The Effect of Concanavalin A on Bone Marrow Colony Formation *in Vitro*[1] (36692)

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(Introduced by G. Meiklejohn)

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It has been shown that nucleated cells from bone marrow of a number of species including humans can give rise *in vitro* to colonies of mature granulocytic and mononuclear cells in semisolid media (1–3). These colonies arise from either single hemopoietic stem cells or cells committed to granulocytic differentiation which undergo division and maturation to yield colonies of 1000 or more cells (1).

In order for the process of maturation and division to occur *in vitro*, a specific stimulus is required. This stimulus for mouse marrow cells can be extracted from animal (including human) serum and urine, embryonic extracts, and a number of other tissue sources (1). The urinary colony stimulating factor (CSF) has been purified and appears to be a glycoprotein of molecular weight of 35,000 (1). Although there is heterogeneity among the other described CSF, most appear to be similar biochemically to that described in human urine (1).

Although factors stimulating human marrow cells are essentially the same, the quantitative differences among sources of CSF are large. While human urinary CSF stimulates 100–200 colonies from mouse marrow cells, it will stimulate only 10–20 colonies from the same number of human marrow cells (1, 3). A much more potent stimulus for human bone marrow is feeder layers of peripheral white blood cells (WBC) from humans (3). CSF from peripheral WBC has not been extracted or isolated but appears to be a protein factor secreted by metabolically intact cells (4, 5).

The mechanism and means by which various CSF initiate and stimulate colony formation is unknown. It is clear that CSF does not act merely as a triggering substance, but is necessary for every cell division in the maturation pathway (1). The present studies were done to determine whether the action of CSF is a cell surface related phenomenon.

In order to investigate the interaction of CSF with target cells the effect of concanavalin A[2] on the CSF was chosen for a number of reasons. First, concanavalin A is a cell surface acting agent and in the fibroblast culture system is capable of reverting transformed cells to normal cells (6, 7). Secondly, concanavalin A binds specifically to certain sugar moieties with a configuration such as alpha methyl-glucopyranoside (MGP) and is capable of binding proteins which contain these carbohydrates (8, 9). Thirdly, concanavalin A is a phytohemagglutinin and is capable of lymphoblastic transformation (10). These experiments show that concanavalin A is inhibitory to the formation of colonies at a very low concentration and the nature of this inhibition is discussed.

Materials and Methods. Tissue culture. Human marrow cells. The basic components of this system are a feeder layer of peripheral leukocytes and an overlay of human bone marrow cells aspirated from the posterior iliac crest of hematologically normal individuals (3).

Feeder layers were prepared from peripheral leukocytes obtained from normal laboratory individuals. Blood was collected

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[2] Calbiochem, Los Angeles, CA.

by venipuncture into heparinized tissue culture tubes and the red cells were allowed to sediment by gravity for 1–2 hr at room temperature. The buffy coat was then sterilely removed and incorporated into the tissue culture media. This cell suspension, consisting of 0.5% agar, McCoy's media with fetal calf serum, and cells at a concentration of 1×10^6/ml was pipetted at a volume of 1 ml/plate into 35 mm plastic petri dishes and allowed to jell.

Bone marrow cells obtained by aspiration through a Westerman-Jensen needle were collected into heparinized tissue culture tubes and allowed to sediment at room temperature by gravity for 1–2 hr. Buffy coat was collected and incorporated into the same media used for the preparation of feeder layers; with the exception of using 0.3% agar and 200,000 nucleated cells/ml. One milliter was then pipetted gently over the solidified feeder layers and allowed to jell.

The plates were then incubated at 37° in a humidified atmosphere at 7.5% CO_2 in air. At the end of 18 days plates were removed and colonies were scored.

Mouse marrow cells. The basic components of this system are unpurified human urine and mouse marrow cells obtained from femurs incorporated into an agar tissue culture media.

Human urine was collected from normal individuals and dialyzed 72 hr with 3 changes of distilled water, centrifuged and supernatants were filtered through Millipore filters. Mouse marrow cells obtained from femurs of C57/Bl mice were suspended at a concentration of 75,000/ml in the same tissue culture media with 0.3% agar. Urine was pipetted at a volume of 0.1 ml/plate into 35 mm petri dishes and mixed. The previously prepared suspension of marrow cells was then pipetted into the plates in 1 ml volumes, mixed with the urine and allowed to jell.

Plates were then incubated at 37° in a humidified atmosphere of 7.5% CO_2 in air. At the end of 7 days plates were removed and colonies were scored.

