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Transfer Factor: a Murine Model

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Transfer factor has been studied extensively in humans, but a satisfactory subprimate model has not been established. Using BALB/c mice immunized with complete Freund adjuvant, we show that a low-molecular-weight substance derived from disrupted spleen cells transferred sensitivity to purified protein derivative (PPD) to recipient nonimmunized BALB/c mice. Transfer was confirmed by footpad swelling to PPD in vivo and by splenic lymphocyte transformation to PPD in vitro. In recipients of transfer factor, an inverse correlation was noted between the splenic lymphocyte response to PPD and to concanavalin A. Material obtained from spleens of saline-treated BALB/c mice did not transfer sensitivity to PPD to recipient mice.

Lawrence reported in 1955 that disrupted leukocytes from a sensitized human donor yielded a low-molecular-weight dialyzable substance designated transfer factor (TF), which could transfer delayed-type hypersensitivity (DTH) to a nonimmune recipient (13). The existence of TF in humans has been confirmed repeatedly by other workers (2, 10). TF has been used therapeutically in patients with tumors (14), disseminated fungal infections (11), and certain immunodeficiency diseases (22). However, investigation of the mechanism of transfer and standardization of the potency of various TF preparations has been slowed by the lack of a suitable animal model.

Extensive study by several workers in the 1960s failed to show dialyzable TF in guinea pigs (1), although Jeter et al. described transfer of sensitivity with disrupted leukocytes in guinea pigs in 1954 (9). More recent studies using cows (12), rats (15), dogs (21), and guinea pigs (3) have led to renewed interest in a subprimate model for the study of TF. Rifkind et al. (19) have shown that a TF-like substance derived from murine spleen cells can transfer DTH as measured by footpad swelling. Our studies confirm the existence of a low-molecular-weight substance derived from murine spleen cells which is able to pass DTH to recipient mice; we further show that transfer is associated with the development of in vitro lymphocyte transformation to the antigen to which TF donors have been sensitized.

MATERIALS AND METHODS

Animals. Female BALB/c mice (Charles River Laboratory, Wilmington, Mass.) were entered into the experiment at 7 weeks of age and were sacrificed by cervical dislocation at 10 to 12 weeks of age.

Immunization. Mice were immunized on days 1 and 12 of the experiment by subcutaneous inoculation with 0.2 ml of complete Freund adjuvant (CFA) (Difco Laboratories, Detroit, Mich.) mixed 1:1 with 0.9% saline. Control mice received 0.9% saline on the same schedule.

Cell transfer. Three weeks after immunization, spleens from either CFA-immunized or saline-treated control mice were removed aseptically, and cell preparations were made by repeatedly injecting RPMI 1640 medium (GIBCO, Grand Island, N.Y.) into the spleens, using a 22-gauge needle and a 1-ml syringe. The cells were washed twice in RPMI 1640 and suspended to a concentration of 5×10^8 mononuclear cells/ml, and 1 ml was injected intraperitoneally (i.p.) into each recipient. Cell preparations consistently showed >95% viability by trypan blue dye exclusion.

TF. TF was prepared from spleen cells of either CFA-immunized mice (TF_S) or saline-treated mice (TF_N) 3 weeks after immunization. For each batch of TF, spleens of 60 mice were removed, pooled, added to 25 ml of 0.9% saline, and disrupted in a Virtis homogenizer for 10 min. The suspension was frozen in a Dry Ice-acetone bath and thawed in a 37°C water bath 10 times. Dense fibrin-like material was removed; the suspension was spun at $40,000 \times g$ for 60 min; and the supernatant was collected, diluted with 30 ml of distilled water, and filtered through an Amicon ultrafiltration apparatus and a UM 10 membrane (Amicon, Inc., Lexington, Mass.). The filtrate (molecular weight, <10,000) was lyophilized and resuspended in sterile distilled water so that 1 ml was the equivalent of 5×10^8 spleen cells. Each recipient animal received 1 ml of TF i.p.

