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Dextran Potentiation of the Canine Lymphocyte Response to Plant Mitogens[1] (38515)

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(Introduced by Roger O. McClellan)

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Peripheral blood lymphocytes are widely used for a variety of *in vitro* immunologic testing procedures. This use has led to the development of numerous methods for the separation of mononuclear cells from blood. Techniques employing dextran either alone or in combination with other materials in high density flotation or density gradient systems have been frequently employed (1–4). Although the effect of using separated and unseparated peripheral blood leukocytes has been addressed frequently in the literature (5), relatively little attention has been given to the effect of the separation medium on the responses of the treated lymphocytes. Since dextran has recently been reported both to be a mitogen (6) and to alter immunological responses to antigenic stimuli (7), it was felt that the use of dextran as a separation medium for lymphocytes deserved further attention. In our laboratory, we have used 6% dextran 70 in isotonic saline for the purpose of concentrating canine peripheral blood lymphocytes for use in lymphocyte transformation studies. This paper reports the finding that relatively small quantities of dextran in the final culture medium markedly potentiates the *in vitro* response of canine lymphocytes to plant mitogens.

Materials and Methods. Blood from 19 clinically healthy, 1- to 2-yr-old Beagle dogs raised in our colony was used for these experiments. The colony breeding and management procedures have been described (8, 9). Aseptic techniques were used in all procedures. Thirty ml of blood was drawn in sterile sodium heparin from each dog for culture. A smaller blood sample was always drawn concurrently in sodium EDTA for hematological examination. Separation of lymphocytes in all cases was accomplished by addition of 10 ml of 6% dextran 70 (mol wt 60,000–90,000) in normal saline (McGaw Laboratories, Glendale, CA) to 20 ml of whole blood. The mixture was gently but thoroughly mixed and incubated at 37° for 60 min. The suspension was then centrifuged at 37 *g* for 5 min. The leukocyte-rich plasma (LRP) was separated from the erythrocyte pack and total, differential and viability counts made on the separated cells. The LRP contained an average of 60% lymphocytes and less than 10% monocytes. The LRP was then centrifuged at 500 *g* for 5 min. The remaining 10 ml of the untreated whole blood was centrifuged at 900 *g* for 20 min to yield dextran-free plasma. In order to test the effect of dextran on the mitogenic response of the lymphocytes, two groups of cultures were set up from each dog. In one group, the LRP cells were washed three times in Hank's balanced salt solution (HBSS) to remove all the free dextran and then were resuspended in dextran-free autologous plasma. In the second group, the unwashed cells were resuspended in autologous plasma containing dextran. A parallel experiment involving a group of six dogs was run to evaluate the effect of the cell washing procedure. A third group of cultures was added to the first two in which the cells were not washed but the original plasma was decanted and the cells were resuspended in dextran-free autologous plasma. The concentration of resuspended cells in the plasma was adjusted so that 1 ml plasma, when added to 5 ml medium, would yield a suspension of 0.5×10^6 viable lymphocytes per milliliter in the final cultures. The culture groups with and without dextran were further subdivided into two or three subgroups. One

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subgroup had phytohemagglutinin (PHA) added to each culture and a second group had HBSS added as a control. In cells from four dogs, a third subgroup with pokeweed mitogen (PWM) added was included. The medium was Eagle's Minimal Essential Medium, Spinner Modification (Flow Laboratories, Inglewood, CA) supplemented with 10% inactivated fetal calf serum, 4 μM L-glutamine/ml, 100 units penicillin G/ml and 100 μg streptomycin sulfate/ml. The pH was adjusted to 7.2 $\pm$ 0.1 with sodium bicarbonate. Cultures were grown in glass screw-cap culture tubes containing 2 ml of medium. Five replicates were set up for each subgroup. Mitogens were reconstituted as directed by the manufacturer. Cultures were supplemented with 10 μl/ml of either PHA-m (Difco, Detroit, MI), PWM (Grand Island Biological Co., Grand Island, NY) or HBSS. Cultures were agitated and incubated at 37° in a humidified atmosphere of 5% CO_2 and air.

Mitogenic response was evaluated by measuring DNA synthesis. After 72 hr of incubation, 2 μCi tritiated thymidine (Amersham–Searle, 24–29 Ci/mM) was added to each tube. After 24 hr labelling time, a 100-fold excess of cold thymidine in HBSS was added to each tube. The tubes were incubated for 10 min, then centrifuged at 900 g and the supernatant discarded. The cells were washed three times with HBSS and then precipitated twice with 2 ml cold 2% perchloric acid in isotonic saline. Next, 2 ml of 10% aqueous perchloric acid was added to the precipitate and this was incubated at 70° for 30 min to hydrolyze the DNA. After centrifugation, a 1.0 ml aliquot of the supernatant was added to 10 ml of a toluene-based scintillation cocktail containing Triton X-100 (Packard Instrument Co., Downers Grove, IL) and counted by liquid scintillation. Results were evaluated for quench and, since this was a negligible factor, they are expressed as the gross counts per minute (cpm) per culture. Differences between the groups were evaluated using the Student's t test.

