

Tuberculin-Specific Transfer Factor in Dogs

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Five mongrel dogs were sensitized to tuberculoprotein with Freund adjuvant, as determined by responses to tuberculin skin tests and by in vitro lymphocyte cultures. These animals served as blood donors for production of transfer factor. Nine mongrel dogs received single doses of transfer factor that was tuberculin specific. Successful transfer of tuberculin sensitivity as measured by lymphocyte transformations was achieved in five of nine animals. Canine lymphocyte cultures frequently showed relatively low transformations to mitogens as compared with responses noted in other animals. Dissociation between skin test reactivity and in vitro lymphocyte transformation to tuberculoprotein was noted in two of nine animals. Increases in lymphocyte transformations to phytohemagglutinin were seen in three of nine recipient animals after transfer factor administration. Successful transfer from an animal sensitized by prior transfer factor administration was achieved in one instance. These data indicate that transfer factor-like substances are present in dogs. The reasons why successful transfer was achieved in only half of the recipient animals needs further explanation.

Leukocyte extracts, as described by Jeter et al. (4), can transmit delayed hypersensitivity from sensitized guinea pigs to unresponsive animal recipients. Lawrence (6) transferred antigen-specific, cell-mediated immunity of the delayed type from sensitive humans to unresponsive human recipients. Dialysates of these leukocyte extracts have been termed transfer factor. Other transfer factor-like substances are being discovered in an increasing number of experimental animals. Dogs have become an important laboratory animal, especially in the setting of experimental transplantation surgery. This study was undertaken to confirm the presence of transfer factor-like substances in dogs. Two reports have described the existence of canine transfer factor. One study used coccidioidomycosis antigen as the primary immunological marker to prove successful transfer from one animal to another (11). Tuberculin, in that study, was a less satisfactory antigen in terms of skin test reactivity and lymphocyte transformation in the determination of the status of cell-mediated immunity. The other study, reporting only upon recipient skin test reactivity to tuberculin, demonstrated transfer of delayed hypersensitivity with transfer factor in dogs (W. S. Jeter, T. C. Soli, and R. E. Reed, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1976, E109, p. 81).

MATERIALS AND METHODS

Sensitization with Freund adjuvant. Mongrel dogs weighing approximately 25 kg were randomly selected to serve as donors and recipients of transfer factor. Five donor animals were injected once with 2 ml of complete or incomplete Freund adjuvant. They were then skin tested weekly over a 6-week period until intradermal, delayed hypersensitivity skin tests became positive with mammalian tuberculin (MT; U.S. Department of Agriculture, Lansing, Mich.) antigen at 48 h. Skin test sites were only used once.

Production of transfer factor. Venous blood (400 to 500 ml) was collected into acid-citrate-dextrose solution from immunized dogs once skin tests and lymphocyte transformation responses indicated demonstrable hypersensitivity to tuberculoprotein. Erythrocytes were lysed by diluting 1:5 (vol/vol) with sterile 0.87% NH₄Cl. The leukocytes were then sedimented by centrifugation and washed with Hanks balanced salt solution, and their concentration was determined. The cells were then suspended in 5 to 10 ml of Hanks balanced salt solution and frozen (-70°C) and thawed (22°C) 10 times.

Four transfer factor preparations (for recipients labeled A, D, F, and G) were placed in a membrane tubing with a molecular weight cut-off of 15,000 and dialyzed against two changes of 400 ml of distilled water at 4°C for 24 h each. Resultant dialysates were then lyophilized over 48 h. All lyophilized material was then dissolved in 5 ml of distilled water and stored at -70°C until administered to recipient dogs within 2 to 3 months.