General experimental design. Animals were divided into six groups as follows: negative controls treated with saline alone (21 mice); positive controls treated with CFA (20 mice); normal-cell recipients (15 mice); sensitized-cell recipients (14 mice); TF_N recipients (24 mice); and TF_S recipients (36 mice).

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Twenty-four hours after passive cell transfer or TF administration, DTH footpad testing was performed. After the 48-h reading, 2 ml of mineral oil was injected i.p. Three days later, spleens were harvested for lymphocyte transformation studies and peritoneal macrophages were harvested for macrophage migration inhibitory factor (MIF) studies.

DTH footpad tests. Lyophilized preservative-free purified protein derivative (PPD) (Parke-Davis, Inc., Detroit, Mich.) was reconstituted in phosphate-buffered saline (PBS), pH 7.2, to a concentration of 800 μg/ml; 0.025 ml (20 μg) was injected into the plantar surface of the right hind paw. The left hind paw received 0.025 ml of PBS. At 0, 24, 30, and 48 h, right and left footpads were measured by vernier calipers by one observer, blinded as to which preparations the mice had received. The data are presented as 0.1-mm increments from the 0-h readings in PPD-tested and PBS-tested footpads.

Lymphocyte transformation. Spleen cell suspensions were prepared as described for cell transfer studies. Cells were washed twice in RPMI 1640 with 2 mM L-glutamine, 10% heat-inactivated human serum, and 100 μg of streptomycin per ml. Samples of 0.2 ml of cell suspension (25×10^5 cells/ml) were added to flat-bottomed microtiter wells (Linbro, Inc., Hamden, Conn.). Triplicate samples of the following cultures were set up for each animal: controls, using 0.01 ml of PBS; concanavalin A (ConA) (Pharmacia, Inc., Piscataway, N.J.), 2.5 μg in 0.01 ml; and preservative-free PPD, 5, 10, and 25 μg, each in 0.01 ml.

After incubation at 37°C in a 5% CO_2–95% air, humidified atmosphere for 72 h for ConA cultures and 144 h (6 days) for PPD-treated cultures, cells were pulse-labeled for 4 h with 1 μCi of tritiated thymidine (specific activity, 6 Ci/mmol; Schwarz/Mann, Inc., Orangeburg, N.Y.). Cultures were harvested by using a multiple automated sample harvester (MASH; Microbiological Associates, Inc., Rockville, Md.), and isotope incorporation was measured in a liquid scintillation counter. The maximal response to PPD was calculated for each animal. Results were expressed as counts per minute or as the stimulation index (SI), which is the ratio (mean counts per minute in stimulated cultures)/(mean counts per minute in unstimulated cultures).

MIF assay. Peritoneal exudate cells were collected 3 days after injection of oil by washing of the peritoneal cavity with Hanks balanced salt solution with penicillin and streptomycin. Cells from three mice were pooled, washed three times with Hanks balanced salt solution, and adjusted to a concentration of 2×10^8 cells/ml in minimal essential medium with 10% heat-inactivated horse serum (GIBCO), 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Fifty microliters of cells was incubated with either 10 μl of minimal essential medium or 10 μl of PPD (150 μg) at 37°C for 30 min with frequent shaking.

Migration studies, using a modification of the method of Carpenter et al. (6), were conducted in 1% agarose containing 10% 10× medium 199 with Earle salts (GIBCO), 2 mM L-glutamine, 10% heat-inactivated horse serum, and penicillin and streptomycin in plastic petri dishes. Twelve microliters of the cell suspension was placed into 3-mm wells in the agarose, and the cells were allowed to migrate for 20 h at 37°C in a 5% CO_2 atmosphere. The cells were heat fixed to the dish, the agarose was removed, and the area of migration was measured with a planimeter. The well area was subtracted from the total area, and percent inhibition of triplicate sets of wells was calculated by the following formula: percent inhibition = 1 − [(area with PPD)/(area without PPD)] × 100.

Statistical methods. All data were analyzed with the use of Student's *t* test. Correlation coefficients were also calculated for the lymphocyte transformation tests.