Concanavalin A. Concanavalin A was diluted to various concentrations with saline and mixed into the feeder layers or overlays at a volume of 0.05 ml to yield the concentrations indicated. Alpha MGP was used to test the reversibility of concanavalin A and was prepared in distilled water. Alpha MGP was incorporated into the feeder layers or overlays at a volume of 0.05 ml to yield the concentrations indicated.

Agglutination studies with concanavalin A were performed by the method of Aub, Sanford and Wong (11). Several normal individuals were used and their buffy coats were tested. Bone marrows of normal individuals were also tested for agglutination with concanavalin A.

Results. Concanavalin A was first incorporated at various concentrations into feeder layers of peripheral leukocytes obtained from normal individuals. The results of these studies shown in Fig. 1 indicate that concanavalin A inhibited CSF at concentrations greater than 12.5 μg/ml. At lower concentrations of concanavalin A, CSF gradually increased until it equaled that of controls without added concanavalin A. Parallel studies were done in which concanavalin A was incorporated into the mouse marrow system using 0.10 ml of human urine as the source of CSF. In these studies, shown in Fig. 2, concanavalin A inhibited CSF at approximately the same concentrations as found with the human marrow system. If alpha MGP was added together with concanavalin A to the stimulus in both the mouse and human systems, the inhibitory effect of concanavalin A was not present, Fig. 1. These results suggest that concanavalin A inhibits CSF in both mouse and human systems and that this inhibition by concanavalin A can be reversed by a specific hapten inhibitor, alpha MGP.

In order to elucidate further the mechanism of inhibition of CSF by concanavalin A, concanavalin A was incorporated into the marrow cell overlays of the human culture system. Feeder layers were prepared in advance and allowed to incubate for at least 2 days prior to the addition of the target cell overlay; thus allowing maximal secretion of CSF by peripheral WBC. Concanavalin A incorporated into the overlay inhibited the expression of CSF at concentrations similar to the previous study, Fig. 3. These results

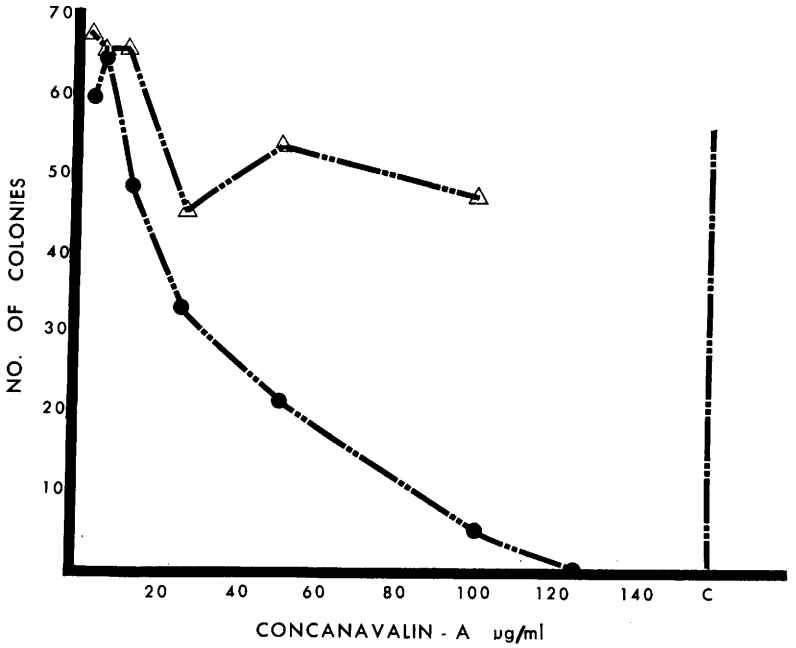

FIG. 1. Effect of concanavalin A on colony formation by normal human bone marrow cells stimulated by human white blood cell feeder layers. Concanavalin A incorporated into the feeder layers. (●) The studies in which concanavalin A alone was used; (△) the studies in which alpha MGP was added along with concanavalin A. Each point on the curves represents the mean colony count of 3 plates at the concentrations shown. The control value, the number of colonies stimulated by feeder layer cells without concanavalin A or alpha MGP is shown as a broken bar over (C).

suggest that concanavalin A does not inhibit the release of CSF from peripheral leukocytes as CSF is maximally released at 2 days and that concanavalin A therefore, must inhibit CSF by interacting with the released factor or with the target bone marrow cell.