Another five preparations of transfer factor were placed in a tube of dialysis membrane with a 15,000-molecular-weight cut-off and were vacuum dialyzed for 24 h (ProDiFilt Unit, Bio-Molecular Dynamics,

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Beaverton, Ore.). Dialysates (7- to 10-ml volumes) were then frozen at -70°C until used. These various preparations of transfer factor were not mixed and were administered subcutaneously to recipient dogs. One dose of transfer factor was the amount derived from 1.2×10^6 to 9.6×10^6 peripheral blood lymphocytes.

Lymphocyte transformation assay. Antigenic and mitogenic sensitivity was assayed with the lymphocyte transformation assay by modification of the method of Oppenheim and Schecter (8). Leukocytes were obtained from 20 ml of heparinized blood by centrifugation after the erythrocytes had been lysed by diluting 1:5 (vol/vol) with 0.87% NH_4Cl . Leukocyte suspensions were adjusted to 5×10^5 lymphocytes/ml, and portions of 1×10^5 lymphocytes were cultured in 0.2-ml microtiter wells in triplicate with 20% autologous serum and 80% RPMI 1640 medium (containing 100 μg of penicillin and streptomycin per ml and 25 mM glutamine). Cultures were incubated for 5 days at 37°C in a 5% CO_2 humidified atmosphere. Positive control cultures were incubated with phytohemagglutinin (PHA-P; Difco Laboratories, Detroit, Mich.), 7.5 and 75 $\mu\text{g}/\text{ml}$ or 7.5 and 25 $\mu\text{g}/\text{ml}$. Experimental cultures were incubated with purified protein derivative (PPD; Parke, Davis & Co., Detroit, Mich.) in varying concentrations ranging from 180 to 3,600 U/ml. Cultures were also incubated without antigens or mitogens and served as indicators of background reactivity.

Cultures were pulsed with 1 μCi of [*methyl*- ^3H]-thymidine on day 5 of incubation for 4 to 6 h and then harvested with a multiple automated sample harvester. The harvested cultures were counted for 1 or 5 min on a Beckman 25-100C scintillation counter. In this system, counts per minute with unstimulated cultures containing 10^5 lymphocytes produced scintillation counts of 50 to 400/min.

Stimulation indexes were derived by dividing the mean of the counts obtained per minute with mitogen- or antigen-stimulated cultures by the mean of counts obtained per minute with cultures containing no antigen or mitogen. Stimulation indexes in response to PPD were always less than 2 in 16 unsensitized animals. Therefore, any stimulation index greater than 3 was considered to be evidence of sensitization to tuberculo-protein. All assays were performed at weekly intervals beginning 96 h after subcutaneous administration of transfer factor.

Skin tests. Sensitivity to MT was assayed by the

Mantoux technique of intradermal skin testing. It was found that 2,500 U of MT gave negative skin tests in unsensitized animals and positive skin tests in sensitized animals. Positive skin tests were considered to be those that exhibited at least a 4-mm diameter of erythema or induration when none had been present before transfer factor administration. Skin tests were read as negative if no erythema or induration occurred at 48 h. Excluded from the study were five unsensitized animals noted to have MT skin tests of 10-mm induration associated with lymphocyte stimulation indexes between 2 to 3 to PPD.

RESULTS

Experimental controls and sensitization of donor animals. Four of the transfer factor donor dogs (labeled 1 through 4) were sensitized with 2 ml of complete Freund adjuvant (Table 1). Donor animal 5 was sensitized with 2 ml of incomplete Freund adjuvant followed by three repeat skin tests with MT. Animal 6 was sensitized with transfer factor from animal 5 and in turn served as a donor for a unit of transfer factor that was given to recipient I.

Dogs donating transfer factor also served as test controls in this study, as their skin test status and lymphocyte transformation indexes were monitored for 4 to 6 weeks, enabling comparison with the transfer factor recipient animals. Skin tests were applied weekly to dogs that donated transfer factor. Between 4 and 6 weeks were required after administration of Freund adjuvant for the skin tests to become positive in all five donor animals. As seen in Table 1, 4 weeks elapsed before a positive lymphocyte transformation stimulation index could be found in four of the five animals. Only 3 weeks were required for positive stimulation indexes to PPD to occur in donor 4. Stimulation indexes became positive (≥ 3) before or simultaneously with the skin test reactions.