RESULTS

Mice receiving TF_S showed a significant response to PPD when swelling of the PPD pad was compared with swelling of the PBS pad ($P < 0.001$) (Table 1). In addition, the difference between swelling of the PPD pads in the TF_S group and the saline control group was significant ($P < 0.001$). Mice receiving TF_N did not exhibit any change in footpad swelling when the PPD pad was compared with the PBS pad or when the response to PPD in the TF_N group of mice was compared with that in the saline-treated group. The PPD footpad response in the TF_S group was significantly greater than that in the TF_N group ($P < 0.005$). Positive controls (CFA-treated mice and recipients of immune spleen cells) showed a greater response to PPD than was shown by the TF_S recipients. Negative controls (PBS-treated mice and recipients of normal spleen cells) did not show swelling to PPD. In all groups with a positive response, the maximal swelling was observed at 24 h; swelling had decreased to approximately one-half of the maximal amount by 48 h.

Specific transformation of spleen cells to PPD was performed in all groups of mice (Fig. 1). Control groups (saline-treated mice, recipients of normal spleen cells, and mice receiving TF_N)

TABLE 1. *Footpad swelling in response to PPD*

Group	Footpad response[a]		*P* value[b]
	PPD pad	PBS pad	
Saline	0.2 ± 0.1	0.2 ± 0.1	NS
Normal-cell recipients	0.3 ± 0.2	0.2 ± 0.2	NS
TF_N	0.4 ± 0.1	0.3 ± 0.2	NS
CFA	11.8 ± 0.9	0.6 ± 0.2	<0.001
Immune-cell recipients	3.7 ± 0.4	0.4 ± 0.2	<0.001
TF_S	1.2 ± 0.1	0.4 ± 0.1	<0.001

[a] Footpad swelling in 0.1-mm increments at 24 h (mean ± standard error of the mean). Mice received 20 μg of PPD in 0.025 ml in the right hind footpad and 0.025 ml of PBS in the left hind footpad.

[b] Student's *t* test comparing PPD pad with PBS pad. NS, Not significant.

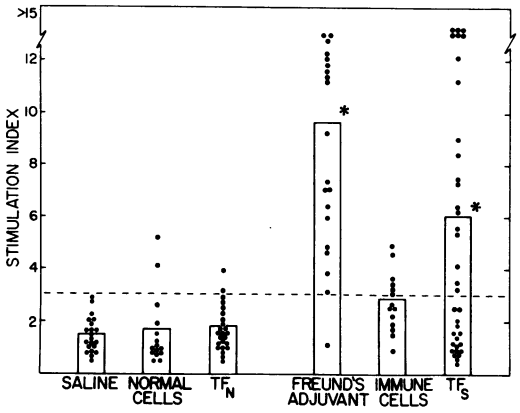

FIG. 1. *Lymphocyte transformation to PPD performed 6 days after mice received TF preparations or passive cell transfer. SI of ≥3.0 was considered positive. The mean SI for each group is represented by the top of the bar. *, $P < 0.001$ when compared with saline control mice.*

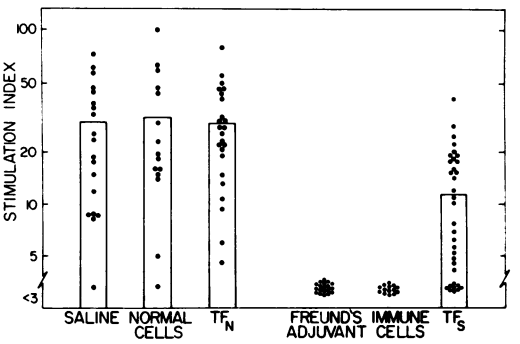

FIG. 2. *Lymphocyte transformation to ConA performed 6 days after mice received TF preparations or passive cell transfer. The mean SI for each group is represented by the top of the bar.*

