

Effect of Development and Aging on the Response of Canine Lymphocytes to Phytohemagglutinin

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The stage of development and age were found to effect the responsiveness of dog T-lymphocytes to phytohemagglutinin. T-lymphocytes from beagles 0 to 4 weeks of age showed significantly less response to phytohemagglutinin ($P < 0.001$) than T-lymphocytes from these same dogs at 6 to 12 weeks of age. Peak response to phytohemagglutinin occurred between 6 weeks to 6 months of age, after which there was a significant correlation ($P < 0.02$) between increase in age and decrease in phytohemagglutinin responsiveness.

Recent studies in this laboratory have been concerned with how modified live measles virus vaccine protects dogs against canine distemper. The demonstration of delayed hypersensitivity to measles and canine distemper virus and of cross-reactions (8) suggest that cell-mediated immune (CMI) response is the primary mechanism by which measles vaccine protects pups against canine distemper.

Much attention has been focused on the ability of the fetal, neonatal, and adult dog to synthesize antibodies to various antigens (16, 23, 28, 41) and to convey passive maternal immunity (3, 19, 34). Although the CMI response is known to be important in protecting against viral (1, 6, 44) and fungal (14, 26) infections and is considered to be the principal protection against natural measles infection in man (12, 20), its relationship to the protection of dogs has received little attention. Dennis et al. (17) studied skin allograft rejection in fetal, newborn, and adult dogs and the effect of thymectomy on this response. Bryant et al. (11) related the responsiveness of canine thymocytes to phytohemagglutinin (PHA) to the development of the response to an allograft.

Because pups younger than 6 weeks of age are not satisfactorily protected against canine distemper by measles vaccine (9), it seemed important to reexamine the ability of the developing pup to produce a CMI response. It is generally accepted that PHA selectively stimulates T-lymphocytes (5, 24, 31) to divide and serves to measure CMI capability. Initial studies were designed to establish optimal conditions for PHA stimulation of canine T-lymphocytes. The purpose of this study was to determine the effect of development and aging on the CMI response as measured by this test.

MATERIALS AND METHODS

Animals. A total of 51 beagles born in the colony at Norden Laboratories were used in this study. These included 16 pups, 10 dogs 6 months of age, 10 dogs 16 months of age, and 15 dogs 5 and 6 years of age. In addition, 53 beagles, 2 years and older, were from Laboratory Research Enterprises in Kalamazoo, Mich. None of the dogs were used for other experiments prior to this study. The 16 pups were first bled when 0 to 3 days of age, once a week until 8 weeks of age, and then once every 2 weeks until 12 weeks of age. Dogs older than 6 months of age were bled once.

Lymphocyte cultures. The method used for evaluating lymphocyte response to PHA was a modification of that described by Park and Good (36). Whole blood was cultured in RPMI 1640 (Flow Laboratories, Rockville, Md.) containing 1% penicillin-streptomycin (Grand Island Biological Company, N.Y.), 1% glutamine (General Biochemical, Chagrin Falls, Ohio), and either fetal calf serum (Kansas City Biologicals, Lenexa, Kan.) or heterologous dog serum. The effects of serum supplementation, culture duration, PHA concentration, and labeling time with tritiated thymidine ($[^3\text{H}]\text{Tdr}$; specific activity 15 Ci/mmol, Amer-sham/Searle, Arlington Heights, Ill.) were determined. Heat-inactivated (56 C for 30 min) and noninactivated heterologous normal dog serum and fetal calf serum were used at 5, 10, and 15% final concentrations. PHA (Difco, Detroit, Mich.) was reconstituted with sterile distilled water.

Blood was drawn from the jugular vein of dogs and transferred to 2-ml heparinized vacutainer tubes (Becton-Dickinson, Rutherford, N.J.) or collected directly in 10-ml heparinized vacutainer tubes. Tests were conducted in upright glass tubes (16 by 125 ml) fitted with stainless-steel enclosures containing 1.4 ml of culture medium and 0.1 ml of blood. Triplicate cultures with and without PHA were prepared from each blood sample. The cultures were incubated at 39 C in a 5% CO_2 and air mixture. All cultures received 1 μCi of $[^3\text{H}]\text{Tdr}$ in 0.5 ml of culture medium.