Effects of transfer factor. All donor animals had positive MT skin tests and positive lymphocyte transformation with PPD at the time of transfer factor donation. Transfer factor (9 U) was given to nine canine recipients designated

TABLE 1. *Effects of sensitization with Freund adjuvant in donor canines*

Donor	Type of sensitization	Diam of MT ^a skin test (mm)	No. of weeks after adjuvant injection	Lymphocyte transformations to PPD (stimulation index) ^b	No. of weeks after adjuvant injection
1	CFA ^c	7 × 10	6	6.0	4
2	CFA	10 × 13	4	9.0	4
3	CFA	8 × 8	4	4.3	4
4	CFA	7 × 10	4	3.3	3
5	IFA ^d and intradermal MT	5 × 7	5	6.4	4

^a MT, Mammalian tuberculin.

^b First positive lymphocyte transformations.

^c CFA, Complete Freund adjuvant.

^d IFA, Incomplete Freund adjuvant.

TABLE 2. *Effects of transfer factor (TF)*

Animal	Stimulation index		MT ^a skin test induration
	PHA	PPD	
Donor 1 ^b	203	3.6	7 × 10 mm
Recipient A, pre-TF ^c	3	0.8	0
4 days post-TF	44	0.8	
6 days post-TF			5 × 5 mm ^d
Recipient B, pre-TF	29	1.4	0
7 days post-TF	7		
10 days post-TF			10-mm erythema only ^d
14 days post-TF	29	1.0	
Donor 2	249	3.3	12 × 15 mm
Recipient C, pre-TF	37	1.6	0
7 days post-TF	26		
10 days post-TF			4 × 4 mm ^d
14 days post-TF	48	3.2 ^d	
Donor 3	202	4.5	10 × 10 mm
Recipient D, pre-TF	19	0.8	12-mm erythema
4 days post-TF	89 ^d	2.7	0
25 days post-TF		3.6 ^d	0
Recipient E, pre-TF	4.5	2.0	0
14 days post-TF	15	2.1	0
Donor 4	416	7.1	9 × 10 mm
Recipient F, pre-TF	14	0.8	0
11 days post-TF	7	0.4	0
Donor 5	100	6.4	5 × 7 mm
Recipient G, pre-TF	31	1.5	3-mm erythema
13 days post-TF			2-mm induration
14 days post-TF	191 ^d		
21 days post-TF		4.9 ^d	
Recipient H, pre-TF	1.0	0.6	0
7 days post-TF			11- × 14-mm erythema ^d
18 days post-TF	69 ^d	7.5 ^d	
Donor 6 (recipient G)	53	4.9	2 mm
Recipient I, pre-TF	2.8	1.1	10-mm erythema
4 days post-TF		4.5 ^d	10-mm erythema
30 days post-TF	23.5		10-mm erythema

^a MT, Mammalian tuberculin.^b Data for all donors are those obtained at time of blood donation for TF preparations.^c TF, Transfer factor.^d Significant TF effect.

A through I (Table 2). Conversion of MT skin tests (≥ 4 mm at 72 h) was noted after transfer factor administration in four animals (recipients A, B, C, and H). Two of these four animals (recipients C and H) also had positive responses in lymphocyte transformations to PPD after transfer factor administration (from donors 2 and 5). A total of five animals developed positive stimulation indexes in response to PPD (recipients C, D, G, H, and I), three of which had negative skin tests (recipients D, G, and I). An additional finding was that an increased PHA responsiveness occurred in three of the recipient animals (recipients D, G, and H) after transfer factor administrations. Dog donors 3 and 5 had elevated values of stimulation indexes to PHA (indexes of 202 and 100, respectively) at the time of blood donation for transfer factor preparation. In recipients A and B, dissociations occurred

between skin test reactivity and lymphocyte transformation; i.e., positive skin tests to MT occurred when lymphocyte transformations showed no positive responses to PPD.