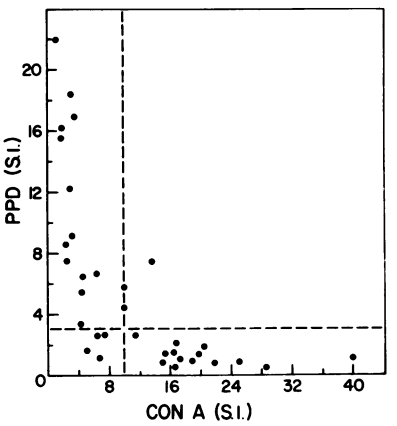

FIG. 3. *Correlation between lymphocyte response to PPD and to ConA in animals receiving TF$_S$. Correlation coefficient = −0.65 ($P < 0.001$).*

did not respond in vitro to PPD. Among 60 animals, only 4 had an SI of >3.0, and the mean SI for the three groups was <2.0. Animals receiving TF_S showed a mean SI to PPD of 6.0; 19 of 36 mice responded to the antigen. The difference between the response to PPD of TF_S and TF_N recipients was highly significant ($P < 0.001$). All CFA-immunized mice except one responded to PPD in vitro. Only 6 of 14 recipients of immune spleen cells showed stimulation to PPD, possibly reflecting poor recirculation from the peritoneal to the splenic pool of cells. Among the TF_S animals there was no correlation between footpad response to PPD and in vitro lymphocyte transformation to PPD. Mean background deoxyribonucleic acid synthesis in counts per minute in 6-day cultures without antigen was as follows: saline-treated mice, 3,094; normal spleen cell recipients, 7,044; TF_N recipients, 7,702; CFA-treated mice, 1,838; sensitized cell recipients, 7,365; and TF_S recipients, 5,262.

Transformation to the mitogen ConA was studied simultaneously with the PPD response in all groups (Fig. 2). The mean SI was approximately 30 in all control groups. None of the CFA-immunized animals or the recipients of immune spleen cells responded to ConA. Among TF_S recipients, 17 of 34 had a very poor mitogenic response (SI < 10), whereas 17 had an SI between 10 and 40. When individual TF_S animals were scrutinized for their responses to PPD and to ConA, there was an inverse correlation noted between the two tests (correlation coefficient = −0.65; $P < 0.001$) (Fig. 3). Those animals that showed specific transformation to PPD, in general, did not respond to ConA. The best ConA response was found in those animals that did not respond to PPD. Mean background deoxyribonucleic acid synthesis in counts per minute in 3-day cultures without mitogen ranged between 2,550 and 3,500 for saline-treated mice, normal-cell recipients, sensitized-cell recipients, and TF_N recipients. Mean spontaneous transformation was 6,070 for TF_S recipients and 12,034 for CFA-immunized mice.

MIF activity was assessed in all groups of animals (Table 2). The TF_S recipients as well as the positive control groups showed inhibition of macrophage migration. However, the specificity of this inhibition is questionable since four of four groups of normal-cell recipients and four of eight groups of TF_N recipients also showed >20% inhibition of migration.

DISCUSSION

In the present study, we have shown that a

TABLE 2. *Direct MIF assay in response to PPD*

Group	No. positive/no. tested[a]	% Inhibition[b]
Saline	2/7	13.5 ± 2.8
Normal-cell recipients	4/4	28.2 ± 5.6
TF_N	4/8	24.5 ± 4.6
CFA	6/7	29.6 ± 3.4
Immune-cell recipients	5/5	44.0 ± 2.3
TF_S	7/9	33.2 ± 4.3

[a] Mice were pooled in groups of three to perform this test; ≥20% inhibition is considered positive.

[b] Mean ± standard error of the mean.

low-molecular-weight substance derived from spleen cells of BALB/c mice immunized to CFA can transfer PPD sensitivity to nonimmunized BALB/c recipients. Very few previous studies have demonstrated transfer of DTH with TF in mice. Rifkind et al. found that a TF-like substance prepared from spleen cells of CF1 mice was capable of transferring antigen-specific footpad responsiveness to recipient mice (19). They also confirmed Crowle's earlier work showing that footpad swelling is a valid indicator of DTH in mice (7, 17). Both the study of Rifkind et al. and ours used complex microbial antigens. Both documented transfer by in vivo footpad swelling. Additionally, we showed development of in vitro responsiveness to PPD after TF administration.