Upon termination of incubation, 0.2 ml of the culture was added to 3 ml of distilled water to lyse the

erythrocytes. The mixture was filtered by negative pressure through a GF/A glass filter paper (Whatman, W & R Balston, Ltd., England) cut to a diameter of 13 mm. The filter was washed successively with two washes of phosphate-buffered saline and two washes of 3% glacial acetic acid. The filter paper was transferred to a glass scintillation vial and to the vial was added 12 ml of PCS solubilizer (Amersham/Searle, Arlington Heights, Ill.). The radioactivity of each sample was measured in a liquid scintillation counter (Isocap/300, Searle Analytical, Des Plaines, Ill.). Data were reported as disintegrations per minute (dpm) or as the stimulation index (SI). The stimulation index was determined as follows:

$$\frac{(\text{dpm, PHA-stimulated cultures}) - (\text{dpm, blank})}{(\text{dpm, unstimulated cultures}) - (\text{dpm, blank})} = \text{SI}$$

For statistical purposes, results were analyzed by either the T-statistic or correlation-regression tests.

RESULTS

Serum supplements. The greatest response of T-lymphocytes to PHA stimulation was obtained when 10% heat-inactivated normal heterologous dog serum was used as a supplement in RPMI 1640.

Concentration of PHA. Maximal response depended on the concentration of PHA in the cultures (Fig. 1). Lymphocyte incorporation of [³H]Tdr was greatest when a PHA dilution of 1:400 was used. Diminished amounts of [³H]Tdr uptake occurred when higher and lower concentrations were used.

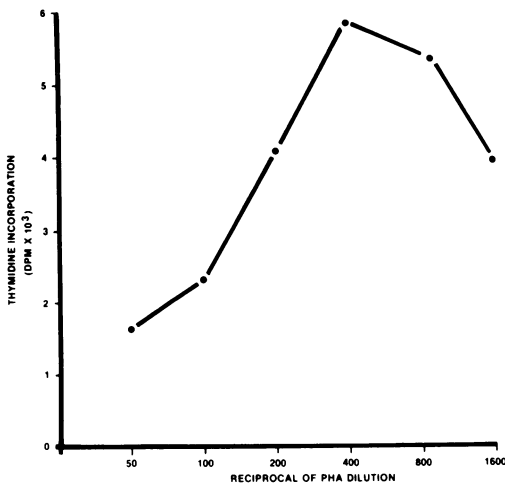


FIG. 1. Responsiveness of dog T-lymphocytes to varying concentrations of PHA. Greatest lymphocyte stimulation and subsequent thymidine incorporation was obtained using a 1:400 dilution of PHA. At higher and lower dilutions of PHA thymidine incorporation was diminished.

Culture duration. The maximal isotope incorporation occurred after incubation of cultures for 5 days in the presence of a 1:400 dilution of PHA (Fig. 2). Diminished responsiveness occurred with shorter or longer culture times.

Labeling time. The greatest disintegrations per minute were obtained when cultures were harvested 16 h after the addition of [³H]Tdr. Labeling lymphocytes 6 h before harvesting resulted in a twofold decrease in [³H]Tdr incorporation. Incorporation of the radioactive label when added to cultures 24 h before harvesting was 20% less than when the label was added 16 h before.

Having defined the conditions under which dog T-lymphocytes are most responsive to PHA, the effect of development and age of dogs on this response was determined.

T-lymphocyte responsiveness to PHA increased markedly between 4 and 6 weeks of age (Fig. 3). The response of dogs 0 to 4 weeks of age was significantly different ($P < 0.001$) from the response of these same dogs between 6 to 12 weeks of age. There were no significant differences between weekly responses within either group. The large standard deviation of stimulation index values at 5 and 6 weeks of age indicates that not all pups became responsive to PHA at the same age.

The responsiveness of dog T-lymphocytes to PHA diminished with age (Fig. 4). Peak response occurred from 6 weeks to 6 months of age. Analysis of these data indicate a significant correlation ($P < 0.02$) between increase in age after 6 months and decrease in the responsiveness of T-lymphocytes to PHA.

DISCUSSION

CMI is considered to be of paramount importance in protecting man against natural measles infections (12, 20). The demonstrations of delayed-type hypersensitivity to measles and canine distemper virus and of cross-reactions between these viruses by Brown and McCarthy (8) suggests that CMI also protects against canine distemper, and that CMI is the mechanism by which measles vaccine protects pups against distemper.

