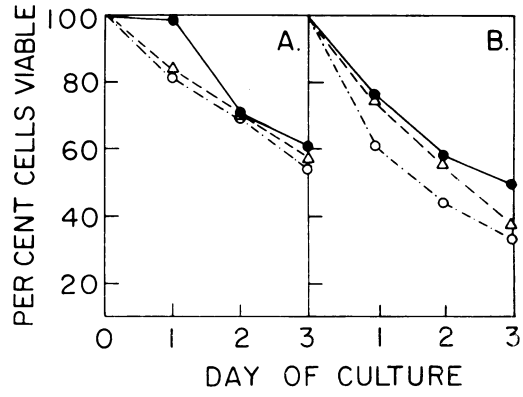

FIG. 1. *Viability of hamster lymph node (A) and spleen (B) cells in RPMI-1640 medium supplemented with various concentrations of heated fetal calf serum. Cells were incubated for 1 to 3 days, and viability was determined by trypan blue exclusion test. The values shown here are averages of two to four tubes.*

one million lymph node cells were cultured for 24 to 96 hr in the presence of arbitrarily selected doses of mitogen: 0.5 and 10 μg of Con A per ml. Lymphocyte cultures which did not receive any Con A served as unstimulated controls. Before harvesting, the cultures were pulsed for 12 hr with 1 μCi of ^3H-thymidine. Replicate cultures were withdrawn at indicated time intervals and assayed for incorporation of radioactivity as described above. The results presented in Fig. 2 show that at 48 hr there was a sharp increase in incorporation of ^3H-thymidine which reached maximum at 72 hr followed by a decline at 96 hr in cultures stimulated with 10 μg of Con A per ml. With 0.5 μg of Con A, the maximum stimulation also was observed at 72 hr. Unstimulated cultures incorporated very little radioactivity. On the basis of these results, a 72-hr period was chosen for lymphocyte stimulation. Similar results were obtained by using PHA-P.

Effect of varying Con A concentration. The results in Fig. 3 show the response of splenic and lymph node lymphocytes to various concentrations of Con A. When cultures were harvested at 72 hr, maximum stimulation occurred with 1 μg of Con A per ml. There was an inhibition of stimulation when 10, 20, or 50 μg of Con A per ml were used. Based on these results, a concentration of 1 μg of Con A per ml was chosen as the optimum concentration for stimulation of hamster lymphocytes.

Effect of varying cell concentration. Lymph node cells in varying concentrations (4×10^5, 10^6, 2×10^6, 4×10^6, 10^7, and 2×10^7) were stimulated with 1 μg of Con A per ml, and the incorporation of radioactivity in trichloroacetic

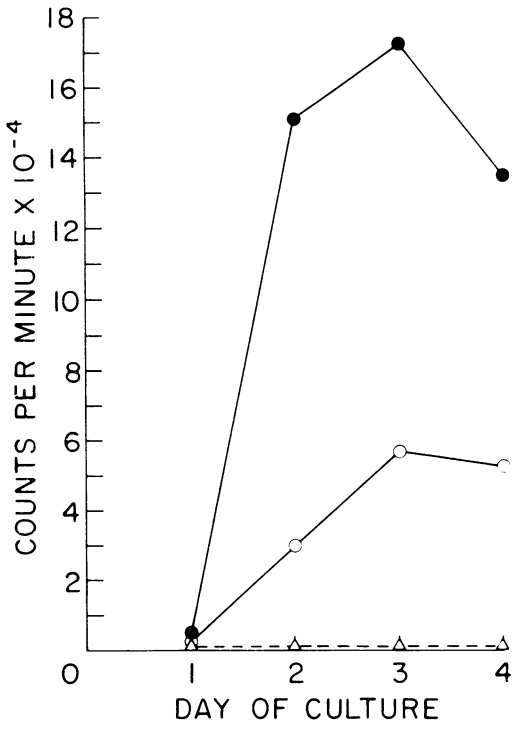

FIG. 2. *³H-thymidine incorporation by concanavalin A-stimulated hamster lymph node cells harvested at various time intervals. Symbols: (○) 0.5 μg of Con A per ml; (●), 10 μg of Con A per ml; (△), no Con A.*