Results. There was a striking difference in mitogenic response of the lymphocyte cultures depending on the presence or absence of a relatively small amount of dextran (0.34%) in the final culture medium. Table I illustrates the difference in response to PHA due to the dextran supplementation. There is a more than sevenfold increase in the DNA synthesis in cultures containing dextran, a difference which is highly significant. Unwashed lymphocytes resuspended in dextran-free plasma reacted similarly to washed lymphocytes indicating that the washing procedure itself did not cause the difference. Furthermore, Table I shows that dextran alone, without any other mitogen present, acts as a weak mitogen at the concentration used. There is about a 1.5-fold increase in mean response in the saline control cultures containing dextran which is significant. The response of individual dogs was quite variable. Means of cultures stimulated with PHA only ranged from 360 to 5400 cpm in individual dogs, whereas those stimulated with PHA plus dextran ranged from 2100 to 59,000 cpm. Lymphocytes from four dogs were also stimulated with PWM. The results were similar to those seen with PHA stimulation. There was over a ninefold increase in the mean cpm when dextran was present. Many investigators express results of lymphocyte transformation studies as the stimulation index (SI) or the ratio of the response of the stimulated to unstimulated or control cultures. The mean SI of the PHA cultures was 6.5 $\pm$ 1.2 SE while that of the PHA plus dextran cultures was 35.1 $\pm$ 6.5 SE. This difference was also highly signifi-

TABLE 1. EFFECT OF DEXTRAN ON MITOGENIC RESPONSE OF CANINE LYMPHOCYTES *In Vitro*.

Mitogen	Washed lymphocytes (no dextran)	Unwashed lymphocytes (0.34% dextran)	P value[a]
Phytohemagglutinin	1410 $\pm$ 310[b]	10730 $\pm$ 3110[b]	<0.01
Saline	220 $\pm$ 20[b]	320 $\pm$ 40[b]	<0.05

[a] Difference between cultures with and without dextran.
[b] Mean CPM $\pm$ SE ^{3}H-Thymidine per culture.

cant ($P < .001$). This experiment was repeated with another group of dogs as well as some of the same dogs with essentially similar results.

Discussion. Mitogens, such as the plant lectins PHA and PWM, are proteins which bind specifically to saccharides on the cell membranes stimulating lymphocyte transformation (10). Dextran is a high molecular weight polysaccharide which has been reported to be a thymus-independent antigen and a B cell mitogen in mice (6). Dextran has also been shown to markedly alter the *in vivo* antibody response to heterologous antigen in the mouse and guinea pig (7). Other workers, however, have reported that although the polyanion dextran sulfate activated spleen lymphocyte transformation, the polysaccharide dextran itself could not do so (11). Our studies indicate that dextran itself can act as a lymphocyte mitogen although in the system tested it is certainly a weak one. More important is the finding that dextran has a synergistic effect with the two plant mitogens tested. Synergism between two different mitogens or between a mitogen and some other material is not unknown. Plant mitogens with hemagglutinating properties have enhanced transforming ability in the presence of erythrocytes, presumably through absorption of the mitogen onto the erythrocyte surface allowing optimal presentation of the mitogen to the lymphocyte (12, 13). The mitogens Concanavalin A (ConA) and PHA also have been shown to act synergistically with lipopolysaccharide (LPS) which is a thymus independent antigen and B cell mitogen (14). Furthermore, dextran sulphate and PHA have been shown to have a synergistic effect on thymus cells although non-ionic dextran plus PHA did not (11).

There are two major possibilities that might be advanced to explain how dextran potentiates the mitogenic response to plant mitogens. First, two different cell types may be involved in the response to the two substances. Since the response is not simply additive, other factors must be involved. This hypothesis is supported by the work of Schmidtke and Najarian (14) who found that changing the concentration of either ConA or LPS had opposite effects on their synergistic action. Such an action could be effected by production of a B cell stimulating factor by the PHA transformed T cells which makes the B cell more susceptible to dextran mitogenesis. Evidence is available for the existence of such factors (15).

Second, the action of both substances might be on a single cell. Dextran in some way might shift the configuration of receptors on the cell membrane thus making them more available for PHA binding. There is good evidence for such receptor redistribution (16). Raff and De Petris (16) have suggested that a single lectin-receptor binding is not sufficient to trigger and activate lymphocytes but that multivalent binding is required. It is possible that the plant mitogens and dextran interact in solution and then attach to the cell surface as a multivalent complex. Control experiments showed that not enough dextran was bound to the cells during a 1-hr incubation to effect transformation and the free dextran in the plasma was necessary. Andersson *et al.* (17) suggest that more than one receptor-mitogen interaction is necessary for cell activation. They postulated that triggering requires a second type of receptor that will interact with the first complex. Dextran, reacting with another type of receptor, could augment this secondary interaction with the plant mitogen-receptor complex.

Further work will obviously be required to fully evaluate the mechanisms involved, however, the importance of the finding of a synergistic effect between dextran and plant mitogens should be recognized by those working with lymphocyte stimulation systems.

Summary. Peripheral blood lymphocytes from Beagle dogs were separated using a dextran sedimentation technique. Lymphocyte cultures were stimulated by the plant mitogens phytohemagglutinin (PHA) and pokeweed mitogen (PWM), both in the presence of dextran and after dextran had been removed from the medium. It was found that dextran acted synergistically with both PHA and PWM and greatly potentiated the mitogenic response of the lymphocytes. The routine use of dextran in separating blood lymphocytes for culture purposes should be reevaluated in light of these findings.

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