The results of measuring T-lymphocyte stimulation by PHA in pups supports the vaccination and challenge studies previously reported (9), in which it was shown that protection against canine distemper by measles virus was dependent upon the age at which the measles vaccine was inoculated. When measles vaccine was inoculated in 3-week-old pups, it was difficult to distinguish between vaccinated and

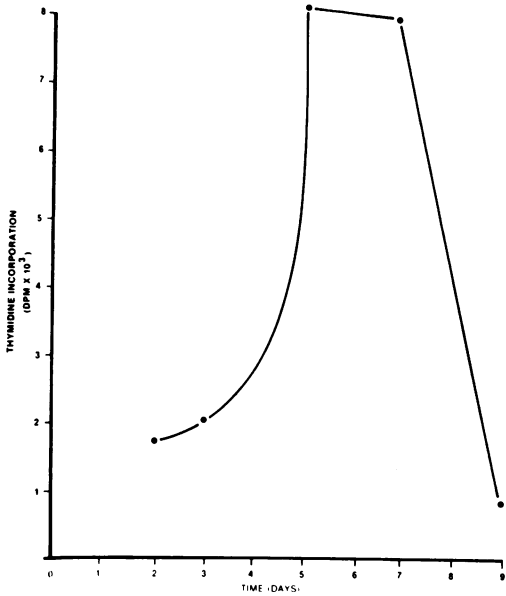


FIG. 2. Responsiveness of dog T-lymphocytes in cultures of different duration times with a 1:400 dilution of PHA. Greatest lymphocyte stimulation and subsequent thymidine incorporation occurred at 5 days. Shorter or longer culture times resulted in diminished responsiveness.

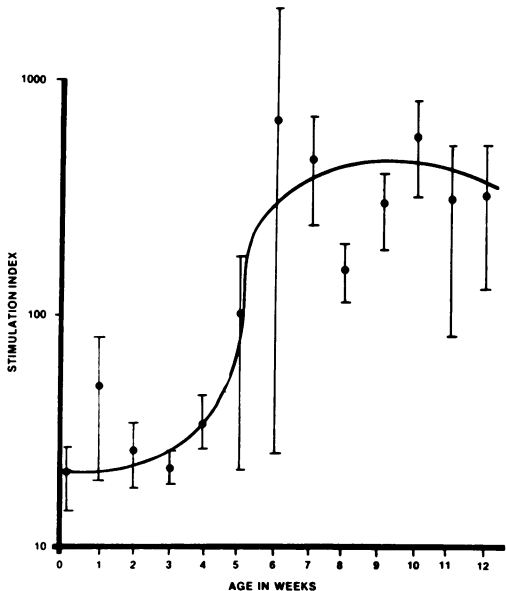


FIG. 3. Development of the response of T-lymphocytes of 16 dog neonates to PHA. Responsiveness to PHA increases significantly between 4 and 6 weeks of age. Each point represents the mean and standard deviation.

nonvaccinated pups. At 4 weeks, acceptable protection was obtained in only 64% of the vaccinated pups. This increased to 93% at 6

weeks. Pups 8 weeks of age or older when inoculated with measles virus were afforded protection approaching that which occurs after canine distemper vaccination.

In the present study, T-lymphocytes of pups 6 to 12 weeks of age showed significantly greater responsiveness to PHA ($P < 0.001$) than when they were less than 4 weeks of age (Fig. 3). This increase in PHA responsiveness parallels the increased ability of the pup to respond to vaccination with measles virus after 3 and 4 weeks of age. Such change in PHA responsiveness may be due to the stage of differentiation of the thymus gland in newborn pups. The weight of the thymus at birth in beagle pups is approximately 100 mg. The thymus increases 200-fold during the first 12 weeks of age and represents 0.5 to 0.7% of the body weight, several times the percentage in the adult dog (2). However, size is not the only consideration.

The appearance of T-lymphocytes that respond to PHA is dependent on the differentia-

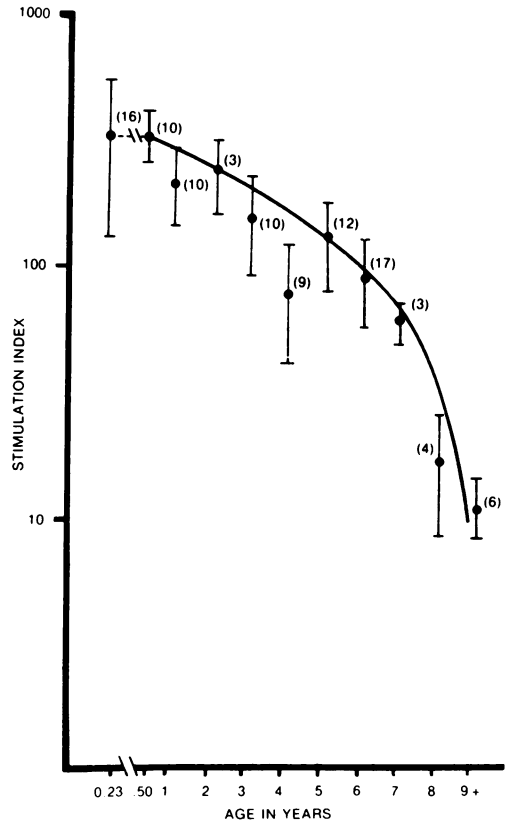


FIG. 4. Effect of age on the response of dog T-lymphocytes to PHA. Responsiveness of T-lymphocytes decreases significantly ($P < 0.02$) with age. Each point represents the mean and standard deviation of the number of dogs indicated in parenthesis.