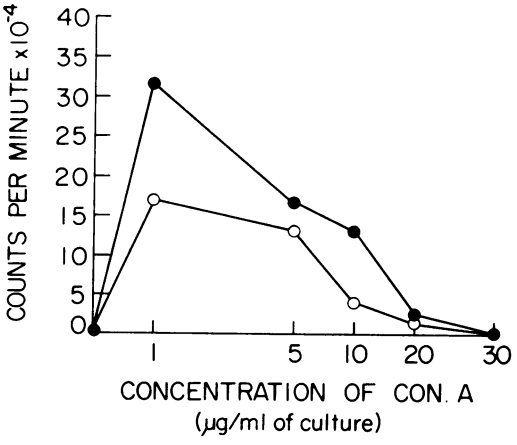

FIG. 3. *Effect of varying concanavalin A concentration on ³H-thymidine incorporation by hamster spleen (○) and lymph node (●) cells.*

acid-precipitable material was determined at 72 hr. Results presented in Table 1 show that there was an increased incorporation of ³H-thymidine with increasing cell concentration. It should be noted that unstimulated cultures containing greater than 10^6 cells incorporated significant amounts of radioactivity. This increase in unstimulated cultures was dependent upon the cell concentration. Maximal ratio of stimulation (Con A to control) was achieved at a concentration of 10^6 cells per culture. Based on this experiment, a concentration of 10^6 cells per culture was chosen for further experimentation.

TABLE 1. *Effect of varying cell concentration on stimulation of hamster lymph node cells by concanavalin A*[a]

No. of cells per culture	³H-thymidine incorporation (mean counts/min/culture)[b]		Ratio of stimulation[c]
	Unstimulated	Stimulated	
4×10^5	81	29,682	366
10^6	120	222,658	1,856
2×10^6	7,486	394,370	53
4×10^6	79,493	397,015	5
10^7	157,498	523,440	3
2×10^7	159,327	495,566	3

[a] Various concentrations of hamster lymph node cells were cultured for 72 hr with 2 μg of Con A in 2 ml of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (lot no. 57A). Twelve hours before harvesting, the cultures were treated with 1 μCi of ³H-thymidine and assayed as described in Materials and Methods.

[b] Average of two to three replicate samples.

[c] Mean counts per minute in the Con A-stimulated cultures divided by the mean counts per minute in the unstimulated cultures.

The data showing the reproducibility of stimulation of hamster lymphocytes by Con A are presented in Table 2. Even though the stimulation ratios vary considerably among lymphocytes derived from different donors, the stimulation ratios are all significantly high. It should be noted that stimulation ratios are also affected by the batch of FCS used to supplement the medium. Higher ratios (377, 764, and 1,856) were obtained when RPMI medium was supplemented with lot 57A of FCS as compared to the ratios obtained with lot 59A. The higher ratios of stimulation obtained with lot 57A are attributable to the low amount of ³H-thymidine incorporation in the unstimulated cell cultures.

Stimulation of lymphocytes with PHA-P. Preliminary experiments showed that maximum stimulation of hamster lymphocytes was obtained with 0.05 ml of PHA when 10^6 cells per culture were used and the culture was harvested after 72 hr. The results in Table 3 show that PHA-P consistently stimulated hamster lymphocytes. However, the stimulation ratios obtained with PHA-P

TABLE 2. *Responses of hamster lymphocytes to concanavalin A*[a]

Expt no.	Fetal calf serum (lot no.)	^{3}H-thymidine incorporation (mean counts/min/culture)[b]		Ratio of stimulation[c]
		Unstimulated	Con A-stimulated (2 μg/culture)	
1	57A	415	317,275	764
2	57A	120	222,658	1,856
3	57A	390	146,872	377
4	59A	4,372	146,990	34
5	59A	4,168	190,956	46
6	59A	1,768	163,621	92
7	59A	3,092	172,803	56
8	59A	3,743	128,587	34
9	59A	2,845	104,687	37
10	59A	2,620	120,538	46
11	59A	1,627	96,550	59
12	59A	762	6,746	9
13	59A	2,994	173,262	58
14	59A	4,457	237,528	53
15	59A	1,501	64,602	43
16	59A	854	58,517	68

[a] Hamster lymph node cells (10^6) were cultured for 72 hr with 2 μg of Con A in 2 ml of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. Twelve hours before harvesting, the cultures were treated with 1 μCi of ^{3}H-thymidine and assayed as described in Materials and Methods.