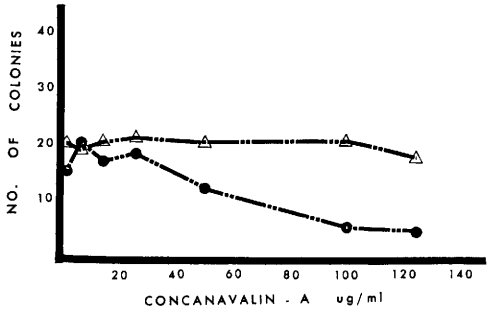

FIG. 2. Effect of concanavalin A on colony formation by mouse bone marrow cells as stimulated by human urine. (●) The effect of concanavalin A at various concentrations; (△) the effect of concanavalin A with added alpha MGP. Each point represents the mean colony count of 3 plates.

Agglutination studies were carried out by the method of Aub, Sanford and Wong (11) to determine the ability of concanavalin A to interact with cells. After 30 min of incubation with concanavalin A peripheral leukocytes showed extensive clumping of nucleated cells. There was variance in the size of clumping with various individuals, but no attempt was made to correlate differential counts with degree of agglutination. Addition of alpha MGP resulted in reversal of agglutination. Aliquots of the concanavalin A–leukocyte suspensions were tested for cell death and showed no increase in trypan blue uptake or lysis compared to controls without concanavalin A. Bone marrow cells from aspirated human marrow also showed a similar degree of agglutination and a reversal with alpha MGP. Trypan blue studies and cell

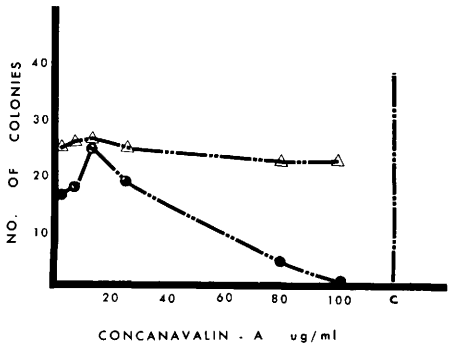

FIG. 3. Effect of concanavalin A on colony formation by human bone marrow cells grown on human white blood cell feeder layers. Concanavalin A added to the feeder layers 48 hr after their preparation at which time maximal secretion of CSF had occurred. (●) addition of concanavalin A only; (△) represent the addition of concanavalin A and alpha MGP. Each point represents the mean colony count of 3 plates. The control value, number of colonies grown from this marrow on white blood cell feeder layers without addition of concanavalin A or alpha MGP is shown as a bar over (C).

counts again revealed no significant increase in uptake in concanavalin A treated cells as compared to controls without concanavalin A. The agglutination by concanavalin A of both peripheral leukocytes and nucleated marrow cells indicate that these cells have receptor sites for interaction with concanavalin A. This interaction is reversible with alpha MGP and concanavalin A alone does not cause any significant degree of cell death. Although this indicates that concanavalin A interacts with cell membranes it does not rule out the possibility that concanavalin A also interacts with CSF.

Preliminary studies were undertaken to determine the effect of concanavalin A on human urine CSF. Human urine was concentrated fourfold and 1.0 ml mixed with concanavalin A (1.0 ml) at a final concentration of 1000 μg/ml and allowed to incubate at room temperature for 1–2 hr. The mixture was then centrifuged at 105,000*g* for 1 hr and the supernatant removed. The supernatant was then tested at volumes of 0.05, 0.10, 0.15, and 0.20 ml for CSF using the mouse bone marrow system. No CSF was found at any of these dilutions, Table I. In order to rule out the effect of any residual concanavalin A in the supernate on marrow cells, alpha MGP was added to the mouse marrow to a final concentration of 10^{-2} *M* to reverse the effect of concanavalin A and then each of the above fractions again was tested at 0.05, 0.10, 0.15, and 0.20 ml. The addition of alpha MGP did not increase CSF. These results suggested that urinary CSF was precipitated by concanavalin A. The precipitate was then resuspended by the addition of alpha MGP and then tested for CSF. The activity recovered was 30% of the original activity.

Discussion. This study shows that concanavalin A, a phytohemagglutinin, is capable of inhibiting the stimulation of granulocyte colony growth *in vitro* from mouse and human bone marrow cells. The process of maturation and cell division of precursor cells into mature granulocytic and mononuclear cell colonies does not occur in the presence of concanavalin A at concentrations of 12.5 μg/ml or higher. This inhibitory action of concanavalin A is blocked if alpha MGP is added to the culture system simultaneously with the concanavalin A.