tion of the medulla (25, 35), and since lymphocytopoietic thymus along with Hassal's corpuscles have been noted in the canine fetus (2, 10, 41), the fetus should be able to elicit a CMI response. Indeed, this seems to be true, for Bryant et al. (11) have shown beagle thymus lymphocyte responsiveness to PHA on gestation day 50, increasing to postnatal day 3 before declining, and Dennis et al. (17) have observed the ability of the fetus to reject a skin allograft, although at a delayed rate.

Although beagle thymocytes (11) showed a diminished responsiveness to PHA at the time of birth and remained unresponsive after birth, T-lymphocytes from the peripheral circulation of the beagle, which were relatively nonresponsive to PHA at birth, became very responsive at 6 weeks of age. This change in T-lymphocyte responsiveness to PHA may be due to one or several causes. First, more than one subpopulation of T-lymphocytes may be involved. Several subpopulations of T-lymphocytes, each with characteristic mitogen receptors, have been delineated in the rats (27), mice (33, 42), and sharks (29). Second, the diversity of responsiveness of T-lymphocyte populations to PHA with time may be due to continuing differentiation (29, 38, 46) or, as Mosier (33) suggests, an interaction and regulation of the responsiveness of one subpopulation by another. This interaction may be altered continually throughout life by physiological changes. Third, the relative nonreactivity of T-lymphocytes of the newborn to 4-week-old dog to PHA may be due to suppressive factors derived from the mother. In dogs, almost all molecular transfer is by colostrum (7), and many factors that may be transferred in the colostrum, including passive antibody, are known to effect the CMI response (4, 13, 15, 30, 32, 39, 47). The subsequent diminishing of suppressive maternal factors with time may result in the acquisition of PHA responsiveness.

Many investigators have noted age-related impairment of CMI and a diminishing effect of PHA on T-lymphocytes from mammals (18, 21, 22, 37, 40, 43). Dogs older than 6 months begin to show a significant age-related decrease in PHA responsiveness ($P < 0.02$) (Fig. 4). Such age-related impairment of cellular immunity could be due to a diminished number of T-lymphocytes or to decreased reactivity of these cells. Gelfand et al. (18) and Stutmen et al. (43) have shown evidence that age-related decline in CMI in NZB mice was due to the loss of the recirculating T-lymphocyte subpopulation, possibly through alterations in thymic function. Walford's work (45) supports this, as he noted

an anatomic involution of the thymus and thymus-dependent tissues in aging mammals. Andersen (2) showed that there is a gradual diminishing of total leukocyte counts in dogs with advancing age. He showed that dogs 7 to 8 years of age have approximately 25% fewer leukocytes as dogs 5 to 6 years of age.

This decreased responsiveness of dog lymphocytes to PHA with age, therefore, may be due to a diminution of the number of T-lymphocytes resulting from involution of the aging thymus. Hori's group (22), however, experimenting with two strains of mice, showed no decline in the number of thymic-dependent T-cells in the spleen with age and concluded that the decrease in PHA responsiveness with age must be due to a decreased responsiveness of individual T-cells. Such decreased responsiveness may be caused by age-related alterations in the concentration of certain serum factors which affect particular aspects of the CMI response. Hallgren et al. (21) suggested nonresponsiveness of T-cells to PHA in aging humans could be due to hyperglobulinemia which, along with the increased frequency of autoantibodies, are characteristic of older adults. Rodey et al. (40) found strains of mice susceptible to autoimmune diseases that showed loss of *in vitro* cellular immune responses.

Although responsiveness of T-lymphocytes to PHA does not reflect the total cellular immune capabilities of the dog, it seems reasonable to suggest that acquisition of PHA responsiveness of dog T-lymphocytes during development and diminishing responsiveness with age does reflect a changing in the CMI capability in dogs. In inaugurating successful immunization procedures for dogs, such variations in immunological responsiveness should be considered.

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