[b] Average of two to three replicate samples.

[c] Mean count per minute in the Con A-stimulated cultures divided by the mean counts per minute in the unstimulated cultures.

TABLE 3. *Response of hamster lymphocytes to phytohemagglutanin-P (PHA-P)*

Expt no.	^{3}H-thymidine incorporation (mean counts/min/culture)[b]		Ratio of stimulation[c]
	Unstimulated	PHA-P-stimulated (0.05 ml/culture)	
1	390	12,962	33
2	4,168	57,917	14
3	3,743	40,475	11
4	2,845	40,468	14
5	2,620	22,485	9
6	1,501	16,940	11
7	762	11,128	15
8	2,994	26,687	9
9	4,457	44,902	10
10	598	16,434	28
11	1,393	16,558	12
12	854	14,322	17
13	637	4,935	8

[a] Hamster lymph node cells (10^6) were cultured for 72 hr with 0.05 ml of PHA-P in 2 ml of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (lot no. 59A). Twelve hours before harvesting, the cultures were treated with 1 μCi of ^{3}H-thymidine and assayed as described in Materials and Methods.

[b] Average of two to three replicate samples.

[c] Mean counts per minute is the PHA-stimulated cultures divided by the mean counts per minute in the unstimulated cultures.

were significantly lower than the ones obtained with Con A.

Stimulation of cells from various lymphoid tissues. Lymphoid cells from spleen, lymph nodes, thymus, and bone marrow of adult hamsters were compared for their ability to be stimulated by Con A and PHA-P. Spleen and lymph node cells were stimulated to a significantly higher degree with Con A as evidenced by ratios of 74 and 58, respectively, for spleen and lymph node cells (Table 4). Thymus cells did not react to any significant degree to Con A. The total counts incorporated were only 897 as compared to 173,262 in lymph node cells and 208,310 counts in spleen cells. Bone marrow cells also failed to be stimulated by Con A. Both stimulated and unstimulated cultures incorporated high amounts of radioactivity. This is because the bone marrow cells were undergoing replication. With PHA-P, only the lymph node cells could be stimulated to a significant degree in this experiment.

Effect of MAG on stimulation of hamster lymphocytes. It was of interest to determine whether continuous binding of Con A to lymphocytes is required for stimulation by the mitogen as measured by ^{3}H-thymidine incorporation. MAG (0.1 M), which preferentially binds Con A, was added to the cultures at the time of addition of Con A and also after various intervals. The results in Table 5 show that MAG (0.1 M) added to the cultures containing 1 μg of Con A per ml inhibited stimulation of cultures by 97%. The stimulation ratio in the presence of MAG and Con A was 1.4 as compared to 53 in the absence of MAG but in the presence of Con A. The addition of MAG did not significantly affect the stimulation of hamster lymphocytes by PHA-P, indicating that the action of MAG was on Con A rather than the lymphocytes and also that receptors for Con A and PHA-P on lymphocyte surface are different.

Experiments were then carried out to determine whether MAG could reverse lymphocyte stimulation by Con A when added at various time intervals after addition of Con A. The results shown in Fig. 4 clearly indicate that MAG (0.1 M), when added as late as 18 hr after the addition of Con A, inhibited lymphocyte stimulation by about 75%. Addition of MAG 30 hr after addition of Con A

TABLE 4. *Stimulation of spleen, lymph node, thymus and bone marrow cells from adult hamsters with concanavalin A and phytohemagglutinin (PHA)*