This inhibitory action of concanavalin A can be the result of the interaction of concanavalin A at a number of sites in the *in vitro* culture system. Concanavalin A may be inhibitory in the culture systems (a) because of toxicity of cells, both peripheral WBC and bone marrow cells; (b) by inhibiting release of CSF by feeder layer peripheral WBC; (c) by combining with the released

TABLE I. Precipitation of Urinary CSF by Concanavalin A.[a]

Sample tested	CSA (no.[b] of colonies stimulated)
Control urine alone	48
After addition of concanavalin A	
Supernatant	0
Supernate + alpha MGP	0
Precipitate + alpha MGP	15

[a] Human urine with known CSF mixed with concanavalin A, centrifuged, and then tested for CSA in supernatant and sediment fractions using mouse bone marrow cells.

[b] Mean number of colonies on 3 plates stimulated by 0.10 ml urine.

CSF; or (d) by acting at the cell surface of the target cell.

Toxicity studies with trypan blue have shown that concanavalin A does not significantly alter the uptake of trypan blue by either bone marrow cells or peripheral WBC in suspension culture. These studies were carried out for 2–4 days without any significant change in the percentage uptake of trypan blue by bone marrow cells treated with concanavalin A compared to those cells not treated. Hence, concanavalin A does not appear to inhibit CSF by a cellular destructive phenomenon involving target colony forming cells.

If concanavalin A were to inhibit the release of CSF, feeder layers of WBC incubated longer than 48 hr before the addition of concanavalin A should not have decreased CSF since the release of CSF by peripheral WBC reaches a maximum at 48 hr and remains the same thereafter.

Similarly, if concanavalin A were to inhibit release of CSF from peripheral WBC, there should be no inhibition of CSF by concanavalin A in the mouse system; as CSF is already in the elaborated form. However, this interpretation of the action of concanavalin A in the mouse system may not be entirely correct as the mouse system may be different. But, similarities in patterns of colony growth and types of colonies would suggest that the two systems are alike. Assuming that both mouse and human systems are alike, the inhibition of CSF by concanavalin A in the mouse system would also suggest that concanavalin A does not inhibit CSF by inhibition of CSF release from peripheral WBC.

Concanavalin A must, therefore, inhibit CSF by interaction with either the cell surface of target cells or by binding the released CSF. In view of the former possibility concanavalin A does bind with cell surfaces as shown by agglutination studies. However, concanavalin A does not appear to show cell specificity in its binding and will agglutinate equally well peripheral WBC and bone marrow cells. This agglutination by concanavalin A of both peripheral WBC and bone marrow cells does not indicate whether concanavalin A may be binding only to mature forms of hemopoietic cells since aspirate bone marrow samples are always liberally admixed and consist largely of mature cell forms. Agglutination studies on cell fractions of bone marrow are required to show whether concanavalin A is binding to colony forming precursor cells and thereby inhibiting CSF by a cell surface phenomenon.

Studies in which alpha MGP was added to suspensions of bone marrow cells before adding to feeder layers may also suggest that concanavalin A is acting at the cell surface of bone marrow target cells. Alpha MGP, when added to the bone marrow cells, protects the cells from the inhibitory action of concanavalin A. This protective action of alpha MGP could be the result of binding to concanavalin A and consequently blocking the binding of concanavalin A to cell surfaces.

Although the inhibitory action of concanavalin A could be explained by a cell surface phenomena, an alternate explanation is that concanavalin A is binding to CSF. In fact it is highly likely that the protective action of alpha MGP alluded to, is really secondary to diffusion into the feeder layer and reversal of the binding of concanavalin A to the released CSF. Preliminary studies with urinary CSF suggest that concanavalin A binds reversibly to the CSF. This is certainly not surprising since urinary CSF is a glycoprotein. From these studies it appears that concanavalin A may be inhibitory by binding to CSF. Thus, further work is being carried out to clarify this question.

However, recent studies by McNeil and Fleming (12) suggest an entirely different possibility of concanavalin A mediated inhibition of CSF. McNeil and Fleming produced interferon *in vivo* by poly I·poly C injection into mice and separated the interferon fraction in the serum by G200 column chromatography. This fraction produced marked inhibition of CSF in the mouse system. Concanavalin A is known to stimulate lymphoblast transformation (13) and has also been shown to induce interferon formation simultaneously. Concanavalin A could, therefore, stimulate lymphoblast transformation and interferon production by lymphocytes in both mouse marrow and human marrow and/or

peripheral WBC. The interferon produced could act as an inhibitor of CSF rather than by the direct action of concanavalin A. The concentrations which inhibit CSF are definitely in the same range as those used for lymphocyte transformation. Further work is being carried out to investigate this possibility.

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