The TF-like substance we found in BALB/c spleen cells has not been biochemically characterized; the preparation of Rifkind et al. from CF1 spleen cells was resistant to deoxyribonuclease, ribonuclease, and trypsin and sensitive to pronase and phosphodiesterase, properties shared with human TF (18). It is possible that antigen could have been transferred to recipients in the TF_S preparation and that active sensitization occurred in these animals. However, this seems unlikely in view of the short time between TF_S administration and evaluation of footpad and lymphocyte transformation responses. It has been shown by others using animal systems that TF does not transfer antigen (3).

That TF is unique to humans and other primates seems untenable since so many immunological phenomena are shared by most mammals (4). Besides the above studies in mice, TF has now been demonstrated in cattle (12), rats (15), dogs (21), and guinea pigs (3). Although several of these models used Lawrence's method to prepare dialyzable material from disrupted leukocytes (15, 21), others used material released into the supernatant by sensitized cells incubated at 37°C for 4 h (3, 12). Assays for transfer of sensitivity have included both skin test reactivity and in vitro lymphocyte transformation in several of these models (12, 21) as well as in our murine model.

In this study, transfer of sensitivity appeared to be specific in that TF_N recipients did not show a response to PPD. However, specificity should be evaluated further by testing recipient animals with footpad responses and lymphocyte transformation to several different antigens. In both footpad swelling and transformation assays, the responsiveness shown by CFA-immunized animals was greater than that exhibited by the TF_S recipients. The poor transformation response to PPD noted by recipients of immune spleen cells may be related to poor circulation to the spleen by the cells given i.p. (5); these animals responded the best of all groups in the MIF assay, which used cells from the peritoneum.

Not all animals given TF_S reacted to PPD by footpad swelling or lymphocyte transformation; it appeared that some preparations of TF_S were more active than others in transferring immunity. Without an adequate method to standardize the quantity of TF given, this has been a recurrent problem in most TF studies (8).

Spleen cells of CFA-immunized mice did not respond to ConA, whereas saline-treated mice responded normally to this mitogen. Likewise, recipients of immune spleen cells showed no response to ConA, whereas recipients of nonimmune cells reacted well. This inhibition of spleen cell mitogenic response could reflect activation of a population of suppressor cells in the spleens, as has been found in mice given *Mycobacterium bovis* BCG i.p. or intravenously (25) or *Corynebacterium parvum* (20). Those animals receiving TF_S that showed transfer of sensitivity to PPD by in vitro lymphocyte transformation responded poorly to ConA, implying that the process responsible for suppression occurred in these animals as well. TF_S animals that did not respond to PPD responded well to ConA, and TF_N recipients responded well to ConA.

In the system we used, PPD was a specific antigen and not a mitogen for murine spleen cells. No saline-treated animals responded to PPD, whereas all except one animal immunized with CFA responded to PPD. Sultzer et al. have reported that PPD is a mitogen for murine spleen cells (23, 24), but this effect was noted at 48 to 72 h, not 144 h, and occurred at larger doses of PPD than we used. Other workers have not found PPD to be a nonspecific mitogen in mice (16).

The direct MIF assay as used in this study appeared to be nonspecific. Intraperitoneal administration of either whole cells or TF, either normal or sensitized, led to inhibition of macrophage migration. Although recipients of TF_S

showed greater inhibition of migration, this was not significantly different from results with recipients of TF_N. The cause of this nonspecificity is not known, but may relate to the use of the peritoneal cavity for administration of cells or TF as well as the procurement of macrophages. This problem could be obviated by an indirect MIF assay using other animal's macrophages as indicator cells.

These studies establish that a TF-like substance does exist in murine spleen cells, and that sensitivity to at least one microbial antigen can be transferred with this moiety. Biochemical characterization of this substance and its relation both to the material obtained by Rifkind et al. (19) in a different inbred mouse strain and to human TF will be of interest. Further studies with other antigens will also be important. Finally, this model should be quite useful in investigating the mode of action of TF.

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