Serial transfer was also accomplished by using transfer factor derived from donor 5 and administered to recipient G. This latter animal exhibited a significant increase in PHA reactivity (stimulation index, 191) and also developed a positive stimulation index in response to PPD (stimulation index, 4.9). Transfer factor was then prepared from recipient G and successfully transferred in vitro PPD responsiveness to recipient I.

Transfer factor recipients' skin tests became positive to MT before lymphocyte transformation responses became positive to PPD in the two animals in which both were positive (recipients C and H). This contrasts with the results

obtained in the animals sensitized primarily with complete Freund adjuvant and incomplete Freund adjuvant, which showed that the lymphocyte response became positive at the same time as or before conversion of the skin tests in all five donor animals (see Table 1). Tuberculin reactivity was not successfully transferred in two recipients (E and F).

DISCUSSION

Other investigators have encountered difficulties in using the dog as an immunological model in the study of delayed hypersensitivity. One difficulty encountered is the relatively low counts per minute and stimulation indexes noted with canine lymphocyte cultures that use PPD as an antigen. Shifrine et al. (11) found in dogs a maximum stimulation index of 6 with PPD, compared with a stimulation index of 75 when coccidioidomycosis antigen was used. In our study, up to 40,000 cpm with 1×10^5 lymphocytes were found after PHA stimulation. Benjamin and Ferris (1) reported a PHA stimulation of 1,410 cpm, and Bryant et al. (2) reported a PHA stimulation of 5,000 cpm with 5×10^5 lymphocytes.

Another difficulty has been in eliciting a delayed hypersensitivity response to tuberculin in the skin of dogs. The high dose (2,500 U) of MT used in the skin tests of this study has been found to be the dose most likely to elicit positive reactions in dogs infected with mycobacteria (9).

Administration of an antigen without Freund adjuvant can cause specific, delayed hypersensitivity to that injected substance (10). Intradermal injection of antigen has been shown to be the most effective route of immunization for eliciting a delayed hypersensitivity response (7). Consequently, it was not surprising that donor 5 became sensitized to tuberculin after intradermal injections of 2,500 U of MT at four weekly intervals. Sensitization was indicated by positive lymphocyte transformations to PPD and a positive MT skin test 1 week later.

As was found in our study, Shifrine et al. have also noted increased PHA responsiveness in dogs after administration of canine transfer factor. Successful transfer of tuberculin reactivity was accomplished in five out of nine attempts, using dialyzable transfer factor as determined by positive lymphocyte culture stimulation indexes. Two of the recipient animals had definitely negative MT skin tests that became unequivocally positive. Animals with initial skin tests that were marginally positive may have been sensitized with naturally occurring mycobacteria in soil or water. The dissociation between positive skin tests and negative blast transformation noted in

our study has been previously observed in humans with chronic mucocutaneous candidiasis (5).

The in vitro and in vivo reactivities to tuberculin found after transfer factor administration was unlike that seen after sensitization with Freund adjuvant and repeated skin testing. In the former case, the positive skin test preceded the finding of lymphocyte reactivity, whereas in the latter case, positive lymphocyte transformation preceded, or was cotemporal with, the finding of positive skin tests. It is very unlikely that skin testing the recipient animals was alone responsible for this sensitization, because different temporal patterns in onset of positive tests were noted, and also because the interval between transfer factor administration and the finding of tuberculin sensitivity was shorter. Although the onset of action of transfer factor has been noted to be as rapid as 1 day (3, 6), David (3) has noted greater reactivity at 6 days, and Lawrence (6) noted initial evidence of transfer of cutaneous reactivity as late as 30 days after administration of transfer factor. These findings in the human system are consistent with our observations in dogs.

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