Source of cells	^{3}H-thymidine incorporation (mean counts/min/culture)			Ratio of stimulation Con A/control	Ratio of stimulation PHA/control
	Unstimulated	Con A-stimulated	PHA-stimulated		
Spleen	2,814	208,310	3,255	74	1.2
Lymph node	2,994	173,262	26,687	57.9	8.9
Thymus	254	897	198	3.5	0.8
Bone marrow	48,938	30,658	69,258	0.6	1.4

TABLE 5. *Effect of 0.1 M methyl-α-D-glucopyranoside (MAG) on stimulation of hamster lymph node cells with concanavalin A and phytohemagglutinin (PHA)*

Supplement to lymphocyte culture at zero hr	^{3}H-thymidine incorporation (mean counts/min/culture)	Ratio of stimulation	Per cent reduction of stimulation by MAG
None	4,457		
Con A	237,578	53	
Con A and MAG	6,467	1	97
PHA	44,902	10	
PHA and MAG	36,092	8	20

was not effective in inhibiting the incorporation of ^{3}H-thymidine, indicating that lymphocytes become irreversibly committed to synthesize deoxyribonucleic acid (DNA) between 18 and 30 hr after the addition of Con A. Removal of Con A by MAG up to 18 hr results in the reversal of this process.

Transformability of lymphocytes from normal, immune, or tumor-bearing hamsters. This experiment was carried out to determine the responsiveness of lymphocytes from adult hamsters bearing tumor transplants induced by SV40-transformed cells and to compare it to lymphocytes from normal animals and animals immune to tumor transplants of the same age. Lymphocytes from four hamsters belonging to each group (normal, immune, and tumor-bearing) were stimulated with PHA-P and Con A. The results of such an experiment are presented in Fig. 5. Stimulation ratios with PHA-P when lymphocytes from normal animals were used ranged from 9 to 11, whereas ratios of 34 to 46 were obtained in cultures stimulated with Con A. Lymphocytes from hamsters immune to tumor isograft showed relatively lower ratios with Con A. A generally good stimulation of lymphocytes from tumor-bearing hamsters was obtained both with PHA-P and Con A except animal K, which gave ratios of 5 and 18 with PHA-P and Con A, respectively. It should be noted, however, that the lower ratios

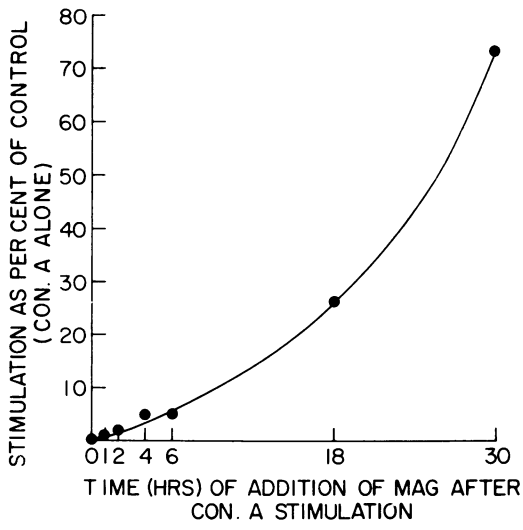

FIG. 4. *Reversal of stimulation of concanavalin A by methyl α-D-glucopyranoside (MAG). At various time intervals after the addition of concanavalin A, 0.1 M MAG was added to the cultures, which were then incubated for a total of 72 hr.*

observed with lymphocytes from animal K were due not to less incorporation of ^{3}H-thymidine in stimulated cultures but to higher incorporation of ^{3}H-thymidine in unstimulated cultures, suggesting that lymphocytes from this animal are already stimulated, probably by the tumor-specific transplantation antigen present at the surface of SV40 tumor cells.

DISCUSSION

Experiments carried out in this study have clearly shown that hamster lymphocytes can be stimulated to undergo DNA synthesis after interaction with nonspecific mitogens Con A and PHA-P.

The optimum conditions for stimulation of hamster lymphocytes in vitro consisted of at least 10^6 cells per 2 ml of culture and the presence of 10% heat-inactivated FCS as a supplement.

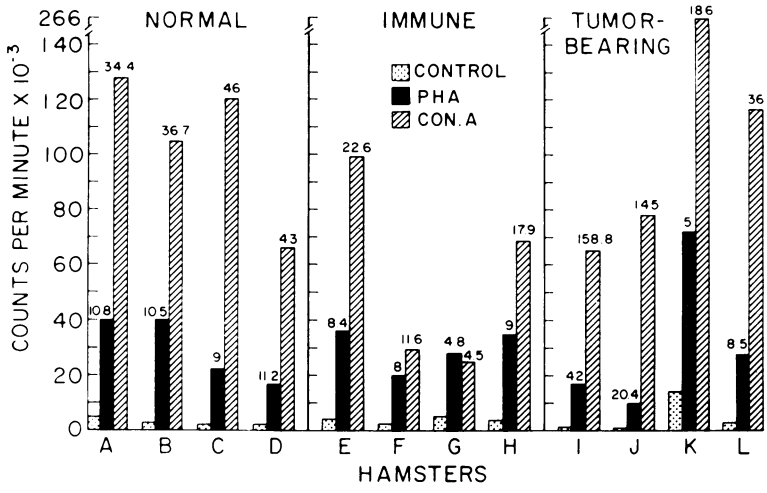

FIG. 5. *Response of lymph node cells from normal, immune, and tumor-bearing hamsters to phytohemagglutinin (PHA) and concanavalin A (Con A). The numbers over the bars denote the ratio of stimulation.*

Initial experiments had shown that unheated FCS was toxic to hamster lymphocytes. The hamster lymphocytes could also be stimulated in the presence of homologous sera. Although the total amount of radioactivity incorporated was low in homologous sera, the stimulation ratio was higher when compared to FCS (Tevethia, *unpublished data*).

The high amounts of radioactivity incorporated in Con A-stimulated cultures when FCS was used as a supplement can perhaps be explained by the presence of natural antibody in FCS against hamster lymphocytes which may potentiate the lymphocyte response to Con A. The participation of natural agglutinins present in human sera in mouse lymphocyte response to PHA has been demonstrated (1).

The results obtained with Con A in stimulated hamster lymphocytes are in agreement with those obtained with human lymphocytes (21). Con A reacts with carbohydrate receptors on the lymphocyte surface since the stimulation by Con A can be inhibited by the addition of MAG, which preferentially binds to Con A. The data obtained in this study also show that continuous binding of Con A to the lymphocyte surface is required only up to the point of initiation of DNA synthesis. Addition of MAG up to that point results in the reversal of the lymphocyte transformation process.

Our studies also have shown that hamster lymphocytes can be stimulated with PHA. Although PHA consistently stimulated hamster lymphocytes, the stimulation was considerably lower than that obtained with Con A. It is possible that Con A is a much more potent mitogen for hamster lymphocytes than PHA or that there are separate lymphocyte populations reacting to Con A and PHA. The lower stimulation with PHA of hamster lymphocytes may be due to the inhibitory effect of FCS in the medium. In a recent report (8) the hamster lymphocytes responded very poorly to PHA in medium supplemented with FCS but responded well when either homologous or human serum was used as supplement.

Lymphocytes derived only from spleen and lymph nodes could be stimulated with Con A. Bone marrow cells could not be stimulated at all either with PHA or with Con A. Thymic cells, however, responded with Con A, but response was poor. The low stimulation of thymus cells could be explained on the basis that thymus contains a small portion of cells that are capable of responding to PHA or Con A. Such a cell population has been demonstrated in mouse thymus and was shown to be cortisone-resistant (5).

In vitro lymphocyte response to PHA in cancer patients has been shown to be markedly depressed (9, 16). Adler et al. (2) recently reported that spleen cells from mice bearing transplanted methylcholanthrene-induced sarcomas were unresponsive to PHA. These authors explained this unresponsiveness on the basis that lymphocytes from tumor-bearing animals are already stimulated, probably by the tumor-specific antigens, and therefore could not be stimulated further by mitogens. However, it is entirely possible that the unresponsiveness in tumor-bearing hosts varies with the stage of tumor development.

Now that the conditions for culturing hamster lymphocytes are well worked out, attempts are

being made to use this test for the assay of cellular immunity directed against tumor-specific transplantation antigen in SV40-induced tumors in hamsters.

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